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VOLUME 131

VOLUME ONE HUNDRED AND THIRTY ONE

ADVANCES IN IMMUNOLOGY

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ADVANCES IN IMMUNOLOGY

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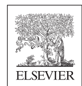

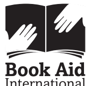

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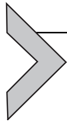

CHAPTER ONE

Malondialdehyde Epitopes as Targets of Immunity and the Implications for Atherosclerosis

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Abstract

Accumulating evidence suggests that oxidation-specific epitopes (OSEs) constitute a novel class of damage-associated molecular patterns (DAMPs) generated during high oxidative stress but also in the physiological process of apoptosis. To deal with the potentially

[1] Contributed equally.

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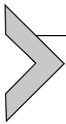
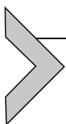
harmful consequences of such epitopes, the immune system has developed several mechanisms to protect from OSEs and to orchestrate their clearance, including IgM natural antibodies and both cellular- and membrane-bound receptors. Here, we focus on malondialdehyde (MDA) epitopes as prominent examples of OSEs that trigger both innate and adaptive immune responses. First, we review the mechanisms of MDA generation, the different types of adducts on various biomolecules and provide relevant examples for physiological carriers of MDA such as apoptotic cells, microvesicles, or oxidized low-density lipoproteins. Based on recent insights, we argue that MDA epitopes contribute to the maintenance of homeostatic functions by acting as markers of elevated oxidative stress and tissue damage. We discuss multiple lines of evidence that MDA epitopes are proinflammatory and thus important targets of innate and adaptive immune responses. Finally, we illustrate the relevance of MDA epitopes in human pathologies by describing their capacity to drive inflammatory processes in atherosclerosis and highlighting protective mechanisms of immunity that could be exploited for therapeutic purposes.



1. OVERVIEW

Cellular stress, senescence, and cell death are tightly associated with oxidative stress. A major consequence of increased oxidative stress is the peroxidation of membrane lipids resulting in the generation of various oxidation-specific epitopes (OSEs). OSEs and the immune responses targeting them have been implicated in many acute and chronic inflammatory diseases, most prominently atherosclerosis. Studies of the biological activities of oxidized LDL (OxLDL), which is a key pathogenic driver of atherosclerosis, have helped identify OSE as a novel class of damage-associated molecular patterns (DAMPs). In this chapter, we will particularly focus on a certain group of OSEs, namely malondialdehyde (MDA) epitopes. MDA epitopes have been documented on the surface of dying cells and in damaged tissues. Recent studies have identified them as major targets of various immune responses that modulate homeostatic processes, eg, the clearance of apoptotic cells. In atherosclerosis, which is characterized by impaired resolution and chronic inflammation, MDA epitopes have been identified as mediators of inflammation and therefore serve as interesting potential targets for immunological therapeutic interventions in cardiovascular diseases (CVDs).



2. BIOCHEMISTRY AND GENERATION OF MDA IN VITRO AND IN VIVO

Oxygen is a fundamental prerequisite for energy production by cellular respiration in aerobic organisms. However, this also results in the constant

generation of reactive oxygen species (ROS) as potentially damaging by-products, which are produced endogenously in mitochondria, peroxisomes, the endoplasmic reticulum, and even in the plasma membrane of cells, but can also be induced exogenously by UV light, heat, bacterial, and environmental agents, such as tobacco smoke and ionizing radiation (Bae, Oh, Rhee, & Yoo, 2011; Nathan & Cunningham-Bussel, 2013). Newly generated ROS can attack membrane lipids containing carbon–carbon double bonds (eg, polyunsaturated fatty acids (PUFAs) of phospholipids), and damage them by a process called lipid peroxidation. Lipid peroxidation of free fatty acids occurs through both enzymatic and nonenzymatic mechanisms. If not efficiently controlled this emanates in the perturbed integrity of different cellular structures potentially leading to cellular death (Nathan & Cunningham-Bussel, 2013). Enzymatic mechanisms involve the activation of lipoxygenases, myeloperoxidases, cyclooxygenases, and cytochrome P450 (Niki, 2009). After the enzymatic removal of hydrogen from the double allylic-activated CH_2 group of PUFAs, oxygen is added, generating a peroxydienyl radical. This is then transformed into an anion and the reaction is terminated by back-transfer of the proton generated in the first reaction step, resulting in the formation of a lipid-hydroperoxide molecule (LOOH). Nonenzymatic mechanisms are mediated by free radicals, which can be indirectly generated by nicotinamide adenine dinucleotide phosphate oxidases and nitric oxide synthases (Niki, 2009). In turn, free radicals are able to remove hydrogen from a CH_2 group of PUFAs, resulting in the generation of LOOH and new dienyl radicals, which propagate this chain reaction. LOOHs that are generated by both reactions then decompose and during their degradation a great variety of secondary products such as MDA, 4-hydroxynonenal (4-HNE), and the remaining core aldehyde of oxidized phospholipids, such as 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine, are produced (Yin, Xu, & Porter, 2011). These end products of lipid peroxidation are highly reactive aldehydes, which can further propagate oxidative damage and are therefore considered to be downstream mediators of oxidative stress. Under normal conditions, lipid peroxidation is inhibited enzymatically by the activities of glutathione peroxidases, superoxide dismutases and catalases, or nonenzymatically by antioxidants, such as vitamin C and vitamin E (Niki, 2009). It has been extensively shown that an imbalance between oxidative stress and antioxidant mechanisms results in pathological oxidation (Finkel & Holbrook, 2000; Nathan & Cunningham-Bussel, 2013). Therefore, total levels of end products of lipid peroxidation, especially of free and adducted MDA, are widely used as indicators of oxidative stress in higher

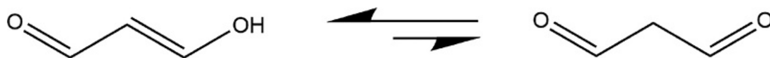


Fig. 1 Chemical structure of malondialdehyde. MDA is an aldehyde with the chemical formula $C_3H_4O_2$. At physiological pH, it exists mainly in its enol form (*left structure*). MDA has a molar mass of 72.06 g/mol, a density of 0.991 g/mL, and its melting point and boiling point are at 72°C and 108°C, respectively. It is produced by acid hydrolysis of 1,1,3,3-tetramethoxypropane at room temperature in vitro (Nair et al., 2001), and due to its high reactivity usually not observed in a pure form.

eukaryotic organisms and have been shown to correlate with the extent of tissue damage in acute injury and chronic diseases (Imai et al., 2008; Pamplona et al., 2005; Weismann et al., 2011; Yla-Herttuala et al., 1989).

Here, we will mainly focus on the biology of one of these reactive terminal degradation products: MDA. MDA (also known as Malonic aldehyde; Propanedial; 1,3-Propanedial) is an aldehyde with the chemical formula $C_3H_4O_2$ that at physiological pH mainly exists in its enol form (Nair, O'Neil, & Wang, 2001). The chemical structure of MDA is given in Fig. 1.

Under physiological conditions, newly generated MDA can modify free amino acids, proteins, nucleotides, and phospholipids creating stable covalent epitopes. Major in vivo carriers of MDA epitopes identified so far are apoptotic/necrotic cells (Amir et al., 2012; Chang et al., 1999, 2004; Chou et al., 2009; Weismann et al., 2011), microvesicles (MV) (Huber et al., 2002; Liu, Scalia, Mehta, & Williams, 2012; Tsiantoulas et al., 2015), and oxidized lipoprotein particles (Palinski et al., 1989; Shao, Tang, Heinecke, & Oram, 2010).

The main substrates for the generation of MDA in vivo are arachidonic acid (AA, 20:4) and docosahexaenoic acid (DHA, 22:6) present in membrane phospholipids. Already in the 1970s Pryor et al. (Pryor & Stanley, 1975) proposed possible mechanisms of MDA generation from these two PUFAs. He suggested the formation of intermediates that are bicycloendoperoxides, which are then broken down by thermal- or acid-catalyzed reactions giving rise to free MDA. Additionally, it was observed that certain amounts of MDA can be generated as a side product of thromboxane A2 synthesis. The enzymes cyclooxygenase 1 (COX-1) or COX-2 metabolize AA to prostaglandin H_2 (PGH_2), and newly formed PGH_2 is then metabolized by the thromboxane synthase, to thromboxane A2, 12-hydroxyheptadecatrienoic acid, and MDA (Hecker & Ullrich, 1989). Several reports suggested that MDA can also formed by oxidation of spermine (Quash et al., 1987), UV-irradiation of the skin surface lipid squalene (Dennis & Shibamoto, 1989), or by gamma irradiation of carbohydrates (Cheeseman, Beavis, & Esterbauer, 1988).

3. GENERATION OF MDA EPITOPES

Free MDA exists as a bifunctional electrophile and its reactivity is pH dependent. At physiological pH MDA is present in the enolate ion form. MDA reactivity can be increased by lowering the pH, which favors the formation of beta-hydroxyacrolein, thereby increasing its affinity toward nucleophilic molecules in the vicinity (Esterbauer, Schaur, & Zollner, 1991). In addition, it has been demonstrated that at high concentrations of MDA in an acidic milieu (pH range 4–7) long oligomers of MDA are formed, which results in hydrolytic cleavage of newly formed MDA oligomers, generating MDA and acetaldehyde (AcA) (Gomez-Sanchez, Hermosín, & Maya, 1990). This observation is very important, as AcA and MDA can react together and create a more complex form of immunogenic epitopes, malondialdehyde-acetaldehyde (MAA). Due to their chemical nature free AcA and MDA possess the ability to create epitopes on major cellular macromolecules in different tissues serving as mediators of oxidative stress (Fig. 2). Forming stable epitopes with biomolecules has been suggested to increase the half-life of MDA in vivo (Siu & Draper, 1982). Furthermore, MDA modifications of lipids, nucleotides, free amino acids, and proteins may result in their loss of function, loss of structural integrity, and may lead to altered cellular responses (Fogelman et al., 1980; Hyvarinen, Uchida, Varjosalo, Jokela, & Jokiranta, 2014; Wallberg, Bergquist, Achour, Breij, & Harris, 2007). Modifications of each type of macromolecules will be discussed separately.

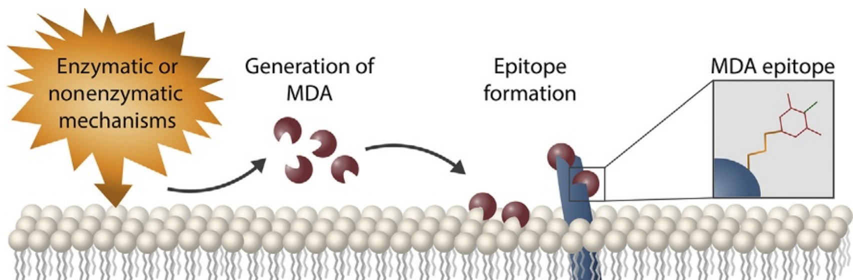


Fig. 2 Mechanism of MDA epitope formation. Lipid peroxidation of polyunsaturated fatty acids by enzymatic or nonenzymatic mechanisms results in the generation of highly reactive aldehydes such as MDA, acting as mediators of oxidative stress. Covalent attachment of MDA to amino groups present in, eg, Lys of biomolecules nearby results in the formation of MDA epitopes.

3.1 MDA Modification of Amino Acids and Proteins

MDA-modified biomolecules such as free amino acids or proteins have been identified in different tissues and body fluids in humans and animals in vivo (Akalin, Baltacioglu, Alver, & Karabulut, 2007; Gonenc, Ozkan, Torun, & Simsek, 2001; Imai et al., 2008; Pamplona et al., 2005; Weismann et al., 2011). Indeed, at neutral pH in the presence of free lysine (Lys) and arginine (Arg), MDA can hydrolyze and form epitopes on their side chains. Additionally, at lower pH MDA has also been shown to form epitopes on another group of amino acids, including glycine (Gly), leucine (Leu), valine (Val), histidine (His), arginine (Arg), tryptophan (Trp), tyrosine (Tyr), serine (Ser), and cysteine (Cys) (Esterbauer et al., 1991; Hadley & Draper, 1988; Nair et al., 2001). Proteins are typically modified by MDA on the ε-amino groups of their side chains forming various epitopes by Schiff base reaction (Slatter, Bolton, & Bailey, 2000; Watson, Preedy, & Zibadi, 2012). These include preferentially side chains of Lys, but modifications of His, Tyr, Arg, ethionine (Met), glutamine (Gln), and Cys have also been observed (Esterbauer et al., 1991; Gurbuz & Heinonen, 2015; Watson et al., 2012). Moreover, it has been demonstrated that both glycosylation and the acetylation of proteins render them more susceptible for MDA modification, possibly as a result of slight alterations in the tertiary structure of the protein that facilitate access for MDA (Mooradian, Lung, & Pinnas, 1996; Tuma, Thiele, Xu, Klassen, & Sorrell, 1996).

In human and animal models of disease MDA epitopes have been detected on proteins found in serum and urine, as well as on proteins of the extracellular matrix, and many intracellular ones (Akalin et al., 2007; Draper, Csallany, & Hadley, 2000; Gonenc et al., 2001; Imai et al., 2008; Weismann et al., 2011; Yla-Herttuala et al., 1989). Based on available literature we have generated an extensive list of endogenous proteins that had been reported to be modified by MDA, and these are presented in Table 2. In four different species, MDA modifications of a total of 107 proteins have been reported, of which five were found in at least two different organisms. These five proteins are ATP synthase (subunit β), NADH dehydrogenase Fe–S protein 2, cytochrome *b*–*c*1 complex (subunit 1), albumin, and actin. Interestingly, the majority of the proteins have mitochondrial origin, strengthening the notion that mitochondria, being major reservoirs of ROS, could be one site within the cell where MDA epitopes are preferentially generated.

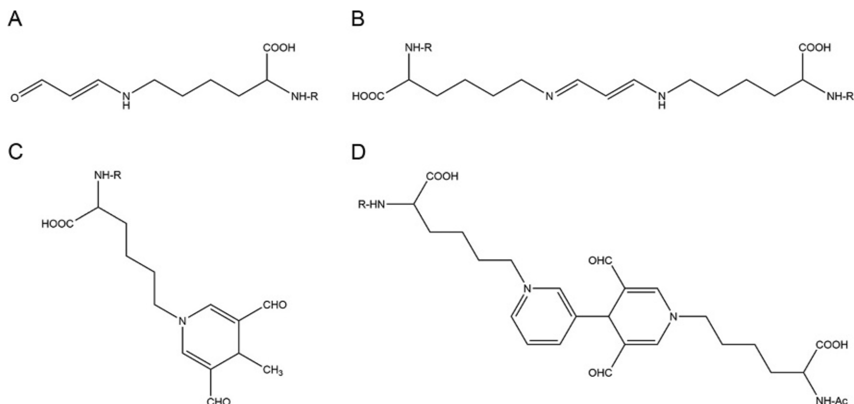


Fig. 3 Chemical structures of MDA epitopes found on Lys side chains of protein (Gonen et al., 2014; Uchida, 2000). *R*, protein backbone. (A) *N*-ε-(2-propenal)Lys. (B) 1-Amino-3-iminopropene. (C) 4-Methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative. (D) Pyridium–dihydropyridine.

Structurally different types of epitopes between MDA and primary amines have been reported, including fluorescent and nonfluorescent ones as well as cross-linking or noncross-linking ones. Important examples are presented in Fig. 3.

The most common type of adduct is *N*-ε-(2-propenal)lysine (Fig. 3A), which is a linear nonfluorescent adduct without the ability to generate cross-links and forms in the absence of AcA at pH 7 (Uchida, Sakai, Itakura, Osawa, & Toyokuni, 1997). It has been identified as the major form of endogenous MDA excreted in rat and human urine using ion exchange and high-performance liquid chromatography (Draper, McGirr, & Hadley, 1986; Piche, Cole, Hadley, van den Bergh, & Draper, 1988) and has been detected on in vitro MDA-modified keyhole limpet hemocyanin (KLH), MDA-modified LDL, and MDA-modified Apoprotein A-I (ApoAI), as well as copper-oxidized LDL (CuOx-LDL) (Shao et al., 2010). More complex MAA epitopes on ε-amino groups of Lys are formed by MDA and AcA in a 2:1 ratio (Tuma et al., 1996). The two major MAA epitopes are a 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative (MDHDC adduct) and a 2-formyl-3-(alkylamino) butanal derivative (FAAB adduct). In contrast to FAAB, MDHDC is a fluorescent adduct with $\lambda_{ex} = 395$ nm and $\lambda_{em} = 485$ nm. It requires the close proximity of two intermediates, including an FAAB adduct that is generated when one molecule of MDA and one of AcA react with free amino groups of a protein side

chain, and an MDA Schiff base (MDA-enamine) generated by the reaction of MDA with another free amino group. After the generation of these two intermediates, the FAAB moiety is transferred to the nitrogen of the MDA-enamine followed by ring closure yielding the MDHDC adduct (Fig. 3C) (Tuma et al., 2001). The formation of MDHDC epitopes has been demonstrated to be favored by acidic conditions (pH 4). Using NMR spectroscopy (Kearley, Patel, Chien, & Tuma, 1999) and performing chemical analysis (Xu et al., 1997) it has been shown that MAA epitopes are different from those epitopes formed when both aldehydes are allowed to react alone. Several lines of evidence suggest that this type of MAA epitopes possesses potent biological activity (Tuma et al., 1996; Uchida et al., 1997), although *N*-ε-(2-propenal) lysine epitopes have also been shown to be highly immunogenic (Gonen et al., 2014). Moreover, epitopes with the ability to form cross-links between different Lys residues in vitro have been found as well. These epitopes form cross-links within the same domain of a protein or domains in close proximity. In these reactions, one lysine residue forms a Schiff base with MDA, which is stabilized by equilibration to an enolate, and reacts then with a second Lys residue to form a diimine cross-link (Requena et al., 1996). Both simple MDA cross-links, such as 1-amino-3-iminopropenes (Fig. 3B), and more complex ones that are also fluorescent, such as pyridium–dihydropyridine epitopes have been described (Fig. 3D) (Itakura, Uchida, & Osawa, 1996). Stable cross-linking MDA epitopes were first demonstrated under semi-physiological conditions using NMR and EI-MS (Slatter, Murray, & Bailey, 1998). Such epitopes have been proposed to play a role by affecting structural proteins, such as collagen in the vessel wall where this could contribute to vascular stiffening and development of vasculopathies. In addition, in vitro MDA cross-links distant Lys residues of ApoAI (Shao et al., 2010) as well as purified ApoE3 and ApoE4 molecules (Montine et al., 1996), and cross-links apoprotein(a) to Apolipoprotein B100 (ApoB100) (Haberland, Fogelman, & Edwards, 1982). Finally, endogenous cross-linking MDA epitopes of proteins are formed on platelet proteins upon platelet activation and have been found to be increased in diseases associated with platelet activation, such as metabolic syndrome and sickle cell anemia (Zagol-Ikapite et al., 2015).

3.2 MDA Modification of Nucleotides and Nucleic Acids

Free MDA has been shown to form covalent epitopes with nucleotides, such as deoxy-guanosine, deoxy-adenosine, and deoxy-cytidine, in a

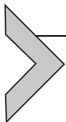
reaction that is enhanced in the acidic milieu. The major epitopes that can be formed by these reactions are pyrimido-[1,2-α]purin-10(3*H*)-one deoxyribose (M_1G), N6-(3-oxopropenyl)deoxyadenosine (M_1A), and N4-(3-oxopropenyl)deoxycytidine (M_1C) (Marnett, 1999). Of these, M_1G are five times more abundant than M_1A adducts in the liver, and the amounts of M_1C are generally very low (Chaudhary, Reddy, Blair, & Marnett, 1996). MDA is considered to be a potent mutagen (Esterbauer et al., 1991), and MDA modifications of nucleotide bases of DNA have been shown to induce genotoxicity by various mechanisms. MDA epitopes on nucleotides can cause template changes (Maddukuri et al., 2010; VanderVeen, Hashim, Shyr, & Marnett, 2003), result in the formation of interstrand cross-links (Niedernhofer, Daniels, Rouzer, Greene, & Marnett, 2003), or create DNA–protein cross-links (Voitkun & Zhitkovich, 1999). Interestingly, it has been shown that DNA isolated from human and rat liver exposed to enhanced lipid peroxidation contains increased amounts of MDA epitopes on DNA (Chaudhary et al., 1994). Nucleotide excision repair has been suggested to be the main mechanism of repair for M_1G-caused damage in DNA (Marnett, 1999). Moreover, mitochondrial DNA has been found to have a higher abundance of MDA-DNA epitopes compared to nuclear DNA. This increased amount of MDA epitopes in mitochondrial DNA is thought to be due to the lack of nucleotide excision repair in mitochondria (Cline, Lodeiro, Marnett, Cameron, & Arnold, 2010). Interestingly, increased levels of MDA-adducted nucleotides are found in the serum and urine of patients suffering from cancer as well as diseases associated with increased ROS production (Gungor et al., 2010; Munnia et al., 2006; Peluso et al., 2011).

3.3 MDA Modification of Phospholipids

In contrast to MDA modifications of proteins or nucleotides, only little data are available on MDA modification of phospholipids, which can be modified as well. Indeed, aminophospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE), have been found to form epitopes with MDA by enaminal derivatization. MDA-modified PS and PE have been identified in aging erythrocytes possibly as a consequence of peroxidative lipid damage (Gil, Farinas, Casado, & Lopez-Fernandez, 2002; Jain, 1988) and as metabolic waste products excreted in urine (Draper et al., 2000; Uchida et al., 1997).

3.4 Metabolism of MDA Epitopes In Vivo

Not much is known about metabolism of MDA epitopes in vivo. Marnett et al. injected intraperitoneally ^{14}C-labeled MDA into male and female Swiss Webster mice and found rapid and uniform distribution of labeled MDA throughout the body (Marnett, Buck, Tuttle, Basu, & Bull, 1985). Moreover, it was observed that conversion of MDA to CO_2 is completed in 4 h. In another study, urine samples obtained from rats challenged with oxidative stress by treatment with iron nitrilotriacetate or carbon tetrachloride or vitamin E deficiency were found to have MDA present in proteins, nucleic acid bases, and phospholipids as measured by HPLC (Draper et al., 2000). A similar excretion pattern, albeit in much smaller amounts, was observed in urine samples obtained from humans (Draper et al., 2000). Additionally, it has been shown that MDA levels in human urine can be modulated by diet, physical exercise, and smoking (Hadley, Visser, & Vander Steen, 2009; Mellor, Hamer, Smyth, Atkin, & Courts, 2010).



4. CARRIERS OF MDA EPITOPES

Tissue stress and subsequent cellular damage result in the generation of dying cells, the release of MV, and the accumulation of cellular debris. A number of studies have documented the presence of MDA epitopes on apoptotic cells and MV. For example, we and others could show that MDA-specific antibodies bind to serum-deprived apoptotic endothelial cells, dexamethasone-treated or UV-irradiated apoptotic T cells, as well as to necrotic cells and apoptotic blebs from retinal pigment epithelial cells (Amir et al., 2012; Aredo et al., 2015; Chang et al., 1999; Chou et al., 2009; Weismann et al., 2011) (see Table 1 for an overview). Furthermore, MDA epitopes have been found on the membranes of oxidized MV released from endothelial cells (Huber et al., 2002), and human monocytic cells treated with unesterified cholesterol (Liu et al., 2012), as well as on in vitro generated platelet-derived MV and on circulating MV collected from human blood (Gil et al., 2002; Tsiantoulas et al., 2015). In addition, MDA has been detected in aging erythrocytes, suggesting the presence of MDA epitopes in their membranes.

MDA epitopes have been identified on plasma lipoproteins and proteins. Among the lipoproteins, oxidized high-density lipoprotein (HDL) and

Table 1 Cellular and Subcellular Carriers of MDA Epitopes

Carrier Type	Induction	Method of Detection	References
Microvesicles (MV)			
Circulating MV		Flow cytometry	Tsiantoulas et al. (2015)
Platelet MV	Ionomycin	Flow cytometry	Tsiantoulas et al. (2015)
Endothelial MV	*tert*-Butyl hydroperoxide, Fe^{2+}	Flow cytometry	Huber et al. (2002)
Monocytic MV	Cholesterol	Flow cytometry	Liu et al. (2012)
Activated or dying cells			
Retinal pigment epithelial cells	Heat	Immunofluorescence	Weismann et al. (2011)
Thymocytes	PMA	Flow cytometry, immunofluorescence	Chou et al. (2009)
Jurkat T cells	UV	Flow cytometry	Amir et al. (2012)
Endothelial cells	Serum deprivation	Flow cytometry, immunofluorescence	Chang et al. (1999)
Thymocytes	Dexamethasone	Flow cytometry, immunofluorescence	Chang et al. (1999)
Jurkat T cells	UV	Flow cytometry, immunofluorescence	Tuominen et al. (2006)
THP-1 cells	Cholesterol	Immunofluorescence	Liu et al. (2012)
Erythrocytes	Aging	HPLC	Gil et al. (2002)
Platelet	Metabolic syndrome, sickle cell anemia	Mass spectrometry	Zagol-Ikapite et al. (2015)

low-density lipoprotein (LDL) eluted from the lesional tissues have been shown to carry MDA epitopes (Haberland, Fong, & Cheng, 1988; Palinski et al., 1989; Shao et al., 2010; Yla-Herttuala et al., 1989). In addition, other proteins modified by MDA have been detected in body fluids and tissues of different organisms listed in Table 2.

Table 2 List of Identified MDA-Modified Proteins

Organism	Tissue	Protein	NCBI (GI)	Detection Method	References
Homo sapiens	Retina	Tubulin, beta chain	5174739	2D gel electrophoresis, immunoblot with anti-MDA antibody	Schutt, Bergmann, Holz, and Kopitz (2003)
		Tubulin, alpha-1 chain	135395		
		ATP synthase beta chain	1145449		
		Ubiquinol-cytochrome *c* reductase core protein I	4507841		
		Pyruvate kinase, muscle	14750405		
		ATP synthase, alpha subunit	4757810		
		Enolase 1, alpha	4503571		
		S-arrestin	14737493		
		NADH dehydrogenase Fe–S protein 2	4758786		
		Guanine nucleotide-binding protein, beta polypeptide 2	4885283		
		Pyruvate dehydrogenase	2144337		
		Annexin A2	16306978		
		Glyceraldehyde-3-phosphate dehydrogenase	31645		

	Porin 31HM (anion channel 1)	238427		
	Voltage-dependent anion channel 2	4507881		
	H119n carbonic anhydrase Ii	2554664		
	Annexin V (Lipocortin V)	999937		
	Prohibitin	4505773		
	ATP synthase, subunit d	5453559		
	Calmodulin 2 (phosphorylase kinase, delta)	14250065		
	Cytochrome *c* oxidase subunit Va precursor	4758038		
	Peroxiredoxin 2	13631440		
	Crystallin, alpha A	4503055		
	Crystallin, alpha B	4503057		
Atherosclerotic lesion	ApoA-I	4557321	Detection with MDA2 antibody on lesional HDL	Shao et al. (2010)
Atherosclerotic lesion	Apo B-100	105990532	Western blot on lesional LDL	Palinski et al. (1989)

Continued

Table 2 List of Identified MDA-Modified Proteins—Cont'd

Organism	Tissue	Protein	NCBI (GI)	Detection Method	References
	Frontal cortex	Superoxide dismutase 2	119568015	2D gel electrophoresis/MS	Dalfo et al. (2005)
		α-Synuclein	80475099		
	Brain cortex	Neurofilament light polypeptide	62511894	2D gel electrophoresis/MS	Pamplona et al. (2005)
		Vimentin	55977767		
		Tubulin beta-2	55977480		
		Tubulin alpha-1B chain	55977471		
		Tubulin alpha-4A	55977476		
		Tubulin alpha-1C chain	20455322		
		Actin, cytoplasmic 1	46397316		
		Actin, cytoplasmic 2	54036665		
		Glial fibrillary acidic protein	121135		
		Gamma-enolase	20981682		
		Alpha-enolase	119339		
		Cytochrome *b*–*c*1 complex subunit 1	92090651		
		ATP synthase subunit beta	114549		
		Creatine kinase B-type	125294		

		Glutamine synthetase	1169929		
		Glutamate dehydrogenase 1	118541		
		Guanine nucleotide-binding protein G(I)/G(S)/G (T) subunit beta-1	51317300		
		60 kDa heat shock protein	129379		
		Dihydropyrimidinase-related protein 2	3122051		
	Plasma	**Albumin**	4502027	LC/MS, Western blot	Odhiambo et al. (2007)
	Aortic tissue	Elastin		Fluorescence of elastin fraction	Yamamoto et al. (2002)
Mus musculus	Broncho-alveolar lavage	Surfactant protein D	6677921	Immunopreciptation and Western blot	McCaskill et al. (2011)
	Blood	Hemoglobin		Sodium borohydrate reduction of MDA adducts	Kautiainen, Tornqvist, Svensson, and Osterman-Golkar (1989)
	Heart and skeletal muscle mitochondria	Aconitase	18079339	SDS-PAGE/MS of MDA positive bands	Yarian, Rebrin, and Sohal (2005)
		Acyl-coenzyme A dehydrogenase	23956084		
		Albumin	163310765		
		ATP synthase	31980648		
		α-ketoglutarate dehydrogenase	21313536		

Continued

Table 2 List of Identified MDA-Modified Proteins—Cont'd

Organism	Tissue	Protein	NCBI (GI)	Detection Method	References
	Kidney mitochondria	NADH dehydrogenase (ubiquinone) Fe–S protein 1	148667767	2D gel electrophoresis/MS	Choksi, Nuss, Boylston, Rabek, and Papaconstantinou (2007)
		ATP synthase subunit alpha	6680748		
		ATP synthase subunit beta	31980648		
		NADH dehydrogenase (ubiquinone) Fe–S protein 2	16359270		
		NADH dehydrogenase [ubiquinone] iron–sulfur protein 4	281485615		
		NADH dehydrogenase [ubiquinone] 1 subunit C2	18859597		
		Succinate dehydrogenase [ubiquinone] flavoprotein subunit	54607098		
		Gamma-glutamyltranspeptidase 1	807201132		
		Isocitrate dehydrogenase 2 (NADP+)	37748684		
		Catalase	157951741		

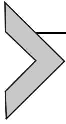
		Cytochrome *b*–*c*1 complex subunit 1	46593021		
		Cytochrome *b*–*c*1 complex subunit 2	22267442		
		Cytochrome *b*–*c*1 complex subunit Rieske	13385168		
		Peroxisomal acyl-coenzyme A oxidase 1 isoform 1	66793429		
		Long-chain-specific acyl-CoA dehydrogenase	31982520		
		Cytochrome *c* oxidase subunit II	34538601		
Rattus norvegicus	Limb and heart muscles	Elongation factor 2	8393296	Immunoprecipitation with anti-MDA antibody, EF2 western	Arguelles, Cano, Machado, and Ayala (2011)
	Gastrocnemius	Beta-enolase	122065177		Marin-Corral et al. (2010)
		Creatine kinase M-type	124056470		
		Carbonic anhydrase 3	5921194		
		Actin, alpha skeletal muscle	61217738		
		Tropomyosin alpha-1 chain	92090646		
		ATP synthase subunit beta, mitochondrial	114562		
	Tibialis anterior	Beta-enolase	122065177		

Continued

Table 2 List of Identified MDA-Modified Proteins—Cont'd

Organism	Tissue	Protein	NCBI (GI)	Detection Method	References
		Fructose biphosphate aldolase A	113609		
		Creatine kinase M-type	124056470		
		Actin, alpha skeletal muscle	61217738		
		Tropomyosin alpha-1 chain	92090646		
	Extensor digitorum longus	Beta-enolase	122065177		
		Fructose biphosphate aldolase A	113609		
		Creatine kinase M-type	124056470		
		Actin, alpha skeletal muscle	61217738		
		ATP synthase subunit beta, mitochondrial	114562		
	Soleus	Beta-enolase	122065177		
		Fructose biphosphate aldolase A	113609		
		Creatine kinase M-type	124056470		
		Carbonic anhydrase 3	5921194		
		Actin, alpha skeletal muscle	61217738		

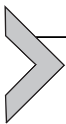
		Tropomyosin alpha-1 chain	92090646		
		ATP synthase subunit beta, mitochondrial	114562		
	Heart	Creatine kinase M-type	124056470		
		Actin, alpha cardiac muscle 1	54036667		
		Tropomyosin alpha-1 chain	92090646		
		Myosin-6	127741		
		Myosin light chain	127151		
		Vacuolar proton pump subunit C1	81882798		
		ATP synthase subunit beta, mitochondrial	114562		
		NADH-ubiquinone oxidoreductase 75 kDa subunit mitochondrial	53850628		
		Aldehyde dehydrogenase mitochondrial	14192933		
	Kidney	Type IV collagen	157821889	IHC, Western blot	Neale et al. (1994)
Oryctolagus cuniculus		Type II collagen	2190238	Western blot	Tiku, Allison, Naik, and Karry (2003)



5. TARGETS OF IMMUNE RESPONSE

As described earlier, MDA modifications of endogenous molecules are typically associated with structural changes and subsequently functional alterations of these macromolecules. Moreover, MDA modifications occur as a consequence of increased oxidative stress and therefore signify tissue damage. Thus, for the host to deal with potentially harmful effects of altered self-molecules it needs to recognize them and respond to their accumulation, eg, by mounting specific immune responses. Already in the 1980s Witztum and colleagues have shown that injection of guinea pigs with glucosylated autologous LDL or glucosylated autologous albumin resulted in the generation of antisera recognizing the glucosylation itself but not the proteins that were modified (Witztum, Steinbrecher, Fisher, & Kesaniemi, 1983).

In a follow-up study, specific antisera could also be generated with differently modified autologous LDL, including methylated, ethylated, acetylated, and carbamylated LDL (Steinbrecher, Fisher, Witztum, & Curtiss, 1984). These pioneering studies demonstrated that even the smallest modifications of autologous proteins can result in the haptenization of self-proteins leading to the formation of neo-self antigens that are recognized by the immune system. This concept has provided insights into the mechanisms by which oxidation of LDL renders it immunogenic, as this process generates several different OSEs that modify OxLDL particles and are recognized by the immune system in a hapten-specific manner. Many different OSEs have been characterized, including 4-HNE, phosphocholine (PC) of oxidized phospholipids, and MDA (Hartvigsen et al., 2009). There is now ample evidence that OSEs are recognized by both innate and adaptive immunity.



6. INNATE IMMUNE RESPONSES TOWARD OSEs

The innate immune system is instantaneously capable of recognizing a broad range of structures via pattern-recognition receptors (PRRs). Depending on whether the ligands originate from a foreign source (eg, microbes) or from within the host, they have been collectively named pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), respectively. Tissue-resident macrophages expressing PRRs on their surfaces are among the first cells of the innate

immune system to sense PAMPs or DAMPs and initiate an inflammatory response to ultimately remove pathogens or the triggering agent, respectively. Several classes of PAMPs, such as bacterial lipopolysaccharide (LPS), bacterial flagellin, lipoteichoic acid from Gram-positive bacteria, double-stranded RNA, unmethylated CpG motifs and DAMPs, such as high mobility group box 1 protein, heat shock proteins, ATP, and uric acid have been described (Chen & Nunez, 2010; Krysko et al., 2012). DAMPs are typically intracellular components that are secluded from the recognition by the immune system and can be released into extracellular environment upon cellular damage. Because DAMPs convey a message of danger to other cells employing specific cellular receptors and triggering innate immunity response, OSEs, including MDA, which fulfill these criteria can be considered a novel class of DAMPs (Fig. 4) (Miller et al., 2011).

Indeed, OSEs are recognized by PRRs present on macrophages, such as the heterogeneous family of scavenger receptors (SRs), which bind and internalize (scavenge) oxidized but not native LDL (Canton, Neculai, & Grinstein, 2013). Among SRs, CD36 and SR-A1 are the two most relevant receptors for the uptake of OxLDL, which leads to the formation of lipid-laden foam cells—a hallmark of atherosclerotic lesions. Importantly, some

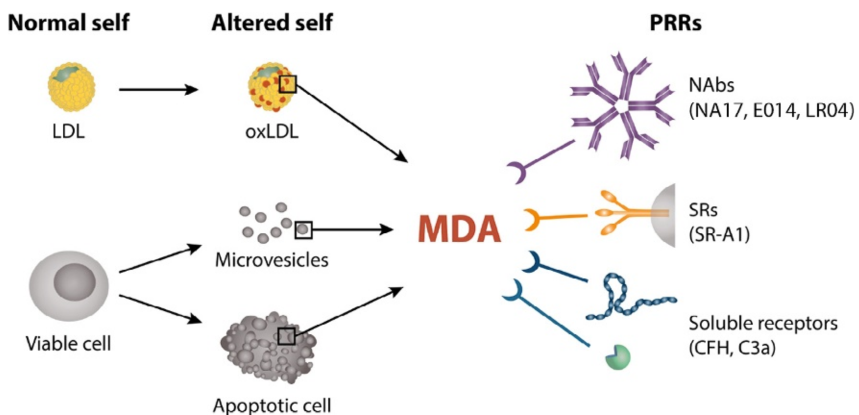


Fig. 4 Overview of MDA epitopes and their interaction with different arcs of innate immunity. MDA epitopes are DAMPs generated on biomolecules such as LDL in atherosclerotic lesions due to oxidative stress and on the surface of cells, cellular debris, or microvesicles during apoptosis and cell activation. Both soluble- and membrane-bound factors of the innate immune system bind to MDA epitopes presented on OxLDL or cell membranes including natural antibodies such as E014 and NA17, the scavenger receptor SR-A1 and two members of the complement system, CFH and C3a. Also other OSEs are recognized by innate immunity (not shown here).

OSEs are not merely taken up by SRs but can also serve as inflammatory mediators alarming the immune system about the damage inflicted by atherogenic lipoproteins. Another important PRR involved in clearing damaged lipoproteins are toll-like receptors (TLRs), which bind to a vast variety of different PAMPs and DAMPs, including OSEs. Certain TLRs have been shown to bind—as part of a multimeric complex—to oxidized phospholipids, OxLDL, and other OSEs, thereby mediating proinflammatory signals. For example, recognition of OxLDL by CD36 on macrophages enables the formation of a heterotrimeric complex consisting of CD36, TLR4, and TLR6, which mediates inflammatory responses resulting in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway activation and expression of chemokines including chemokine (C–X–C motif) ligand 1 (Cxcl1), Cxcl2, chemokine (C–C motif) ligand 5 (Ccl5), and Ccl9 (Stewart et al., 2010). Macrophages lacking any of the three receptors fail to respond to OxLDL stimulation, while TLR2 deficiency does not have any effect. TLR2, however, seems to play a role when macrophages subjected to endoplasmatic reticulum stress are stimulated with several oxidized phospholipids, saturated fatty acids, and OxLDL, which results in a TLR2/CD36-dependent induction of apoptosis (Seimon et al., 2010).

Thus, a number of PRRs recognize OSEs and act as sensors of oxidative stress. Upon ligation of OSEs with their corresponding PRRs, signal transduction events are initiated that culminate in sterile inflammatory responses characterized by chemokine secretion, leukocyte recruitment, or tissue repair. There is accumulating evidence that certain PRRs and pattern recognition proteins (PRPs) are also involved in sensing of MDA epitopes as DAMPs, and in the following section we will review published work both on the recognition by innate immunity and on the proinflammatory effects of MDA epitopes.

6.1 Cellular Receptors of MDA Epitopes

In line with initial studies showing that modified but not native LDL is recognized by macrophages via SRs (Goldstein, Ho, Basu, & Brown, 1979; Henriksen, Mahoney, & Steinberg, 1981), Shechter et al. demonstrated that both acetyl-LDL and fucoidin, a known SR-A1 ligand, inhibited the uptake of MDA-modified LDL (MDA-LDL) by human monocyte–macrophages (Shechter et al., 1981), indicating that MDA-LDL is recognized and degraded also via a SR-dependent mechanisms. Similarly, lipoprotein (a) (Lp(a)) is recognized by human monocyte–macrophages via SRs only

upon modification with MDA, as MDA-LDL, CuOx-LDL, and polyinosinic acid inhibited binding and degradation of MDA-Lp(a) (Haberland, Fless, Scanu, & Fogelman, 1992). In addition, binding of LDL isolated from rabbit atherosclerotic lesions to mouse peritoneal macrophages was competed for by MDA-LDL or polyinosinic acid, suggesting SR-A1-mediated uptake (Yla-Herttuala et al., 1989). In light of this, MDA epitopes present on altered self-molecules or on the surface of dying cells could be sensed and identified by SRs as waste markers and signals of disturbed homeostasis that are required to be scavenged, ie, removed from the system.

More recently, additional groups have confirmed that SR-A1 is involved in binding to MDA-modified proteins in a number of different cell types, including J774 cells (Willis, Klassen, Carlson, Brouse, & Thiele, 2004), bovine or human bronchial epithelial cells (Berger et al., 2014; Wyatt, Kharbanda, Tuma, Sisson, & Spurzem, 2005), murine bone marrow-derived macrophages (Wallberg et al., 2007), mouse peritoneal macrophages, and SR-A1-expressing human embryonic kidney (HEK) cells (Nikolic, Cholewa, Gass, Gong, & Post, 2007), corroborating prior data that SR-A1 is one but not necessarily the only receptor binding to MDA epitopes. Indeed, Zhu et al. accidentally found that CD16, an Fcγ receptor (FcγR) included in the study as a negative control, inhibited the binding of J774 cells to MDA-LDL-coated wells to a larger extent than soluble CD36, which was the original target of interest (Zhu et al., 2014). Similar to SRs, FcγRs have been shown to identify and eliminate microbial pathogens. Binding to the Fc portion of antibodies attached directly to pathogens or to the surface of infected cells allows enhanced uptake and clearance by cells equipped with an FcγR. Four groups of FcγRs exist in mice: FcγRI (CD64), FcγRIIB (CD32), FcγRIII (CD16), and FcγRIV (CD16-2) of which all but FcγRIIB are activating receptors containing an immunoreceptor tyrosine-based activation motif in their intracytoplasmic domain. In the study, the inhibition of MDA-LDL binding occurred in a dose-dependent manner and was competed for by immune complexes, known ligands of CD16. Of note, CD16 did not bind to OxLDL. The authors also observed decreased MDA-LDL and immune complex binding to macrophages after silencing or knocking out CD16, while OxLDL binding was unchanged between wild-type and $CD16^{-/-}$ macrophages. Lack of CD16 resulted also in reduced secretion of tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1), and CCL5 upon MDA-LDL stimulation, which was dependent on Syk phosphorylation. Together, the authors showed that CD16 might be

an additional receptor for MDA epitopes next to SR-A1, possibly implying that a redundancy of uptake and clearance mechanisms could be beneficial for an organism to protect from consequences of oxidative stress.

6.2 Proinflammatory Effects of MDA Epitopes

Inflammation is a physiological process caused by the body's own immune mechanisms that have evolved to sense and handle the undesirable presence of both altered endogenous and exogenous stimuli. Resolution of an inflammatory response can only occur when the inflammatory stimuli are cleared and no longer cause any harm. Impaired resolution and/or continuous generation of inflammatory agents might result in the establishment of a chronic inflammation accompanied by persistent tissue damage. Various studies have addressed the proinflammatory capacity of MDA-modified proteins in different cell types including primary cells and cell lines from different animal models. Stimulation of cells derived from rat heart or liver such as endothelial (Duryee et al., 2008; Hill et al., 1998; Thiele et al., 2004) and stellate cells (Kharbanda, Todero, Shubert, Sorrell, & Tuma, 2001) results in the release of TNF-α, MCP-1, macrophage inflammatory protein 2-alpha (MIP2α), and fibronectin or the expression of intercellular adhesion molecule 1 (ICAM-1), major histocompatibility complex (MHC) class II, and vascular cell adhesion molecule (VCAM-1), MCP-1, and MIP-2. In bovine bronchial epithelial cells, MDA epitopes induce interleukin (IL-8) and urokinase-type plasminogen activator (uPA) secretion via protein kinase C (PKC) activation (Kharbanda, Shubert, Wyatt, Sorrell, & Tuma, 2002; Wyatt, Kharbanda, Tuma, & Sisson, 2001). Indeed, several studies demonstrate that the proinflammatory effects induced by MDA epitopes involve not only the activation of PKC (Kharbanda et al., 2002; Wyatt et al., 2012, 2001) but also phosphoinositide 3-kinase (PI3-kinase), Src-kinase (Nikolic et al., 2007), PL-C/IP3 (Cai et al., 2009), and NF-κB (Raghavan, Subramaniyam, & Shanmugam, 2012; Shanmugam et al., 2008) have been implicated. Interestingly, in analogy to cholesterol crystals, MDA epitopes were shown to induce lysosomal rupture upon uptake (Willis et al., 2004) when stimulating J774 macrophages with MDA-modified hen egg lysozyme (MDA-HEL). The authors found that binding of MDA-HEL to the cells occurs in a SR-A1-dependent manner and results in lysosomal rupture followed by cell death. Other studies have shown that treatment of a human hepatic stellate cell line with MDA-modified horse-serum albumin induces activation of ERK1/2 and NF-κB (Kwon et al., 2014). The same

authors also demonstrated that murine Kupffer cells stimulated with MAA-modified human serum albumin secrete IL-6 after 2 h in the presence of LPS, building on an earlier observation that MAA-BSA-stimulated Kupffer cells exhibited a synergistically enhanced cytokine response in the presence of LPS (Duryee et al., 2004). In contrast, no synergistic effect of the TLR2 ligand Pam3CSK4 with MDA-BSA in macrophages could be observed (Saeed et al., 2014).

Additional validation of the proinflammatory capacity of MDA epitopes in vitro is provided in an unbiased approach, where Shanmugam et al. (2008) analyzed conditioned media of THP-1 cells treated with MDA-modified Lys using hybridization-based cytokine arrays. MDA-Lys was found to increase the levels of 20 cytokines, including CCL11, CCL18, CCL28, tumor necrosis factor superfamily member 14 (TNFSF14), MCP-1 and MCP-2. Further in silico analysis of the networks involved using a pathway analysis software revealed that treatment with MDA-Lys induces targets involved in biological processes such as immune response, cellular movement, and cell-to-cell signaling. Using reporter gene assays or chemical inhibitors, the authors showed that MDA-Lys induced activation of NF-κB and p38 MAPK pathways. Furthermore, they observed that incubation of monocytes with MDA-Lys increases their adhesion to smooth muscle or endothelial cells. Together, they demonstrated that MDA-Lys impacts inflammatory gene expression, activation, and motility of monocytes. This was also observed in a separate study using Jurkat T cells stimulated with free MDA (Raghavan et al., 2012).

Next to the expression of cytokines and adhesion molecules, several groups have also investigated motility, morphological changes, and effects on cell viability caused by MDA epitopes. For example, MDA-BSA-coated plates incubated with SR-A1-expressing HEK cells results a spread morphology within 10 min, accompanied by an increase in cell surface area and the formation of membrane extensions after 2 h in a PI3-kinase- and Src-kinase-dependent manner (Nikolic et al., 2007). Morphological changes were also observed in rat heart endothelial cells stimulated with MAA-BSA (Hill et al., 1998) or in J774 macrophages (Willis et al., 2004) and in some cases these morphological alterations are followed by detachment and cell death (Thiele et al., 2004; Willis et al., 2004; Willis, Klassen, Tuma, & Thiele, 2002). In contrast, MDA-LDL only weakly induced the expression of collagen I, collagen III, and fibronectin in human hepatic stellate cells (Schneiderhan et al., 2001), while the cell morphology was found to be unchanged. Also THP-1 cells stimulated with MDA-LDL

displayed even increased cell proliferation in one study (Suzuki, Sasaki, Kumagai, Sakaguchi, & Nagata, 2010), and other researchers found that free MDA induces migration in peripheral blood mononuclear cells and BMDMs after 1 h of incubation (Geiger-Maor et al., 2012), while rat neurons were damaged by free MDA via induction of Ca^{2+} influx (Cai et al., 2009) or via a MAPK pathway in a dose- and time-dependent manner (Cheng, Wang, Yu, Wu, & Chen, 2011). These apparently inconsistent results may be due to the different cell types used in these studies. Nevertheless, there is little doubt about the proinflammatory properties of MDA epitopes in vitro. However, there are only few studies investigating the in vivo effect of MDA epitopes. One study by us showed that MAA-BSA induces a proinflammatory response in the retinal pigment epithelium of mice upon intravitreal injection characterized by a robust increase in KC expression (Weismann et al., 2011). In another murine model of lung injury, MAA-BSA or MAA-modified surfactant D (SPD) was instilled into the lungs of wild-type mice upon which elevated levels of neutrophils and KC were detected in the lung lavage fluid (Wyatt et al., 2012). Even though the amount of in vivo data is scarce, it clearly corroborates the in vitro data demonstrating that MDA epitopes are DAMPs capable of alerting the immune system of high oxidative stress, inflammation, and potential tissue damage by inducing secretion of cytokines.

6.3 Innate Recognition by IgM Natural Antibodies and Complement

Natural antibodies (NAbs) are predominantly of the IgM class and constitute an important component of humoral innate immunity. In addition to their key role in the first line defense against microbial infections, NAbs also play a vital role in the removal of apoptotic cells and metabolic waste to maintain tissue homeostasis (Manson, Mauri, & Ehrenstein, 2005). They are naturally occurring antibodies that can bind microbial structures and stress-induced self-antigens through an evolutionary conserved repertoire of variable regions made out of nonmutated germline genes. In mice, IgM NAbs are secreted by B-1 cells, which constitute a subset of primordial B cells with different surface marker expression, activation requirements, and a distinct anatomical location (Baumgarth, 2011). NAbs are present at birth and detectable already at the fetal stage. They can be found in germ-free mice, indicating that their occurrence is independent of external antigenic stimuli. Nevertheless, their titers can be enhanced by positive antigenic stimulation (Baumgarth, 2011; Baumgarth, Tung, & Herzenberg, 2005).

First insights into the concept that IgM NAbs bind OSEs came from studies in which spleens of 9 months old Apolipoprotein E (Apoe)$^{-/-}$ mice fed a high fat high cholesterol diet for 7 months, which exhibit high titers of OSE-specific IgM, were used to generate hybridoma cell lines (Palinski et al., 1996). About 30% of the hybridoma cell lines producing IgM antibodies were recognizing epitopes of OxLDL. Interestingly, a large part of the clones identified were found to have specificity for MDA-LDL. Detailed characterization of one of the clones termed E06 that specifically binds to PC of oxidized phospholipids of OxLDL demonstrated its CDR3 region to be encoded by the canonical rearrangement of the identical V-D-J germ line genes that encode a previously characterized IgA NAb, called T15 (Shaw et al., 2000). This suggested that many more OSE-specific IgM could be NAbs. Indeed, we could subsequently show that plasma of mice that were kept under complete germ-free conditions contained IgM antibodies with specificity for several OSEs, including MDA-type epitopes (Chou et al., 2009), demonstrating their natural occurrence in the absence of exposure to foreign antigens. Notably, colonization of the gut of these mice with microbiota of conventionally housed mice resulted in the expansion of some, but not all, OSE-specific IgM. The latter is consistent with the fact that many IgM NAbs possess dual reactivity for microbial and self-antigens (Baumgarth, 2011; Racine & Winslow, 2009). Moreover, we could also demonstrate that IgM antibodies in human umbilical cord blood of newborns contained high titers of IgM with specificity for OxLDL and MDA-LDL. In contrast to IgG, IgM are not transported across the placenta. Therefore, IgM in umbilical cord blood are exclusively of fetal origin and therefore can be regarded as human NAbs. Interestingly, compared to the matched maternal plasma of the newborns, the IgM titers to OxLDL and MDA-LDL were significantly enriched in the umbilical cord plasma. Key evidence for the existence of OSE specific and in particular MDA-specific IgM NAbs came from characterization of the binding specificity of IgM derived from murine B-1 cells, isolated from the peritoneal cavities of wild-type mice. Both supernatants of purified B-1 cells stimulated with LPS or IL-5 as well as plasma of recombination-activating gene 1-deficient mice (Rag1$^{-/-}$) (that do not have any functional B or T cells) reconstituted with purified B-1 cells contained IgM with specificity for MDA epitopes. Importantly, ELISpot studies for the frequencies of IgM-secreting cells in the spleens and antigen absorption studies of the plasma IgM of B-1 cell reconstituted mice revealed that ca. 15% of all B-1 cell-derived IgM NAbs have

specificity for MDA epitopes, as well as more complex MAA epitopes, which constitute the majority of OSE-specific IgM NAbs that represent 30% of the NAb repertoire. Finally, sequence analyses of the variable region of a newly cloned anti-MDA IgM mAb, termed NA17, derived from the spleens of these mice, did not reveal nucleotide variation to germline genes in the V_H rearrangements, and only 1 nucleotide insertion between V_L and J_L germline gene segments (Chou et al., 2009). Similarly, previously cloned anti-MDA IgM NAbs (E014, LR04) have also been found to express unmutated germline variable genes (Lichtman, Binder, Tsimikas, & Witztum, 2013). Thus, several lines of evidence identify MDA epitopes as major targets of IgM NAbs.

The high prevalence of MDA-specific IgM NAbs is consistent with an important role in homeostatic housekeeping functions. Indeed, as described earlier, apoptotic cells as well as MV carry MDA epitopes and are recognized by MDA-specific IgM. NAbs have been shown to play a critical role in the clearance of apoptotic cells via complement-dependent mechanisms (Chen, Park, Patel, & Silverman, 2009; Ogden, Kowalewski, Peng, Montenegro, & Elkon, 2005). We could also demonstrate that the MDA-specific IgM NAb NA17 binds apoptotic thymocytes but not viable cells. Moreover, when injected together with apoptotic thymocytes into the peritoneal cavity of mice, NA17 enhances their uptake by peritoneal macrophages in vivo (Chou et al., 2009). It has been suggested that MDA epitopes exposed on apoptotic cells guide the immunosilent and antiinflammatory clearance of apoptotic cells by recruitment of MDA-specific IgMs and corecruitment of C1q (Chen et al., 2009). Thus, IgM NAbs with specificity for MDA have the capacity to engage certain functions of the complement system for host homeostasis.

The complement system represents another humoral component of innate immunity. It is a complex system consisting of about 40 different proteins organized in three major pathways: the classical, the lectin, and the alternative pathway (Ricklin, Hajishengallis, Yang, & Lambris, 2010). Major functions of complement involve the protection of the host from invading pathogens, the orchestration of innate and adaptive immune responses, and housekeeping functions in promoting clearance of apoptotic cells. Immune complexes deposited on pathogenic surfaces initiate the classical pathway, a range of different carbohydrates exposed on pathogens initiate the lectin pathway, and the alternative pathway has a low grade of constitutive activation. Although each complement pathway has a unique way of initiation, they all converge at the step of C3 cleavage, a central component of the

complement, by C3 convertases into a smaller C3a and a larger C3b fragment. Downstream of C3, all pathways are merged into one resulting in the generation of the terminal complement complex (TCC). Clearly, the proteolytic cascade of complement needs to be tightly controlled at several levels to prevent deleterious consequences for the host, which is achieved by a group of proteins called regulators of complement activity (RCA). RCA family members are divided into two major subgroups, soluble ones, such as complement factor H (CFH) and C4-binding protein (C4BP), and membrane-bound ones, including CD46, CD55, CR1, CR2, CR3, and CR4 (Zipfel & Skerka, 2009).

There is now increasing evidence that MDA epitopes also directly trigger specific aspects of complement. Using an unbiased approach to identify MDA-reactive plasma proteins in pull-down assays using MDA-modified polystyrene beads and plasma from $Rag^{-/-}Ldlr^{-/-}$ mice, we identified CFH as a major binding protein for MDA epitopes (Weismann et al., 2011). CFH is a glycoprotein in plasma with a concentration of 500 μg/mL and acts as major regulator of the alternative complement pathway. CFH plays an important complement inhibitory role both in solution and attached to host cell surfaces. CFH regulates complement in three different ways: it disables the formation of the C3 convertase, facilitates its decay, and enhances proteolytic cleavage of C3b to generate inactive iC3b. Employing different ELISAs, we showed that CFH binds to MDA epitopes directly and independently of their protein carrier, but not to PC or 4-HNE epitopes (Weismann et al., 2011). Furthermore, this binding seems to require the presence of advanced MAA epitopes such as MDHDC and is Ca^{2+}- and Mg^{2+}-independent. When CFH is bound to MAA-modified BSA, its complement regulatory activity in factor-I-mediated C3 cleavage is retained, as demonstrated by the fact that MAA-bound CFH had the capacity to cleave C3b into iC3b in the presence of factor I. This is important as opsonization of apoptotic cells with iC3b has been shown to mediate their antiinflammatory clearance by macrophages, and we have demonstrated that binding of CFH to dying cells and blebs is in part mediated by MDA epitopes. CFH is a multidomain molecule composed of 20 domains named short consensus repeats (SCRs) arranged in a "beads on the string" fashion. Using recombinant constructs of CFH, in which different numbers of SCRs had been deleted, we could demonstrate that only SCR7 and SCR19/20 were able to mediate binding of CFH to MAA epitopes. Notably, SCR7 and SCR20 have been described as clustering sites for mutations in CFH that are associated with many complement-related diseases, such as

age-related macular degeneration (AMD), atypical hemolytic uremic syndrome (aHUS), and C3 glomerulopathies (Ricklin & Lambris, 2013). In this regard, we could demonstrate that CFH of carriers of the common CFH SNP rs1061170 that results in an H to Y exchange on position 402 (H402Y variant) exhibits severely impaired binding to MAA in a gene-dosage-dependent manner with >60% decreased binding for homozygous carriers compared to homozygous controls. These findings provided important insights into the strong association of rs1061170 with the risk to develop AMD—a chronic inflammatory disease of the retina that is associated with increased oxidative stress and the most common cause of blindness in the elderly. One may speculate that binding of MAA on apoptotic and necrotic cells by CFH may allow it to inhibit proinflammatory effects of MAA and regulate complement activation on these surfaces. Indeed, we could show that CFH at physiological concentrations has the capacity to inhibit MAA-induced IL-8 secretion by the human monocytic THP-1 cell line. Moreover, the induction of KC expression in the retinal pigment epithelium of mice injected intravitreally with MAA-BSA was completely abolished when CFH was coinjected. Thus, CFH has been shown to scavenge proinflammatory properties of MAA in vitro and in vivo, and to mediate important cofactor activity on MAA-carrying surfaces. Both properties would favor an antiinflammatory removal of potentially harmful dying cells. Recently, Aredo et al. generated transgenic CFH mice carrying human CFH sequence for SCR6–8 (with either 402Y or 402H), flanked by the mouse sequence for SCR1–5 and SCR9–20 (Aredo et al., 2015). Aged mice transgenic for the human CFH H402 variant, in contrast to wild-type mice, had increased accumulation of MDA-modified proteins, increased microglial/macrophage activation, and induction of proinflammatory gene expression in the retina. Similar results were observed when 11 months old mice of both groups where subjected to another model of chronic oxidative stress using hydroquinone diet and exposure to white fluorescent light for 8 weeks (Aredo et al., 2015). These findings support the notion that CFH binding to MDA may play an important role in AMD pathogenesis by modulating the consequences of oxidative stress and inflammation in the retinal pigment epithelium.

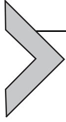
Further insights into the potential importance of CFH binding to MDA were provided by studies investigating the effect of SNPs in SCR19–20 of CFH that predispose to the development of aHUS (Hyvarinen et al., 2014). This disease is characterized by the development of microangiopathic hemolytic anemia and thrombocytopenia leading to renal failure

(Nester et al., 2015). In this study, recombinant CFH SCR19–20 constructs harboring various SNPs were tested for their ability to bind MDA-BSA. Eight out of 12 SNPs were found to alter binding to MDA-BSA. The authors suggested that impaired MDA binding observed in these CFH variants may be involved in pathogenesis of aHUS. In addition, CFH SCR19–20 fragments were found to bind to MDHDC epitopes as it has been shown for full-length CFH and SCR7-containing fragments (Hyvarinen et al., 2014; Weismann et al., 2011). Furthermore, the authors suggested that binding of SCR6–8 to MDA-BSA has a different nature of interaction, in contrast to the binding of SCR19–20, as the SCR6–8 could not be inhibited with increasing concentrations of NaCl (Hyvarinen et al., 2014). Although CFH is a single chain molecule with SCRs organized in a "beads on the string" fashion, structural analyses suggest that when CFH is bound to, eg, C3b on cellular surface it is bent, and SCR4 and SCR9 are brought together in close proximity (Kajander et al., 2011; Wu et al., 2009). It is not known how the 3D structure of CFH looks when it is bound to MDA, but it can be speculated that both domains (SCR7 and SCR19/20) cooperate in mediating binding to MDA, although both SCRs may bind to MDA epitopes in a different manner. The binding affinity of CFH to MAA-BSA, which actually represents the avidity of the whole molecule, was found to be ~62 nM (Weismann et al., 2011).

In addition to SCR-containing complement proteins, it has also been shown that recombinant human C3a, a proinflammatory anaphylatoxin, binds specifically to MDA- and MAA-modified LDL or BSA (Veneskoski et al., 2011). The same authors also showed that MAA- and MDA-modified LDL comigrated with C3a in gel shift assays and facilitated scavenging of C3a by J744A1 macrophages. Using ELISA, they demonstrated that C3a was binding to MDA- and MAA-modified proteins (HDL and LDL) but not to PC-modified proteins, suggesting that MAA/MDA epitopes on OxLDL mediate binding to C3a. Additionally, we also tested the binding of plasma purified C3 to MAA-BSA by ELISA, but could not observe any binding. These data suggest that only upon cleavage of C3, the C3a fragment can bind to MDA (Weismann et al., 2011).

Thus, certain complement regulators and complement effectors bind to MDA and MAA epitopes in particular, which are present on OxLDL, apoptotic cells, and MV (Veneskoski et al., 2011; Weismann et al., 2011). While CFH has been shown to limit MAA-induced inflammation, future studies need to explore the effects of MAA-decorated surfaces on complement activation in general. Because MDA/MAA epitopes are major targets of IgM

NAbs as well as CFH, they may represent important hubs on dying cells and oxidized lipoproteins allowing complement to mediate critical housekeeping functions to maintain host homeostasis. The fact that MDA/MAA epitopes are recognized by membrane PRR, such as SR-A1, as well as soluble PRPs, such as CFH and IgM NAbs, identifies them as potent regulators of tissue homeostasis and as novel DAMPs of innate immunity (Fig. 4).

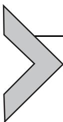


7. ADAPTIVE IMMUNITY RESPONSES ON MDA EPITOPES

In contrast to innate immunity, adaptive immunity is acquired throughout life following exposure to specific antigens. This results in the generation of a nearly unlimited repertoire of receptors with high antigen specificity and the generation of immune memory, which is carried out by various subsets of CD4+ Th and CD8+ Tc as well as B cells and the antibodies they secrete (Lichtman et al., 2013). Different subsets of Th cells, including IFNγ- and TNF-α-secreting Th1 cells; IL-4, IL-5, IL-10, and IL-13-secreting Th2 cells; and IL-17-secreting Th17 cells, have been described.

A multitude of studies exists demonstrating that active immunization with MDA-modified proteins or lipoproteins triggers adaptive immune responses. These characterizations were mainly done in atherosclerosis-prone rabbits and mice. Moreover, adaptive IgG antibodies with specificity for MDA have been documented in humans and several animal models of atherosclerosis as well as models of ethanol-induced chronic inflammatory tissue injury (Tuma et al., 1996; Xu et al., 1997). Based on this, Witztum and colleagues initiated studies to demonstrate the possibility to induce hapten-specific immune response against MDA epitopes. Both antisera (MAL-2) and monoclonal IgG antibodies (MDA2) could be generated in guinea pigs and mice immunized with MDA-modified homologous LDL (Palinski et al., 1989, 1990). These data suggested that the robust production of hapten-specific IgG antibodies occurs in a T cell-dependent manner. We could show that immunization of mice with homologous MDA-LDL induced the robust production of T cell-dependent IgG antibody titers with specificity for MDA epitopes. Moreover, this immunization in which MDA-LDL was emulsified in complete Freund's adjuvant for the primary and incomplete Freund's adjuvant for the boosting injections resulted in a preferential production of Th2-dependent IgG1, whereas Th1-dependent IgG2a/c antibodies were only moderately induced. Antigen stimulation assays of splenocytes of immunized mice led to a secretion of primarily

IL-5, IL-10, IL-13 and to a much lower degree IFNγ following stimulation with MDA-LDL, but not native LDL, consistent with a Th2-biased immune response (Binder et al., 2004; Gonen et al., 2014). The studies also demonstrated the induction of T cells specific for MDA epitopes, which likely are recognized by T cells in the context of a specific peptide sequence. Indeed, Wuttge et al. showed that subcutaneous immunization of nude mice that lack T cells with MDA-modified autologous albumin fail to mount specific IgG responses (Wuttge, Bruzelius, & Stemme, 1999). We have confirmed the requirement of T cells for IgG responses triggered by MDA-LDL, as immunization of MHC class $II^{-/-}$ or T-cell receptor $\beta^{-/-}$ mice with MDA-LDL failed to induce robust IgG antibody titers (Binder et al., 2004). In addition, immunization of $CD4^{-/-}$ $Apoe^{-/-}$ mice with MDA-LDL results in reduced IgG1, IgG2a/c, and IgM antibody titers to MDA-LDL (Zhou, Robertson, Rudling, Parini, & Hansson, 2005). Notably, the remarkable Th2 dominated immune response to MDA-LDL induced by immunization occurs independently of the carrier, and can be observed also when other proteins, eg, mouse serum albumin, modified with other adducts, such as MAA and propanal are used as antigens (Gonen et al., 2014). Nevertheless, MAA-modified proteins have been found to induce the strongest antibody responses. The strong immunogenicity of MAA epitopes in particular has been demonstrated before in several immunization studies of various organisms (bovine, human, rabbit, and mice), as immunization with MAA-modified homologous albumin induced specific antibodies even in the absence of any adjuvant (Thiele et al., 1998). Of considerable interest is the fact that immunization of $SR\text{-}A1^{-/-}$ mice with MAA-BSA resulted in a reduced generation of anti-MAA-BSA antibodies compared to control wild type, suggesting that SR-A1-mediated uptake of MDA-modified proteins by antigen-presenting cells is critical (Duryee et al., 2005). Thus, both endogenous and exogenous MDA/MAA haptens have the capacity to induce robust adaptive immune responses, which are characterized by antibody production and induction of specific Th cells.



8. MDA EPITOPES IN DISEASES

There is a growing list of diseases in which increased levels of MDA have been detected using various methods (Bhuyan, Bhuyan, & Podos, 1986; Brown & Kelly, 1994; Dei et al., 2002; Dexter et al., 1989; Gonenc et al., 2001; Grigolo, Roseti, Fiorini, & Facchini, 2003; Haider

et al., 2011; Imai et al., 2008; Jain, McVie, Duett, & Herbst, 1989; Odhiambo et al., 2007; Pemberton et al., 2004; Schoenberg et al., 1995; Shimizu et al., 2001; Sikar Akturk et al., 2012; Tuma et al., 1996; Valles, Aznar, Santos, & Fernandez, 1982; Wade, Jackson, Highton, & van Rij, 1987; Weismann et al., 2011; Yla-Herttuala et al., 1989). MDA has been documented in both chronic and acute diseases associated with high levels of oxidative stress, such as cardiovascular, neurodegenerative, metabolic, and communicable diseases (Fig. 5). However, the levels of MDA determined in the plasma of healthy individuals have shown great variability, in part due to the method of blood drawing and sample preparation (Del Rio, Stewart, & Pellegrini, 2005). Moreover, the detection methods for MDA still possess a number of limitations. For example, the most commonly used method for MDA detection, the thiobarbituric acid reactive substances assay, has long been known to be nonspecific (Esterbauer et al., 1991). Although more reliable methods for detecting MDA levels with high specificity exist including MDA-specific antibodies or assays based on mass spectrometry (Zagol-Ikapite et al., 2015), it still remains unclear if the detected changes of MDA are reliable biomarkers for all these diseases.

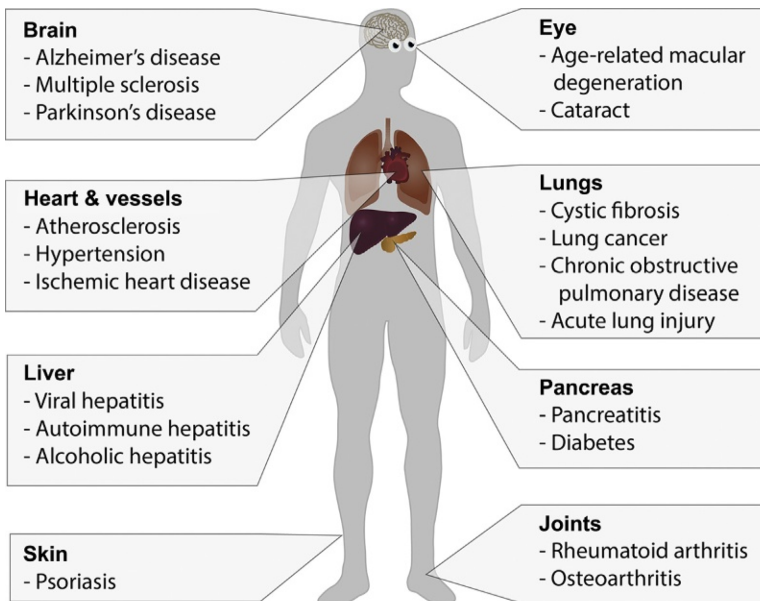
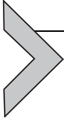


Fig. 5 MDA epitopes in diseases. MDA epitopes can be found in many pathological settings affecting multiple organs including neurodegenerative diseases, metabolic diseases, cancers, and also infectious diseases.



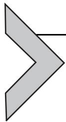
9. RELEVANCE OF OSEs IN CARDIOVASCULAR DISEASE

CVDs are among the most-studied diseases with respect to a role for MDA epitopes. CVDs constitute the major cause of mortality and disability worldwide (Mendis et al., 2011). Generally, CVDs can be divided into two major subgroups, CVDs that arise due to atherosclerosis (stroke, heart attack, hypertension, and peripheral vascular disease) or CVDs that are independent of atherosclerotic process (congenital and rheumatic heart diseases, cardiomyopathies, and arrhythmias). According to the WHO, CVDs with atherogenic origin are responsible for 80% of CVD deaths.

Atherosclerosis is a chronic inflammatory disease of large- and medium-sized arteries and the underlying cause for heart attacks and strokes. Several nonmodifiable and modifiable risk factors, such as hypercholesterolemia, hypertension, diabetes, smoking, gender, and age have been identified. Atherosclerotic lesion formation is sustained by high levels of plasma LDL cholesterol that are deposited in the artery wall, where an inflammatory reaction is triggered. These initial fatty streak lesions can progress to more complex plaques with many inflammatory infiltrates and large acellular necrotic areas. Once these advanced plaques become unstable they are prone to rupture, which triggers a local thrombotic event that can ultimately lead to a heart attack or stroke.

LDL belongs to a group of lipoprotein particles ranging in size from 18 to 25 nm with the major purpose to transport cholesterol in the circulation. It contains a single ApoB100 molecule (with 4536 amino acids, of which 356 are Lys) and a lipid part consisting of phospholipid and cholesterol molecules. Atherogenesis is initiated by endothelial cell dysfunction and intimal retention of LDL. Once LDL is trapped in the subendothelial space of the arteries it undergoes several types of modifications, most prominently enzymatic and nonenzymatic oxidation leading to the generation of OxLDL. OxLDL itself triggers proinflammatory responses in endothelial cells and enhanced recruitment of monocytes into the intima of the artery wall (Gerrity, 1981; Quinn, Parthasarathy, Fong, & Steinberg, 1987). Recruited monocytes then differentiate into macrophages that further propagate the oxidation of LDL through 12/15-lipoxygenase (12/15-LO) and myeloperoxidase (MPO) pathway, thus enhancing the proatherogenic inflammatory cascade. OxLDL is then taken up by macrophages using an array of SRs expressed on their surface, such as CD36 and SR-A1 (Greaves & Gordon, 2009; Moore, Sheedy, & Fisher, 2013). An impaired

balance between cellular uptake and efflux of cholesterol results in the transformation of lesional macrophages into lipid-laden foam cells—hallmark cells of atherosclerosis. Consequently, intracellular accumulation of OxLDL results in the formation of cholesterol crystals that trigger lysosomal rupture and activation of the inflammasome. In addition, intracellular accumulation of free cholesterol can enhance ER stress and promote an unfolded protein response that triggers apoptosis foam cell macrophages (Feng et al., 2003). Usually, lesional apoptotic cells are silently cleared by professional phagocytes, but once the efferocytotic mechanism becomes overloaded either with a multitude of apoptotic cells or excess of OxLDL this emanates in impaired clearance of apoptotic cells. Uncleared apoptotic cells then undergo secondary necrosis, lose membrane integrity, and release cellular debris and DAMPs, further propagating the inflammatory response. Thus, OxLDL, apoptotic cells, and cellular debris accumulate in atherosclerotic lesions and sustain the inflammatory process (Glass & Witztum, 2001; Hartvigsen et al., 2009; Tabas, 2010; Tsiantoulas, Diehl, Witztum, & Binder, 2014). Moreover, in addition to innate immune responses adaptive immunity also plays a role in atherogenesis (Lichtman et al., 2013). T cells—most prominently IFNγ-secreting Th1 cells—have been found in lesions and were demonstrated to promote atherogenesis. Although B cells are rarely found in lesions, their modulatory roles on atherogenic process have recently gained much attention.



10. MDA EPITOPES IN ATHEROSCLEROSIS

During the oxidative modification of LDL, the phospholipid and cholesterol moiety of LDL undergoes lipid peroxidation, which leads to the generation of many different OSEs, including MDA epitopes. OxLDL has been shown to be proinflammatory and immunogenic, suggesting that it is a major driver of the inflammatory process during atherogenesis. Indeed, the characterization of OSEs as novel class of DAMPs has provided important insights into the sterile inflammatory process of atherosclerosis. Accumulating evidence suggests that MDA epitopes represent critical mediators of inflammation in atherosclerosis.

The fact that atherosclerotic lesions contain MDA epitopes has been known for more than 30 years. Using a monoclonal antibody raised against MDA-Lys residues, the presence of MDA epitopes was demonstrated in atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits (Haberland et al., 1988), and later Yla-Herttuala et al. (1989) have shown that LDL in

rabbit and human lesions is in fact oxidized and a major carrier of MDA epitopes. MDA-modified LDL—in contrast to native LDL—possesses a strong chemotactic potential and can be engulfed more readily by macrophages (Yla-Herttuala et al., 1989). Consistent with findings in rabbits and humans, lesions from Apoe$^{-/-}$ mice were shown to contain MDA epitopes in regions rich in macrophages as well as in the necrotic core, which is an area replete with apoptotic cells (Palinski et al., 1994). Possibly, accumulating apoptotic cells prominently contribute to MDA epitopes in atherosclerotic lesions. Indeed, we have recently shown that the levels of plasma MV isolated from the culprit lesion site of patients suffering a myocardial infarction are increased and enriched in MDA epitopes compared to plasma MV of the same patients obtained from the periphery (Tsiantoulas et al., 2015). Additionally, it has been shown that HDL isolated from human atherosclerotic tissues is enriched in MDA epitopes compared to HDL from plasma of healthy donors. Modification of ApoAI, the major protein moiety of HDL, with MDA in vitro results in dysfunctional HDL. MDA epitopes on ApoAI cause structural and conformational changes, thereby impairing its ability to interact with ABCA1 and promoting cholesterol efflux onto HDL, which is a homeostatic mechanism to remove access cholesterol from macrophages. Thus, oxidative modification of ApoAI by MDA impairs an important cardioprotective function of HDL, which may also contribute to atherosclerosis development (Shao et al., 2010).

Inflammation in vascular lesions is considered a key driver of atherogenesis. Major carriers of MDA epitopes, such as OxLDL, MV, and dying cells have been shown to possess robust proinflammatory properties, and there is accumulating evidence that MDA epitopes themselves are mediators of these effects. For example, the cytotoxic effect of OxLDL on endothelial cells can be blocked by a polyclonal antibody against MDA but not by a polyclonal antibody against ApoB100. Moreover, it was shown that MDA epitope-mediated OxLDL cytotoxicity was partially dependent on Akt pathway activity (Yang et al., 2014). Additionally, the direct proinflammatory nature of MDA epitopes has been demonstrated by several groups. As discussed earlier, treatment of THP1 cells with MDA-Lys resulted in strong activation of NF-κB activity as well as the activation of signaling pathways related to inflammation, cellular motility, and cell-to-cell signaling. Furthermore, MDA-Lys also increased monocyte binding to vascular smooth muscle and endothelial cells (Shanmugam et al., 2008). Additionally, MAA-BSA induced expression of proinflammatory cytokines IL-8, IL-1β, TNF-α, and IL-12β in THP-1 cells (Weismann et al., 2011).

All these cytokines have been shown to play an important role in atherosclerotic lesion formation (Ait-Oufella, Taleb, Mallat, & Tedgui, 2011). For example, IL-8 or CXCL1 promotes leukocyte recruitment to the vascular wall and atherosclerosis-prone mice deficient in either Cxcl1 or its receptor Cxcr1 develop significantly reduced atherosclerosis. Interestingly, also MDA-carrying MV were able to induce IL-8 secretion in human monocytes, and this effect could be neutralized in the presence of the MAA-specific IgM NAb LR04, suggesting that MAA epitopes are in part responsible for the proinflammatory effect of MV (Tsiantoulas et al., 2015). All these data strongly support the fact that MDA epitopes act as DAMPs that modulate lesional inflammation and have a fundamentally important role in the process of atherogenesis.

10.1 MDA Immunization Protects from Atherosclerosis

The importance of MDA in atherogenesis has been demonstrated in a large number of immunization studies, which showed induction of atheroprotective immunity by immunization with MDA antigens. These studies indicate that endogenously generated MDA is relevant for atherogenesis, as the immune response against MDA specifically mediates protection. For example, immunization of WHHL rabbits with MDA-modified homologous LDL resulted in increased antibody titers to MDA epitopes and atheroprotection, in contrast to immunization with KLH (Palinski, Miller, & Witztum, 1995). Similar atheroprotective effects as well as increased antibody levels were observed in immunization studies of atherosclerosis-prone Apoe$^{-/-}$ and Ldlr$^{-/-}$ mice (Binder et al., 2004; Fredrikson et al., 2003; Freigang, Horkko, Miller, Witztum, & Palinski, 1998; George et al., 1998; Gonen et al., 2014; Zhou, Caligiuri, Hamsten, Lefvert, & Hansson, 2001; Zhou et al., 2005). Apoe$^{-/-}$ mice immunized with homologous MDA-LDL develop high titers of anti-MDA-LDL IgG antibodies and had 50% less lesional area at the aortic sinus compared to control mice injected with PBS (George et al., 1998). Additionally, Ldlr$^{-/-}$ mice immunized with homologous MDA-LDL or native LDL developed high IgM and IgG titers to MDA-LDL and had attenuated atherosclerosis. Interestingly, immunization with homologous native LDL also protected from atherosclerosis without inducing high titer of antibodies, suggesting a different mechanism for atheroprotection with native LDL (Freigang et al., 1998). Subsequently, seven peptides within human ApoB100 were shown to be immunogenic and were recognized by IgM and IgG autoantibodies in human serum (Fredrikson et al., 2003). In a follow-up study,

immunization of Apoe$^{-/-}$ mice with a mixture of two peptides of human ApoB100 (peptides 142 and 210 with 85–90% similarity to the mouse ApoB100 sequence) resulted in a reduction of plaque area by 60%, in contrast to control immunized mice. Immunization with ApoB100 peptides increased IgG titers against MDA-modified peptides, suggesting that the peptides used as antigens became modified with MDA in vivo (Fredrikson et al., 2003). The importance of MDA and MAA as a key antigen has been demonstrated by the fact that immunization of Apoe$^{-/-}$ mice with plaque homogenates from Apoe$^{-/-}$ mice triggers MDA-specific antibodies and reduces lesion formation. More precisely, immunization with MDA-LDL or plaque homogenates led to increased T cell-dependent IgG antibodies recognizing MDA-LDL, the titers of which were found to negatively correlate with plaque size or serum cholesterol levels (Zhou et al., 2001). In an attempt to identify the exact requirements for the atheroprotective effect of immunization with MDA-LDL, extensive immunization studies with different types of related antigens have been performed. In these studies, Ldlr$^{-/-}$ mice were immunized with MDA-LDL (MDA-LDL), MDA-modified murine serum albumin (MDA-MSA), MAA-MSA, propanal-modified MSA, native MSA, and PBS, and then fed an atherogenic diet for 28 weeks. The most robust reduction in aortic lesion area (40%) compared to PBS control was observed when MAA-MSA was used as an immunogen. Despite similar induction of hapten-specific immune responses with the other antigens, only mice immunized with MDA-LDL and MDA-MSA had a significant reduction of lesion area, albeit to a lesser extent than immunization with MAA-MSA. Notably, immunization with MDA-LDL also induced antibodies that bound to MAA-modified peptides. This observation led to the conclusion that MAA epitopes are the immunodominant epitopes inducing atheroprotective immune responses. Thus, MAA hapten-specific immunity mediates the protective effect of immunization with MDA-LDL, independent of the carrier protein.

Several studies tried to characterize the mechanisms responsible for the protective effect of immunization. For example, as discussed earlier, we could show that immunization of both wild type and atherosclerosis-prone mice with homologous MDA-LDL and MAA-MSA induces a robust Th2-biased immune response that is dominated by MDA-specific IgG1 antibodies (Binder et al., 2004). High titers of antibodies may mediate atheroprotection, as infusion of IEI-E3, a human IgG1 antibody that was selected against MDA-modified peptides of human ApoB100, into

$Apoe^{-/-}$ mice for 4 weeks resulted in decreased atherosclerotic lesion formation compared to infusion of an isotype control (Schiopu et al., 2007). Moreover, infusion of another human recombinant IgG1 (2D03) recognizing the MDA modifications on the same human ApoB100 peptide also reduced atherosclerosis and constrictive injury-induced remodeling in the carotid artery of $Ldlr^{-/-}$ mice (Strom et al., 2007). Using the human-derived anti-OxLDL IgG Fab IK17 specific for MDA epitopes it was demonstrated that IK17 inhibits the uptake of OxLDL by macrophages in vitro (Shaw et al., 2001). In addition, infusion of an adenoviral construct overexpressing recombinant IK17 scFv into cholesterol-fed $Ldlr^{-/-}Rag\text{-}1^{-/-}$ mice resulted in decreased peritoneal foam-cell formation and reduced atherosclerosis (Tsimikas et al., 2011). These antibodies have been suggested to block foam cell formation and protect from atherosclerosis. On the other hand, Th2 cytokines, such as IL-5 and IL-13, induced by MDA-immunization possess important atheroprotective properties. For example, IL-5 stimulates B-1 cells to secrete IgM NAb in a noncognate manner. A large part of IgM NAbs have specificity for OSEs (Chou et al., 2009), and atherosclerosis-prone mice unable to secrete IgM antibodies develop accelerated atherosclerosis (Lewis et al., 2009). Hematopoietic IL-5 deficiency in $Ldlr^{-/-}$ mice leads to increased atherosclerosis and reduced levels of specific atheroprotective IgM NAb, T15/E06. Furthermore, we also showed that IL-13 protects mice from atherosclerotic lesion formation by promoting alternative activation of lesional macrophages that are more efficient in removal of OxLDL (Cardilo-Reis et al., 2012). Moreover, it has been suggested that immunization with MDA-LDL also induces regulatory T cells, which could suppress the secretion of the proatherogenic cytokine IFNγ by specific Th1 cells. Nevertheless, Zhou et al. showed that immunization of $CD4^{-/-}Apoe^{-/-}$ mice that lack Th cells with homologous MDA-LDL still induced atheroprotection (Zhou et al., 2005). On the other hand, adoptive transfer of CD4+ Th cells from MDA-LDL immunized mice into $Apoe^{-/-}$ SCID mice resulted in accelerated atherosclerosis compared to transfer of T cells from control immunized mice. Thus, the true effects of Th-dependent immunity to MDA-LDL remain to be elucidated in detail. Clearly, a feature common to all immunization studies is the production of MDA-specific antibodies at much higher levels than the ones occurring naturally and in the course of diet feeding.

In the past 25 years, many epidemiological studies assessed associations of IgG and IgM antibodies to OxLDL and MDA-LDL epitopes with the development of coronary, carotid, and peripheral artery disease (Salonen et al.,

1992; Tsimikas et al., 2003; Tsimikas, Witztum, et al., 2004; Wu et al., 1997). These studies have often been very inconsistent due to lack of standardized antigens or assays, and because tested cohorts of patients were simply too small or not followed prospectively (Tsimikas et al., 2012). Some of these inconsistencies could be overcome with the use of recombinant peptide mimotopes of MAA that serve as standardized antigens for MAA-specific antibodies (Amir et al., 2012). Nevertheless, studies in humans have provided insights into the role of these immune responses in CVD. While the role of IgG antibodies to MDA-LDL is less clear, there is now substantial evidence that IgM antibodies to MDA-LDL are inversely associated with atherosclerosis or clinical manifestations thereof (Gounopoulos, Merki, Hansen, Choi, & Tsimikas, 2007; Tsiantoulas et al., 2014). These data suggest that MDA-LDL-specific IgM antibodies may mediate protective functions in atherosclerosis. Interestingly, MDA-specific IgM antibodies have been found to be higher in people younger than 65, females, and nondiabetics (Fraley et al., 2009; Tsimikas, Lau, et al., 2004). Moreover, many if not most IgM antibodies with specificity for MDA-LDL may be IgM NAbs.

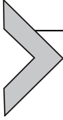
10.2 MDA-Specific Natural Immunity Protects from Atherosclerosis

As discussed earlier, IgM NAbs are important mediators of MDA-specific immunity, and several potential mechanisms on how they can mediate atheroprotective effects have been suggested. First, IgM with specificity for MDA/MAA have been shown to act antiinflammatory. We could demonstrate that the MAA-specific IgM NAb LR04 significantly decreased the ability of platelet-derived MV to induce IL-8 secretion by THP-1 cells and primary human monocytes (Tsiantoulas et al., 2015). IL-8 is a key mediator of leukocyte recruitment to the vascular wall and chief proatherogenic chemokine (Ait-Oufella et al., 2011). Thus, inhibition of MDA/MAA-induced IL-8 secretion by endothelial cells and monocytes may have an important modulatory function in atherogenesis. Furthermore, MDA-specific IgM may also protect by promoting clearance of dying cells. Indeed, defective phagocytic clearance of dying cells in atherosclerotic lesions has also been implicated in the progression of atherosclerosis (Tabas, 2010; Van Vre, Ait-Oufella, Tedgui, & Mallat, 2012). For example, $Ldlr^{-/-}$ mice reconstituted with bone marrow of mice deficient in the milk fat globule-EGF factor 8 protein ($Mfge8^{-/-}$), which facilitates PS-mediated clearance of apoptotic cells, developed increased lesion size with more

accumulating apoptotic cells and increased necrotic areas (Ait-Oufella et al., 2007). A similar effect has been observed in Apoe$^{-/-}$ mice also deficient in MER proto-oncogene tyrosine kinase (Thorp, Cui, Schrijvers, Kuriakose, & Tabas, 2008). The ability of MDA-specific IgM to modulate efferocytosis has been addressed in the studies by Chen et al. (2009) and Chou et al. (2009). For example, incubation of apoptotic thymocytes with wild-type mouse serum in the presence of MDA-BSA resulted in diminished deposition of IgM and C1q on apoptotic cell surfaces and consequently led to decrease in complement-mediated phagocytosis by dendritic cells (Chen et al., 2009). To explain this, the authors suggested that MDA-BSA competed for the binding to MDA epitopes on apoptotic cells, on which they may act as hubs for activation of complement-mediated efferocytosis via IgM and C1q axis. We could directly demonstrate a role for MDA-specific IgM NAbs in apoptotic cell clearance in an in vivo clearance assay in Rag1$^{-/-}$ mice, deficient in B- and T-cell populations. Intraperitoneal injection of apoptotic thymocytes preincubated with the IgM NAb NA17 resulted in significantly enhanced phagocytosis by macrophages compared to apoptotic thymocytes preincubated with control IgM (Chou et al., 2009). The importance of this mechanism is further supported by the fact that soluble IgM deficiency (Lewis et al., 2009) as well as C1q deficiency aggravates atherosclerosis in atherosclerosis-prone mice (Bhatia et al., 2007). Thus, MDA epitopes may act as marker of metabolic waste and dying cells in atherosclerotic lesions, allowing the immune system to mediate housekeeping functions (Chang et al., 1999; Chen et al., 2009; Chou et al., 2009; Weismann et al., 2011).

Not only IgM antibodies have the capacity to protect from MDA-induced inflammation. Additionally, CFH that colocalizes in human coronary lesions with MDA epitopes has been shown to neutralize MDA-induced expression of proinflammatory genes, including IL-8. Interestingly, a few studies have shown an association of the 402H allele, which reduces MDA binding of CFH (Weismann et al., 2011), with increased cardiovascular risk. However, a metaanalysis of eight different study populations failed to find a significant association of this gene variant with CVDs (Sofat et al., 2010). Nevertheless, additional SNPs of CFH that can affect its capacity to bind MDA have been identified and only functional data on MDA binding of CFH in plasma may provide insights into a potential association of this CFH function with CVD. In support of this, we could demonstrate that CFH bound on MDA-decorated surfaces still can mediate cofactor activity for Factor I to inactivate C3b into iC3b. In turn, freshly deposited inactive iC3b promotes antiinflammatory clearance of opsonized

particles (Weismann et al., 2011). Thus, CFH may protect from atherosclerosis by binding to MDA epitopes, thereby reducing inflammation and additionally increasing opsonization with iC3b, which facilitates efferocytosis. Studies in animal models of atherosclerosis may provide critical insights into these functions.



11. MDA EPITOPES AS THERAPEUTIC TARGETS IN CARDIOVASCULAR DISEASE

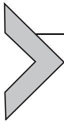
Current therapies in CVD mainly aim at lowering LDL plasma levels by increasing the availability of the LDL receptor at the cell surface using, for example, statins or PCSK9 antibodies. Nevertheless, an important contribution of the innate and adaptive immunity to the development of atherosclerosis has become evident. With two ongoing clinical studies, the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) and the Cardiovascular Inflammation Reduction Trial (CIRT), we are currently witnessing a premiere in regard to clinical trials addressing the role of inflammation in CVD. CANTOS and CIRT are the first studies which aim at preventing recurrent cardiovascular events in patients by reducing inflammation without affecting plasma cholesterol levels using low-dose methotrexate or a neutralizing anti-IL-1β antibody (canakinumab), respectively. Other potentially interesting though preclinical immunomodulatory approaches for therapeutic intervention in atherosclerosis have been reviewed elsewhere (Amir & Binder, 2010; Lichtman et al., 2013; Miller & Tsimikas, 2013; Witztum & Lichtman, 2014).

As OSEs have been implicated as potential antigens triggering innate and adaptive immune responses in atherosclerosis, targeting these structures by active or passive immunization in order to reduce the inflammatory burden could provide a novel therapeutic approach to treat atherosclerosis. Indeed, several lines of evidence point toward a protective effect of increasing antibody titers against OSEs. Interestingly, active immunization using a single OSE, MDA-LDL as an immunogen is sufficient to protect from atherosclerosis as demonstrated by a number of studies in animals (Binder et al., 2004; Freigang et al., 1998; George et al., 1998; Gonen et al., 2014; Palinski et al., 1995; Zhou et al., 2001). Even more intriguing is the fact that this protective effect seems to be independent of the carrier presenting MDA epitopes on its surface as several studies showed that immunization using various carrier proteins all modified with MDA epitopes results in less atherosclerosis, including MDA-modified ApoB100-peptides (Fredrikson et al., 2003), MDA-modified laminin (Duner et al., 2010) and fibronectin

(Duner et al., 2011), or killed *Porphyromonas gingivalis* (Turunen et al., 2012), a pathogen-carrying surface structures that mimic MDA epitopes. Therefore, the identification of MDA epitopes as critical structures conveying atheroprotection through immunization could allow for the development of more standardized immunogens benefiting from lower variability and thus higher reproducibility. Accordingly, using phage display libraries we have identified an MDA mimotope, the P2 peptide, and demonstrated that immunization with P2 induces MDA-specific antibodies, which bind to human atherosclerotic lesions (Amir et al., 2012). On the other hand, several studies reported that passive immunization by therapeutic infusion of anti-MDA antibodies also had beneficial effects by reducing plaque inflammation and regression of atherosclerotic lesions (Schiopu et al., 2007; Tsimikas et al., 2011). Even though animal studies have convincingly shown that vaccination using MDA epitopes is protective in atherosclerosis, translation to human settings has proven to be difficult. Not only do critical differences between rodents and humans exist in terms of lipoprotein metabolism during atherogenesis, which advises against a direct implementation of experimental setups to the clinics, but the ubiquitous presence of MDA epitopes in the body also complicates any human vaccination study because the generation of antibodies against continuously formed MDA epitopes could lead to unwanted side effects of the vaccination in the long term given that we still lack a deeper understanding of the mechanisms involved.

Meanwhile, other approaches offering protection from pathologically elevated levels of MDA epitopes might be worth of consideration. Several studies demonstrated that endogenous production of natural IgMs can be boosted by treatment with IL-18 (Kinoshita et al., 2006), IL-25 (Mantani et al., 2015), and IL-5 (Binder et al., 2004), by genetic deficiency of sialic acid-binding Ig-like lectin G (Siglec G), a negative regulator of the B cell receptor present also on B1a cells (Hoffmann et al., 2007), and by additional strategies yet to be elucidated. While many of these approaches are not specific or even potentially harmful, identification of pathways that specifically regulate IgM NAb production may provide novel therapeutic opportunities. In addition, the MDA-neutralizing properties of CFH could be harnessed for therapeutic purposes as we have shown that CFH protects from MDA-induced inflammation in a mouse model of wet AMD by binding to MDA epitopes (Weismann et al., 2011). Treatment of patients possessing a CFH variant with impaired binding using CFH or truncated CFH constructs which contain only a fraction of the protein including the critical MDA-binding region SCR19/20 could help protecting from

MDA-mediated inflammation. Indeed, such constructs, termed mini CFH, have demonstrated their therapeutic potential in mouse models of C3 glomerulopathy (Nichols et al., 2015) and we speculate that their neutralizing capabilities could be also beneficial in atherosclerosis. Regardless of the successes in animal models, the feasibility of these approaches in human patients has to be taken with caution as the treatment of a chronic disease that develops over decades might result in long-term complications. Nevertheless, in cases where only short-term treatment is required or for individuals at high risk, the benefits could outweigh the disadvantages.



12. CONCLUSIONS

Lipid peroxidation of cellular membranes results in the generation of various OSEs that act as downstream mediators of oxidative stress in tissues. In this chapter, we have discussed MDA epitopes as a prominent and important example. This includes evidence that they: (1) are proinflammatory danger signals for a wide range of cell types, (2) can be recognized by multiple arcs of innate immunity with critical roles in housekeeping functions, and (3) are potent immunogens, which elicit protective immune response in mouse models of atherosclerosis. We speculate that MDA epitopes present on dying cells and biomolecules damaged by oxidative stress serve as waste markers for the immune system, flagging their carriers for complement-mediated rapid and silent clearance by professional phagocytes. However, excessive production of MDA epitopes and/or impaired clearance capabilities results in their nonphysiological accumulation and subsequently elicits proinflammatory responses, which are sustained as long as the carriers of MDA epitopes cannot be neutralized or scavenged—a condition most likely occurring in many if not all chronic inflammatory diseases that are associated with impaired resolution, such as atherosclerosis.

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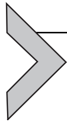

CHAPTER TWO

Factors That Regulate the Generation of Antibody-Secreting Plasma Cells

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Abstract

The generation of antigen-specific neutralizing antibodies and memory B cells is one of the most important immune protections of the host and is the basis for successful vaccination strategies. The protective antibodies, secreted by preexisting long-lived plasma cells and reactivated antigen-experienced memory B cells, constitute the main humoral immune defense. Distinct from the primary antibody response, the humoral memory response is generated much faster and with greater magnitude, and it

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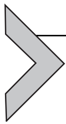
produces antibodies with higher affinity and variable isotypes. Humoral immunity is critically dependent on the germinal center where high-affinity memory B cells and plasma cells are generated. In this chapter, we focus on recent advances in our understanding of the molecular mechanisms that govern fate decision for memory B cells and plasma cells and the mechanisms that maintain the long-lived plasma-cell pool, with emphasis on how the transcription factor Blimp-1 (B lymphocyte-induced maturation protein-1) helps regulate the above-mentioned immunoregulatory steps to ensure the production and maintenance of antibody-secreting plasma cells as well as how it directs memory cell vs plasma-cell fate. We also discuss the molecular basis of Blimp-1 action and how its expression is regulated.



1. GERMINAL CENTER

1.1 Initiation of the Germinal Center Reaction

A successful establishment of humoral immune response is critical for the clearance of invading pathogen infection and forms the basis of vaccination. Immature B cells are produced in bone marrow and migrate to secondary lymphoid organs where they mature and encounter antigens via B-cell receptor (BCR). B cells acquire large antigen in the follicle of lymph nodes. The priming of naïve B cells with large antigen can be assisted by interaction with dendritic cells (DCs) and macrophages located in the subcapsular sinus boundary of lymph nodes or in the marginal zone of the spleen (Balazs, Martin, Zhou, & Kearney, 2002; Carrasco & Batista, 2007). The conduit system comprising the basement membrane-like structures ensheathed by fibroblastic reticular cells can deliver small soluble antigens from the afferent lymph to B cells (Balazs et al., 2002; Roozendaal et al., 2009). After encountering antigens that were either in soluble form or presented by antigen-presenting cells (Batista & Harwood, 2009), B cells process antigens and move to the T-B border, where B cells interact with T follicular helper (Tfh) cells via contact between peptide-MHC class II complexes on antigen-primed B cells and the antigen-specific T-cell receptor (McHeyzer-Williams, Okitsu, Wang, & McHeyzer-Williams, 2012). Pre-germinal center (GC) Tfh cells secrete cytokines, including IL-21, IL-4, and IFN-γ, to induce the activation of B cells and antigen-specific B-cell responses (Breitfeld et al., 2000; King, Tangye, & Mackay, 2008). In addition, pre-GC Tfh cells express a unique panel of effector molecules, including CD40L, ICOS, and cytoplasmic adaptor protein SLAM-associated protein (SAP), that promote B-cell activation, differentiation, and survival (Ma, Deenick, Batten, & Tangye, 2012).

Specifically, CD40L promotes B-cell proliferation and protects these cells from apoptosis (Tangye, Deenick, Palendira, & Ma, 2012). The interaction between ICOS on Tfh cells and ICOSL on B cells facilitates the expression of certain key Tfh-associated molecules, such as IL-21 (Bauquet et al., 2009), which acts in an autocrine manner to maintain Tfh-cell homeostasis. SAP is crucial for the establishment of a stable interaction between Tfh cells and antigen-primed B cells (Qi, Cannons, Klauschen, Schwartzberg, & Germain, 2008). The interaction between antigen-primed B cells and pre-GC Tfh cells also triggers cytokine singling pathways that subsequently instruct different isotypes of class-switch recombination (CSR) in GC, depending on the types of cytokine released by Tfh cells (McHeyzer-Williams et al., 2012).

After contacting Tfh cells, activated B cells either enter the GC reaction or move to extrafollicular medullary cords to become short-lived plasma cells that secrete antibody with low affinity against invading pathogens (Jacob & Kelsoe, 1992). Short-lived plasma cells express germline transcripts of immunoglobulins but may also undergo immunoglobulin CSR and limited somatic hypermutation (SHM) (MacLennan et al., 2003). Although extrafollicular short-lived plasma cells survive for only 3–5 days (McHeyzer-Williams & McHeyzer-Williams, 2005), they produce early protective antibodies during infection. Antigen-specific B cells that are committed to GC precursors subsequently move toward the center of follicles in an orphan G protein-coupled receptor EBI-2-dependent manner and undergo massive and rapid clonal expansion (Gatto, Paus, Basten, Mackay, & Brink, 2009; Pereira, Kelly, Xu, & Cyster, 2009), which results in the formation of the early GC. As vigorous proliferation of GC B cells precedes, the GC increases in size and is polarized into two microenvironments composed of a dark zone and light zone. B cells in the dark zone and light zone show distinct surface markers: the dark-zone B cells are $CXCR4^{hi}CD83^{lo}CD86^{lo}$, whereas the light-zone B cells are $CXCR4^{lo}CD83^{hi}CD86^{hi}$. B cells in the dark zone are known as centroblasts whose extensive proliferation, ranging from 6 to 12 h to complete a cell cycle (MacLennan, 1994), initiates SHM that further increases the diversification of their IgV genes. Dark-zone B cells have distinct gene expression profiles that underlie increased expression of cell-cycle regulators and proapoptotic factors but downregulated expression of negative regulators of the cell cycle and antiapoptotic factors (Klein & Dalla-Favera, 2008; Klein et al., 2003). B cells in the light zone, called centrocytes, exit the cell cycle and are smaller in size. In addition to B cells, several other cell types including Tfh, follicular DCs, and

macrophages (De Silva & Klein, 2015) are also present in the light zone. The expression of CXCR5 along with loss of the T-cell zone-homing chemokine receptor CCR7 permits antigen-specific Tfh cells to move from the T-cell zone to the GC, which provides essential help for the maturation and differentiation of GC B cells (Hardtke, Ohl, & Forster, 2005). Antigen held by follicular DCs can be taken up by GC B cells, after which these cells further process and present the antigen to Tfh cells (El Shikh & Pitzalis, 2012). With support from those cells—particularly antigen-specific Tfh cells—centrocytes undergo affinity maturation based on the affinity of their mutated IgV genes, whereby high-affinity BCR variants are selected to differentiate into memory B cells or plasma cells (Chan & Brink, 2012). Centrocytes that express higher affinity BCR transduce signaling pathways serve to protect against apoptosis (Liu et al., 1989), and the selected centrocytes can reenter the dark zone to reinitiate cell division and undergo additional cycles of SHM, a process known as "cyclic reentry" (Victora et al., 2010). Cyclic reentry is controlled by the biphasic expression of c-Myc in GC B cells, and this expression is suppressed in centroblasts, but c-Myc is reexpressed in a select subset of centrocytes prior to dark-zone reentry (Dominguez-Sola et al., 2012).

1.2 Molecules Important for GC Generation and Function

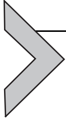
The formation of GC and the GC reaction is regulated by several key molecules, such as B-cell CLL/lymphoma 6 (BCL-6), interferon-regulatory factor 4 (IRF4), and activation-induced cytidine deaminase (AID). BCL-6 was first identified as a proto-oncogene frequently expressed in non-Hodgkin's lymphoma (Ye et al., 1993). BCL-6 is a member of the BTB/POZ/zinc-finger family of transcription factors (Basso & Dalla-Favera, 2012). Upregulation of BCL-6 is essential for the formation of the GC, maintenance of GC B-cell genomic integrity, and prevention of premature differentiation of plasma cells (Fukuda et al., 1997; Phan, Saito, Kitagawa, Means, & Dalla-Favera, 2007; Shaffer et al., 2000). BCL-6 binds to thousands of genes in GC B cells including those involved in B-cell activation, survival, DNA-damage response, cell-cycle arrest, cytokine signaling, Toll-like receptor signaling, and differentiation (Basso et al., 2010). The expression of BCL-6 can be initiated in antigen-experienced GC B precursors, and this is crucial for sustaining the cognate interactions with pre-GC Tfh cells and for their entry into the center of follicles (Kitano et al., 2011). In addition to its importance in GC B cells, BCL-6 is required for the generation of Tfh cells (Johnston et al., 2009; Nurieva et al., 2009). Recent

genome-wide analysis of target genes directly bound by BCL-6 in Tfh cells revealed that BCL-6 represses gene expression associated with T-cell migration pathways, T-cell receptor signaling pathways, and Th1, Th17, Th2, and Treg cell differentiation pathways via interaction with the transcription factor, activator protein 1 (AP-1), and targeting to the AP-1-binding site of endogenous loci (Hatzi et al., 2015).

IRF4 is necessary for the formation of the GC, and mice lacking *Irf4* have an impaired GC and are defective for plasma-cell generation (Mittrucker et al., 1997); specifically, loss of *Irf4* in mature B-cell stages abrogates GC formation in response to T cell-dependent (TD) antigen immunization and *Listeria major* infection (Willis et al., 2014). The function of IRF4 in initiation of the GC and plasma-cell fates is temporally controlled in a concentration-dependent manner (Ochiai et al., 2013), and transient induction of *Irf4* induces the generation of BCL-6-expressing GC B cells in vivo, whereas strong BCR signaling triggered by increased antigen affinity causes high expression of IRF4, which promotes the generation of plasma cells. IRF4 is also essential for Tfh-cell formation (Bollig et al., 2012).

AID was first identified by subtractive hybridization cloning of cDNAs expressed in mouse CH12F3 lymphoma cells that had been induced to undergo CSR (Muramatsu et al., 1999). Mice deficient in *Aicda*, the gene encoding AID, show a complete blockage of SHM and CSR and have a hyper-IgM phenotype, although cytokine- or lipopolysaccharide (LPS)-induced transcriptional activation of the Ig germline transcripts occur normally (Muramatsu et al., 2000). Patients deficient in AID display hyper-IgM syndrome and suffer from recurrent infections (Revy et al., 2000). AID directly converts cytosine into uracil (U) in single-stranded target DNA of both IgS and IgV regions (Chaudhuri et al., 2003; Di Noia & Neuberger, 2007; Pham, Bransteitter, Petruska, & Goodman, 2003). The U:G mismatches created by AID-mediated deamination can be repaired in a number of ways, leading to the generation of transition/transversion mutations in the IgV region in SHM or DNA double-strand breaks in the S region of the IgH gene in CSR (Keim, Kazadi, Rothschild, & Basu, 2013; Odegard & Schatz, 2006).

In addition, epigenetic regulation has also been implicated in GC formation and function. EZH2, which catalyzes methylation of lysine 27 of histone 3, is essential for the generation of the GC (Beguelin et al., 2013). Mice carrying an *Ezh2* deletion in postswitched GC B cells show a loss of proliferative GC B cells and impaired immunoglobulin affinity maturation that results in reduced formation of high-affinity antibodies following immunization.



2. PLASMA CELLS

2.1 Generation of Plasma Cells

The switch from the membrane-bound form of Ig to the secreted Ig form characterizes the terminal differentiation of B cells into plasmablasts, which will eventually mature into noncycling plasma cells. The generation of antibody-secreting plasma cells is initiated by the exposure of mature B cells to a T cell-independent (TI) and TD antigen. Plasma cells generated by TD antigen can be produced by either extrafollicular or GC responses. In the case of an extrafollicular response, T cells outside the follicle, ie, extrafollicular T helper cells, support the proliferation and differentiation of plasmablasts in an IL-21- and ICOS-dependent manner (Odegard et al., 2008). In collaboration with extrafollicular T cells, DCs located in extrafollicular regions also promote the production of switched or unswitched plasmablasts by expressing B-cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) that can be triggered by the interaction between lectin-like oxidized low-density lipoprotein receptor-1 on extrafollicular DCs and its ligands, such as oxidized low-density lipoproteins (Joo et al., 2014). The strength of antigen recognition by BCR determines the pathway choice of antigen-experienced B cells to differentiate into either extrafollicular or GC plasmablasts (Paus et al., 2006). High-affinity or abundant antigens direct B cells to differentiate into extrafollicular plasma cells, whereas low-affinity antigens instruct B cells to undergo GC reactions by which high-affinity clones can be selected by affinity maturation. In GC responses, a subset of GC B cells can express a partial plasma-cell phenotype (Angelin-Duclos, Cattoretti, Lin, & Calame, 2000). It has been proposed that plasma-cell fate is determined by the affinity of BCR for antigens in the GC rather than stochastic selection from GC B cells (Phan et al., 2006). High-affinity GC B cells are more likely to form plasma cells. However, other reports have also provided evidence that cytokine-driven stochastic division regulates the generation of IgG-secreting plasma cells (Duffy et al., 2012; Hasbold, Corcoran, Tarlinton, Tangye, & Hodgkin, 2004). Concordantly, a recent transcriptome analysis of differential gene expression profiles of dividing B cells stimulated to undergo plasma-cell differentiation showed that the expression of plasma-cell-specific genes was accordingly initiated beginning with initiation of the cell cycle (Shi et al., 2015).

2.2 Molecular Signatures of Plasma Cells

Plasma cells express unique surface markers that are distinct from those of B cells. Many B-cell-specific surface proteins, including MHC class II, B220, CD19, CD20, CD21, and CD22, are downregulated on plasma cells (Calame, 2001). Syndecan-1 (CD138), a heparan sulfate-rich integral membrane proteoglycan, is one of the plasma-cell markers (Sanderson, Lalor, & Bernfield, 1989). Plasma cells emigrating from the GC begin to express CXCR4, which prompts the cells to home to splenic red pulp, lymph-node medullary cords, and bone marrow (Hargreaves et al., 2001). With regard to intracellular proteins whose expression level changes during plasma-cell differentiation, sequential waves of functionally related proteins have been demonstrated to be differentially expressed: mitochondrial and cytosolic chaperones are induced first, followed by a surge in expression of metabolic enzymes, endoplasmic reticulum (ER)-resident folding factors, and redox-balance proteins (van Anken et al., 2003). Metabolomics analysis of metabolic profiling in extracellular medium of plasma cells has shown that the metabolome of the antibody-secreting phase is characterized by decreased use of glucose, sustained consumption of glutamine, and release of glutamate and alanine (Garcia-Manteiga et al., 2011). The transition from activated B cells to plasma cells requires the coordinated regulation of gene expression governed by several key transcription factors, including those that drive plasma-cell differentiation, such as B lymphocyte-induced maturation protein-1 (Blimp-1), IRF4, and X box-binding protein-1 (XBP-1), and those that downregulate these drivers, such as PAX5, BCL-6, BACH2, IRF8, and PU.1. Aside from these key transcription factors, plasma cells express a unique panel of transcription factors that govern their cellular functions; these factors include members of the AP-1, NF-κB, NFAT, and octamer-binding factor families (Underhill, George, Bremer, & Kansas, 2003).

2.3 Key Transcription Factors in Plasma-Cell Differentiation

The discovery that Blimp-1 functions in the B-cell lineage will be discussed in more detail in Section 4. Pax5 is expressed throughout B-cell development except in plasma cells (Barberis, Widenhorn, Vitelli, & Busslinger, 1990). Pax5 maintains B-cell identity by controlling the B-cell gene expression program (Cobaleda, Schebesta, Delogu, & Busslinger, 2007; Horcher, Souabni, & Busslinger, 2001). Enforced expression of Pax5 in stimulated

B cells prohibits the generation of plasma cells (Lin, Angelin-Duclos, Kuo, & Calame, 2002; Usui et al., 1997); conversely, loss of *Pax5* in the chicken cell line DT40 is sufficient to induce IgM secretion (Nera et al., 2006). Mechanistically, Pax5 directly suppresses transcription of *Prdm1*, the gene encoding Blimp-1 (Mora-Lopez, Reales, Brieva, & Campos-Caro, 2007), whereas ERK1/2-dependent phosphorylation of Pax5 at serine 189 or 283 abrogates Pax5-dependent suppression of *Prdm1* (Yasuda et al., 2012). Transcription of *XBP-1* is also suppressed by Pax5, whose downregulation during plasma-cell differentiation may at least partly contribute to the induction of *XBP-1* (Reimold et al., 1996). In addition to regulating genes involved in facilitating GC functions, BCL-6 suppresses plasma-cell differentiation at least partly by directly suppressing the transcription of *Prdm1* (Shaffer et al., 2000; Tunyaplin et al., 2004). BCL-6 is phosphorylated by MAPK, MEK1, and MEK2 but not by JNK (Moriyama, Yamochi, Semba, Akiyama, & Mori, 1997). Following BCR activation, BCL-6 is phosphorylation by MAPK, leading to its degradation via the ubiquitin/proteasome pathway (Niu, Ye, & Dalla-Favera, 1998) and, consequently, to derepression of *Prdm1*. Accordingly, LPS-stimulated knockout of *Bcl-6* in splenic B cells leads to enhancement of their differentiation into $CD138^+$ Ig-secreting cells (Tunyaplin et al., 2004). Recently, the SWI/SNF chromatin remodeling complex was shown to interact with BCL-6 to repress *Prdm1* (Choi, Jeon, Choi, Park, & Seong, 2015). Mice lacking the core component of SWI/SNF complex in the B-cell lineage exhibit impaired GC reactions and increased expression of Blimp-1 (Choi et al., 2015). BACH2 is highly expressed from the pro-B to mature B-cell stages through the transcriptional activation by Pax5 (Schebesta et al., 2007), but BACH2 is downregulated in plasma cells (Muto et al., 1998, 2010). BACH2 is also a critical regulator of CSR and SHM (Muto et al., 2004). Mice lacking *Bach2* show impaired IgG responses after immunization with TI or TD antigen. *PRDM1* is an important BACH2 target (Ochiai et al., 2006). In the absence of BACH2, more IgM-secreting plasma cells are produced in stimulated mouse splenic B-cell cultures, which correlate with higher expression of Blimp-1 (Muto et al., 2010). IRF8 and PU.1 cooperatively suppress CSR and plasma-cell differentiation by downregulating *Aicda* and *Prdm1* (Carotta et al., 2014). Deletion of both *Irf8* and *PU.1* in B cells synergistically enhances the production of Ig-secreting plasma cells as compared with B cells lacking either *Irf8* or *PU.1*.

Aside from the aforementioned function of IRF4 in GC formation, IRF4 drives plasma-cell differentiation. Mice with deletion of *Irf4* in GC

B cells that have undergone IgG1 CSR manifest complete loss of IgG1-producing plasma cells (Klein et al., 2006). IRF4 activates *Prdm1* by directly binding to its intron 5 (Sciammas et al., 2006). IRF4-mediated induction of *Prdm1* initiates the plasma-cell gene expression program, and reintroduction of Blimp-1 into IRF4-deficient B cells restores antibody production (Sciammas et al., 2006). Recently, IRF4 was shown to upregulate ZBTB20, a BCL-6 homolog that facilitates the generation of plasma cells (Chevrier et al., 2014). The action of IRF4 in gene regulation requires cooperation with other transcription factors. For instance, IRF4 targets AP-1/IRF composite elements via interaction with the basic leucine-zipper transcriptional factor ATF-like (BATF), which is an AP-1 family transcription factor, in B cells (Glasmacher et al., 2012; Ochiai et al., 2013). Interestingly, genome-wide analysis of the binding motif of IRF4 revealed that IRF4 preferentially binds to the interferon sequence response element motif in plasma cells to activate the gene expression program associated with plasma cells (Ochiai et al., 2013), suggesting that IRF4 concentration determines GC vs plasma-cell fate by targeting a distinct motif in target genes.

XBP-1 not only is a critical transcription factor controlling the secretion of antibodies by plasma cells but also plays an important role in the unfolded protein response. Most secreted proteins fold correctly upon disulfide-bond formation and upon being properly modified by posttranslational N-linked glycosylation in the ER lumen. When the level of misfolded proteins exceeds the ER capacity, the unfolded protein response may be triggered; this allows the ER to either expand protein folding capacity or initiate apoptosis (Ron & Walter, 2007). Upon ER stress, inositol-requiring kinase 1α is activated, leading to the excision of an intron from *XBP-1* pre-mRNA and a translational frameshift that results in the production of an alternatively spliced form of XBP-1 with increased transcriptional activity (Calfon et al., 2002; Yoshida, Matsui, Yamamoto, Okada, & Mori, 2001). This XBP-1 mRNA is rapidly induced in vitro by stimuli that are able to trigger plasma-cell differentiation and is required for the generation of antibody-secreting plasma cells (Iwakoshi et al., 2003; Reimold et al., 2001; Todd et al., 2009). Specifically, XBP-1 is important for the generation of plasma cells in the late phase of B-cell maturation, as B-cell-specific *Xbp-1* knockout mice manifest substantially reduced Ig production but display normal induction of the plasma-cell marker CD138 (Todd et al., 2009). Although the upregulation of XBP-1 level during plasma-cell differentiation has been proposed to be linked with the unfolded protein response resulting from massive and improperly folded Ig (Iwakoshi et al., 2003), it has been

demonstrated that B cells that fail to secrete IgM still upregulate XBP-1 normally following stimulation to induce plasma-cell differentiation (Hu, Dougan, McGehee, Love, & Ploegh, 2009). XBP-1 increases cell size, lysosome content, mitochondrial mass and function, ribosome number, and general protein synthesis at least in part by regulating genes encoding secretory pathway components (Shaffer et al., 2004).

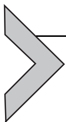
2.4 Maintenance of Plasma Cells

After leaving secondary lymphoid organs, plasmablasts, the dividing antibody-secreting cells, circulate in blood and migrate to bone marrow where they are exposed to survival factors necessary to further differentiate into long-lived plasma cells (Radbruch et al., 2006). It has been demonstrated that phenotypically distinct plasma-cell precursors can be found in mouse bone marrow 2 weeks after immunization (O'Connor, Cascalho, & Noelle, 2002); proliferation of these precursors is required for the generation of long-lived plasma cells. Plasmablasts and plasma cells can be distinguished based on the fact that plasma cells express a relatively higher level of Blimp-1 (Kallies et al., 2004). The chemokine receptor CXCR4 is important for plasma-cell homing to bone marrow and for interaction with stromal cells that express CXCL12 (CXCR4 ligand), which enables their survival in the bone marrow niche (Tokoyoda, Egawa, Sugiyama, Choi, & Nagasawa, 2004). Specifically, IgG^{+} plasma cells are associated with $CXCL12^{hi}$ reticular cells in bone marrow. CD28 is expressed on all plasma cells, including splenic short-lived plasma cells and bone marrow long-lived plasma cells. However, CD28 selectively supports the survival of bone marrow long-lived plasma cells but not splenic short-lived plasma cells. Additionally, bone marrow $CXCL12^{hi}$ reticular cells express CD80, the CD28 ligand, which further provides long-lived plasma cells with the CD28-driven survival signal upon interaction with reticular cells (Rozanski et al., 2011).

Other cells and chemokines in bone marrow also play important roles in supporting plasma-cell survival. An in vivo study showed that APRIL and its receptor, B-cell maturation antigen (BCMA), are required for plasma-cell maintenance (Benson et al., 2008). However, the expression of APRIL is low on $CXCL12^{hi}$ reticular cells, but the bone marrow resident eosinophils, megakaryocytes, and monocytes express high levels of APRIL and CXCL12 (Belnoue et al., 2012; Chu et al., 2011; Winter et al., 2010). In addition to APRIL, IL-6 is also expressed by these cells in bone marrow to sustain

plasma-cell survival (Belnoue et al., 2012; Chu et al., 2011; Winter et al., 2010). Other studies also have indicated that long-lived plasma cells in bone marrow can be sustained by BAFF, APRIL, and IL-6 in vitro, which is associated with the enhanced expression of the antiapoptotic protein, myeloid cell leukemia 1 (Mcl-1), in plasma cells (O'Connor et al., 2004). Accordingly, mice lacking BCMA, the receptor for BAFF, show reduced capacity to maintain long-lived plasma cells in bone marrow after immunization with TD antigen (O'Connor et al., 2004). In addition, CD93 is expressed during early B-cell development but is reinduced during plasma-cell differentiation. *Cd93*-deficient mice are unable to maintain antibody production and the numbers of plasma cells in bone marrow, suggesting that CD93 also participates in plasma-cell maintenance in the bone marrow niche (Chevrier et al., 2009).

B-cell intrinsic transcription factors also support the survival of long-lived plasma cells. The number of plasma cells in bone marrow is reduced when Blimp-1 is deleted after immunization with TD antigen (Shapiro-Shelef, Lin, Savitsky, Liao, & Calame, 2005). The BTB–POZ transcription factor ZBTB20, which is expressed in GC B cells but is most highly expressed in long-lived plasma cells, is also essential for the maintenance of long-lived plasma cells and for durable antibody responses (Chevrier et al., 2014; Wang & Bhattacharya, 2014). ZBTB20 is regulated by IRF4 and antagonizes the effect of BCL-6 to promote plasma-cell differentiation and longevity. Activation of APRIL-mediated BCMA signaling increases the expression of Mcl-1, which controls bone marrow plasma-cell survival (Peperzak et al., 2013). Aiolos, one of the Ikaros family members, is essential for plasma-cell generation. Aiolos-deficient mice fail to generate high-affinity plasma cells in bone marrow and to sustain serum antibody titers after immunization, yet SHM and the generation of both memory B cells and short-lived high-affinity plasma cells in the spleen appear to be normal (Cortes & Georgopoulos, 2004).



3. MEMORY B CELLS

3.1 Properties of Memory B Cells

Memory B cells have several unique features including long lifespan, high sensitivity to low doses of antigen, quick and robust proliferation, and rapid differentiation into plasma cells that produce high-affinity antibodies during the secondary response. CD27 is a marker for human memory B cells (Klein, Rajewsky, & Kuppers, 1998). These CD27^{+} memory B cells also express

IgM or isotype-switched Ig. However, CD27 is not an appropriate marker for mouse memory B cells (Anderson, Tomayko, Ahuja, Haberman, & Shlomchik, 2007). CD80 combined with CD73 and program cell death 1 ligand 2 (PD-L2, also called CD273) were identified as markers for mouse memory B cells and are expressed at different levels among different subsets (Tomayko, Steinel, Anderson, & Shlomchik, 2010). Based on the levels of CD80, CD73, and PD-L2, at least five phenotypic subsets of murine memory B cells can be defined, regardless of whether IgM or class-switched Ig is expressed (Tomayko et al., 2010). The heterogeneity within the murine memory B-cell compartment represents the distinct types of memory B cells with different ontology and maturity (Tomayko et al., 2010). Other reports have also shown that Fc-like receptor proteins 2 and 4 are predominantly expressed by human memory B cells (Davis, 2007). Memory B cells can survive for long periods and can induce faster and stronger humoral responses when they reencounter the same antigen (Pape, Taylor, Maul, Gearhart, & Jenkins, 2011), in contrast to plasma cells that provide the first line of protection against infection but do not respond to the second infection because of low expression of membrane-bound Ig (Manz, Lohning, Cassese, Thiel, & Radbruch, 1998). Although spleen and tonsil are the major reservoirs for antigen-specific human memory B cells, they appear to be dispensable for preserving immunological memory following a reencounter with antigen (Giesecke et al., 2014).

3.2 Generation of Memory B Cells

In general, memory B cells expressing class-switched and high-affinity BCRs develop within the GC (MacLennan, 1994), but growing evidence also indicates the existence of GC-independent memory B cells (Anderson et al., 2007; Kaji et al., 2012; Taylor, Pape, & Jenkins, 2012) and unswitched IgM^+ memory B cells (Dogan et al., 2009; Klein, Kuppers, & Rajewsky, 1997; Pape et al., 2011). Memory B cells from the GC-independent pathway express unmutated and low-affinity BCR. The requirement of BCL-6 for the GC reaction has been utilized to investigate the generation of GC-independent memory B cells (Kaji et al., 2012; Toyama et al., 2002). Mice that receive memory B cells derived from *Bcl-6*$^{-/-}$ donors can still produce antigen-specific IgM or isotype-switched (sw) Ig(G, A, or E) to a lesser extent, whereas mice that receive B cells from wild-type mice produce substantial IgM and sw Ig memory B cells (Taylor et al., 2012).

To generate TD memory B cells, antigen-activated B cells migrate to the borders of the B-cell and T-cell zones of secondary lymphoid organs and form stable interactions with cognate pre-GC Tfh cells to receive helper signals (Qi et al., 2008). Cognate B cells then migrate to the outer follicles and proliferate, where some of them differentiate into short-lived plasma cells (McHeyzer-Williams & McHeyzer-Williams, 2005) and some of the others develop into memory B cells (Anderson et al., 2007; Kaji et al., 2012; Taylor et al., 2012). These memory B cells produced early during an immune response in the extrafollicular area are called GC-independent memory B cells. However, certain B cells that have encountered Tfh cells reenter the B-cell follicle to form the dark zone of the GC, where they undergo clonal expansion and SHM of BCR. After exiting the cell cycle, GC B cells with diverse BCR affinities migrate to the light zone to undergo affinity selection via interaction with antigen-presenting follicular DCs and cognate Tfh cells. The affinity-matured GC B cells then exit the GC to become long-lived post-GC plasma cells or memory B cells, the latter of which are also called GC-dependent memory B cells (McHeyzer-Williams & McHeyzer-Williams, 2005). The signals required for prememory B-cell exit from GC cycle are still largely unknown, but maintenance of the specificity of memory B-cell subsets is dependent on transcriptional regulation (Wang et al., 2012). Conditional ablation of *T-bet* revealed that T-bet is required for generating IgG2a$^+$ memory B cells and steady-state survival, whereas RORα is required for survival of IgA$^+$ memory B cells (Wang et al., 2012).

Distinct from B2 cells, such as marginal zone and follicular B cells, B1 cells are a self-renewing subset of mature B cells that are predominately present in the peritoneal and pleural cavities. B1 cells can also generate memory B cells by a TI pathway (Alugupalli et al., 2004; Yang et al., 2012b). In the context of priming with glycolipid (FtL) from *Francisella tularensis* live-vaccine strain, FtL-specific B1-a memory cells arise in spleen and then rapidly migrate to the peritoneal cavity. Following FtL rechallenge, these memory B cells remigrate to the spleen and produce anti-FtL IgM (Yang et al., 2012a, 2012b).

3.3 Regulation of Memory B-Cell Formation

By which mechanism is memory B-cell fate determined? Little is known about the intrinsic mechanisms that are essential for the formation of memory B cells. The search for master regulators of transcription that control memory B-cell fate via gene expression profiling has proved challenging.

In the GC-independent memory B-cell pathway, CD40 signaling provided by pre-GC Tfh cells alone is sufficient to induce activated B cells to differentiate into memory B cells but not GC B cells (Taylor et al., 2012). IL-21 signaling provided by cognate T cells upregulates the expression of BCL-6 in B cells (Linterman et al., 2010). This suggests that the duration of conjugate formation can steer activated B cells to become GC B cells with adequate T-cell help or become GC-independent memory B cells with a lower level support (Kurosaki, Kometani, & Ise, 2015). In addition, some reports have defined the mechanisms that govern the size of memory B cells in vivo. For instance, studies focusing on the function of the BCL-2 family in B-cell homeostasis have revealed that this family is a major regulator of memory B-cell survival, and therefore, BCL-2 is a key molecule that controls humoral responses (Clybouw et al., 2011; Fischer et al., 2007; Takahashi, Ohta, & Takemori, 2001). Activated human B cells express higher levels of the p53-upregulated modulator of apoptosis (PUMA) and proapoptotic factors BCL-2-interacting mediator of cell death (BIM) but a lower level of the prosurvival factor BCL-2 compared with resting B cells or memory B cells. Overexpression of BCL-2 or deletion of BIM or PUMA leads to an increase in size of the memory B-cell compartment (Clybouw et al., 2011; Fischer et al., 2007; Takahashi et al., 2001). In addition, Syk, the key signal transducer in BCR-mediated signaling in mature B cells, is needed for the survival of memory B cells (Ackermann et al., 2015). Thus, it is likely that memory B cells are supported by multiple signaling pathways that promote survival (Kurosaki et al., 2015). It is also conceivable that memory B cells need to adopt an intrinsic mechanism that can ensure their long-term survival. Ongoing autophagy and limited cell death are characteristic of memory B cells, which ensure the long-term maintenance of memory B-cell pools (Chen et al., 2014). During acute viral infection, the generation of viral antigen-specific memory B cells can be inhibited by the perforin-dependent effects of natural killer cells on the suppression of CD4 T cells and Tfh cells (Rydyznski et al., 2015).

In the context of transcriptional regulation, signal transducer and activator of transcription 5 (STAT5) is activated in human GC B cells, leading to the induction of BCL-6, which may facilitate the commitment to memory B cells by providing memory B cells with self-renewal properties (Scheeren et al., 2005). However, BCL-6 is not expressed in memory B cells, and its expression was found to decrease significantly in an in vitro human memory B-cell differentiation culture system (Kuo et al., 2007). Moreover, ectopic expression of BCL-6 in human memory B-cell differentiation cultures

results in formation of fewer memory B cells, demonstrating that repression of *BCL-6* is required for memory B-cell differentiation. A recent study also indicated that epigenetic regulation plays a role in memory B-cell differentiation. B-cell-specific deletion of monocytic leukemia zinc-finger protein (*MOZ*), a histone acetyltransferase, could alter cell-fate decisions in both primary and secondary immune responses (Good-Jacobson et al., 2014). The absence of MOZ in B cells impairs dark-zone generation with a concomitant decrease in both cell-cycle progression and BCL-6 expression, yet the differentiation to IgM and low-affinity IgG^+ memory B cells is enhanced.

3.4 Activated B-Cell Factor-1 Promotes the Generation of Memory B Cells

We recently showed that the basic helix-loop-helix transcriptional repressor, activated B-cell factor-1 (ABF-1), is prevalently expressed in purified mouse and human memory B cells, and its expression is suppressed by Blimp-1 (Chiu et al., 2014) (Fig. 1). ABF-1 was identified by yeast two-hybrid analysis using the basic helix-loop-helix region of E2-2, another basic helix-loop-helix E protein member, as a bait. ABF-1 expression is restricted to human lymphoid tissue, fetal liver, bone marrow, activated B cells, and Epstein–Barr virus-transformed cell lines (Massari et al., 1998). ABF-1 can form homodimers or a heterodimer with the E protein E12 or E47, which then facilitates its binding to the E box DNA element, resulting in the repression of E47-mediated transcriptional activity (Massari et al., 1998).

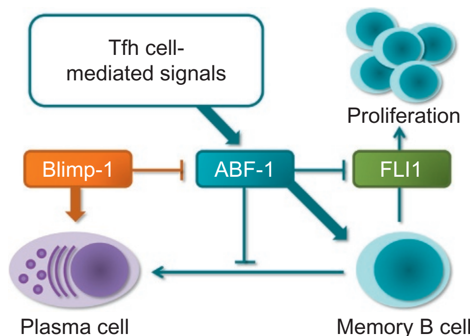
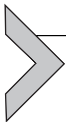


Fig. 1 ABF-1 promotes the generation of memory B cells. In mature B cells, ABF-1 is induced by T follicular helper (Tfh) cell-mediated signals to promote memory B-cell fate. ABF-1 downregulates the expression of *FLI1* to repress cell proliferation and prevents plasma-cell fate. In plasma cells, the expression of *ABF-1* is suppressed by Blimp-1.

ABF-1 coordinates with the protein ID2 to suppress certain B-cell-specific genes by antagonizing the activity of E2A (Mathas et al., 2006). Additionally, B cells treated with *Staphylococcus aureus* strain Cowan or IL-2 express *ABF-1*, linking its function to B-cell activation (Massari et al., 1998). The mouse ortholog of ABF-1 is MyoR (or Musculin, Msc), which is named for its ability to repress myogenesis. Msc is expressed in undifferentiated myoblasts and is downregulated during differentiation in vitro (Lu, Webb, Richardson, & Olson, 1999). We showed that ectopic expression of ABF-1 prevents the production of antibody-secreting plasma cells in an in vitro differentiation system derived from stimulating CD27^{+} human blood memory B cells with IL-21 and anti-CD40. Conversely, knockdown of ABF-1 potentiates the formation of antibody-secreting cells. A transgenic mouse model that expresses inducible ABF-1 after immunization with TD antigen demonstrated that overexpression of ABF-1 during an immune response facilitates the generation of GC and antigen-specific memory B cells but blocks the production of antibody-secreting plasma cells. When we searched for ABF-1 target genes via microarray analysis, we found that ABF-1 downregulates the expression of *FLI1*, which accelerates cell proliferation (Truong & Ben-David, 2000). Consistent with this result, overexpression or knockdown of ABF-1 in human memory B cells results in reduced or enhanced proliferation, respectively. It is plausible that ABF-1-mediated suppression of *FLI1* may at least partly explain why memory B cells manifest cell-cycle arrest (Anderson, Hannum, & Shlomchik, 2006).



4. BLIMP-1 IS A KEY REGULATOR OF PLASMA-CELL DIFFERENTIATION

4.1 Functional Roles of Blimp-1 in B Cells

Blimp-1, initially named PRDI-BF1 (positive regulatory domain I-binding factor 1), was discovered as a transcriptional repressor of *IFN-β* in response to virus infection of human osteosarcoma cell line (Keller & Maniatis, 1991). Blimp-1 is a conserved transcription factor for which homologs have been identified in multiple species including *Caenorhabditis elegans*, zebrafish, *Xenopus*, and *Drosophila melanogaster* but not in unicellular eukaryotes such as *Saccharomyces cerevisiae* (Tunyaplin, Shapiro, & Calame, 2000). Blimp-1 not only plays critical roles in embryonic development of multiple species but also controls the differentiation and function of many cell lineages in adult mice (Bikoff, Morgan, & Robertson, 2009).

Blimp-1 was originally cloned by a subtractive hybridization screen using cDNA from a mouse BCL-1 lymphoma cell line that had been treated with IL-2 and IL-5 (Turner, Mack, & Davis, 1994). Moreover, ectopic expression of Blimp-1 in BCL-1 cells is sufficient to drive the generation of IgM-secreting plasma cells. One study that used immunohistochemistry to detect Blimp-1 expression in lymphoid tissues following immunization revealed that Blimp-1 is expressed in plasma cells derived from either TI or TD immunization in spleen and in long-lived plasma cells in bone marrow (Angelin-Duclos et al., 2000). Studies using reporter mice carrying the GFP gene inserted at the *Prdm1* locus showed that heterozygous mice expressed GFP in antibody-secreting cells following immunization and that bone marrow long-lived plasma cells expressed higher levels of Blimp-1 than the plasmablasts detected in the spleen (Kallies et al., 2004). The importance of Blimp-1 in plasma-cell differentiation is demonstrated by the phenotypes observed in B-cell-specific *Prdm1* conditional knockout mice (Shapiro-Shelef et al., 2003). Mice with B-cell-specific deletion of *Prdm1* undergo normal B-cell development but fail to produce plasma cells and secrete Ig following immunization with TI or TD antigens, with concomitant enhancement of the generation of GC B cells in the case of immunization with TD antigen. The production of $B220^{lo}CD138^{+}$ plasma cells in *Prdm1*-deficient mice is impaired after immunization with TD antigen (Shapiro-Shelef et al., 2003). The $NP^{+}CD79b^{+}B220^{-}CD138^{-}$ preplasma memory B cells are greatly reduced, but $NP^{+}B220^{+}CD138^{-}$ memory B cells are not affected in B-cell-specific *Prdm1*-deleted mice (Shapiro-Shelef et al., 2003). An ex vivo study using Blimp-1-deficient B cells stimulated with LPS revealed that those cells proliferate more vigorously than control cells but produce little IgM-secreting cells and $B220^{int}CD138^{+}$ plasma cells (Shapiro-Shelef et al., 2003). In addition, Blimp-1 is required for antibody secretion by B1 cells, a peritoneal B-cell subset that contributes to the generation of circulating natural antibodies for protection against bacterial and viral infection (Savitsky & Calame, 2006). Although Blimp-1 is essential for the generation and maintenance of plasma cells, it is not required for the initiation of the commitment of plasma-cell fate (Kallies et al., 2007). Besides, Blimp-1 not only is required for the generation of plasma cells but also needs to be continuously expressed in long-lived plasma cells in bone marrow to maintain these populations (Shapiro-Shelef et al., 2005). Inducible deletion of *Prdm1* in vivo following immunization results in the loss of plasma cells in bone marrow and reduced titers of antigen-specific IgG1 in serum (Shapiro-Shelef et al., 2005). It turns out that Blimp-1 maintains the survival of plasma

cells, at least in part, by directly suppressing the proapoptotic gene *ASK1* (apoptosis signal-regulating kinase 1) by binding to 250 bp upstream of the transcription start site (Lin et al., 2012). ASK1 is activated in vitro following treatment of mouse splenic B cells with LPS or cytokine-mimicking TI or TD stimuli-mediated generation of short-lived plasma cells, respectively, which may partly account for the short lifespan of short-lived plasma cells. More importantly, not only antigen-specific short-lived cells but also long-lived plasma cells generated by immunization accumulate in *ASK1*-deficient mice. Thus, long-lived plasma cells may require sustained suppression of *ASK1* by Blimp-1 to avert stress-induced apoptosis.

Blimp-1 is also important for the survival of multiple myeloma (MM) cells, which are transformed plasma cells. MM is an incurable neoplasm characterized by accumulation of a monotypic plasma-cell population in bone marrow, the presence of monoclonal immunoglobulins in serum and/or urine, and osteolytic bone lesions (Hideshima & Anderson, 2002). MM is a genetically clonal malignancy that progresses slowly (Greipp et al., 2005). Normal B cells undergo CSR and SHM via the GC reaction to produce high-affinity antibodies. During this process, AID generates double-strand DNA breaks at the IgH locus, which creates transformation-promoting genetic lesions via a high mutation rate (Gonzalez et al., 2007). Although these mutations are repaired immediately, some aberrant translocations can accumulate, which may result in the generation of monoclonal gammopathy of undetermined significance (Kyle et al., 2007); this is an indolent, asymptomatic condition but has an increased incidence of MM. Chromosomal translocations at the immunoglobulin switch region of the IgH locus is frequently juxtaposed to proto-oncogenes, such as Cyclin D1, Cyclin D3, c-Maf, FGFR3, MMSET, and c-Myc (Bergsagel & Kuehl, 2005; Kuehl & Bergsagel, 2002). Complexity in the activating mutations in the genes *Ras* and *TP53* and inactivating mutations in cyclin-dependent kinase genes leads to the malignant transformation of plasma cells (Palumbo & Anderson, 2011). Pathogenesis may occur upon a secondary genetic abnormality, ie, deletion or translocation, such as an abnormality in the plasma-cell program genes, XBP-1, Blimp-1, and IRF4 (Morgan, Walker, & Davies, 2012). In addition to the intrinsic alteration of genes in MM, the communication between MM and the bone marrow microenvironment that supplies survival signals is also important for MM progression. We previously showed that Blimp-1 is required for the survival of MM cells, and deletion of Blimp-1 results in apoptosis of MM cells (Lin, Kuo, Ying, Yang, & Lin, 2007). Blimp-1 sustains the survival of MM cells by directly suppressing

the expression of *ASK1* (Lin et al., 2012). Inducible expression of ASK1 in MM cells causes apoptosis, which is linked to a significant induction in the level of the proapoptotic protein Bim, especially Bim_{EL}, which is encoded by an alternatively spliced form of Bim pre-mRNA (O'Connor et al., 1998). Furthermore, ASK1 is required for Blimp-1 knockdown-induced apoptosis and -induced Bim elevation. Notably, Blimp-1 is a tumor-suppressor gene whose mutation is linked with activated B cell-like diffuse large B-cell lymphoma (ABC-DLBCL) (Mandelbaum et al., 2010). Accordingly, splenomegaly and lymphoproliferation was frequently observed in aged mice with B-cell-specific deletion of *Prdm1* (Mandelbaum et al., 2010). Using an animal model with constitutive activation of the canonical NF-κB pathway and deletion of *Prdm1* in B cells, Calado et al. (2010) showed that animals developed a lymphoma resembling human ABC-DLBCL. Therefore, in the B-cell lineage, Blimp-1 is not only required for the generation and maintenance of normal plasma cells but also involved in sustaining the survival of malignant plasma cells and suppressing the generation of a subtype of DLBCL.

4.2 Roles of Blimp-1 in Other Cell Lineages

In addition to the B-cell lineage, Blimp-1 has been implicated in other immune responses. In $CD4^+$ T helper cells, Blimp-1 plays a regulatory role to prevent T cells to be overactive because conditional deletion of *Prdm1* in mouse T cells causes the accumulation of effector memory T cells and results in the development of autoimmune colitis (Kallies et al., 2006; Martins et al., 2006). Blimp-1 also optimizes the differentiation of Th2 cells and limits Tfh-cell differentiation (Cimmino et al., 2008; Johnston et al., 2009). *Prdm1* deletion in T cells reduces the differentiation and cytotoxic activity of $CD8^+$ effector cells in vivo, thereby impairing immunity against lymphocytic choriomeningitis virus infection (Kallies, Xin, Belz, & Nutt, 2009; Rutishauser et al., 2009; Shin et al., 2009). In DCs, Blimp-1 is important for restraining the production of cytokines and homeostasis of DC subsets (Chan et al., 2009). Mice lacking Blimp-1 in all hematopoietic lineages, generated by crossing *Prdm1*$^{f/f}$ mice with Tie2-cre transgenic mice revealed new roles of Blimp-1 in DC lineage (Chan et al., 2009). Blimp-1 negatively regulates the homeostatic development of a select subset of cDCs, $CD8^-$ cDCs, and is required for DC maturation. Cytokine- or Toll-like receptor ligand-induced maturation of DCs is impaired both in vivo and in vitro when Blimp-1 is absent (Chan et al., 2009). Moreover, DC-specific deletion

of *Prdm1* resulted in altered DC function and developed systemic lupus erythematosus (Kim, Zou, Goldstein, Reizis, & Diamond, 2011). In addition, the production of several cytokines/chemokines, including IL-2, IL-6, IL-10, Ccl-2, IFN-γ, TNF-α, and TNF-β, has been reported to be either directly or indirectly suppressed by Blimp-1 (Chan et al., 2009; Cimmino et al., 2008; Cretney et al., 2011; Martins, Cimmino, Liao, Magnusdottir, & Calame, 2008; Smith et al., 2010). Blimp-1 is also expressed in keratinocytes, and *Prdm1* deletion in mouse adult keratinocytes results in macrophage/granulocyte-dominant skin inflammation (Chiang et al., 2013). Adult mice lacking Blimp-1 in keratinocytes manifest chronic skin inflammation, active innate and adaptive immune responses in lymphoid organs, and enhanced granulopoiesis in bone marrow. Neutrophilia and skin inflammation in conditional knockout mice at least partly results from an elevated level of G-CSF, which is indirectly regulated by Blimp-1. In agreement with these results, Blimp-1 level is reduced in skin lesions of some cases of human eczema and chronic skin inflammation (Chiang et al., 2013).

4.3 Mechanisms of Transcriptional Repression by Blimp-1

Human and mouse Blimp-1 contain 789 and 856 amino acid residues, respectively, that comprise several functional domains: the acidic region, the PR domain (named from PRDI-BF1 and the Rb-binding protein RIZ1, the first two proteins in which the PR domain was characterized), the proline-rich domain, and a Krüppel-type zinc-finger domain (Tunyaplin et al., 2000). Mouse Blimp-1 contains an additional 31 residues at the N-terminus despite the high sequence identity between human and mouse Blimp-1 (Huang, 1994). *PRDM1* encodes two major isoforms, namely Blimp-1α and Blimp-1β, expression of which is driven from alternative promoters (Gyory, Fejer, Ghosh, Seto, & Wright, 2003). Blimp-1α is transcribed from the promoter immediately upstream of exon 1, which produces the full-length protein. Blimp-1β is transcribed from a distinct promoter and exon using a promoter that is located upstream of exon 4. Compared with Blimp-1α, Blimp-1β lacks the first 101 residues and has only part of the PR domain. Although Blimp-1β retains DNA-binding activity, nuclear localization, and the ability to associate with histone deacetylases (HDACs), it cannot repress target genes (Gyory et al., 2003). Blimp-1β is overexpressed in MM (Ocana et al., 2006), non-GC B-cell DLBCL (Liu et al., 2007), and T-cell lymphoma (Zhao et al., 2008), which displays

a much lower ratio of Blimp-1α/Blimp-1β than normal plasma cells; this may be a consequence of aberrant hypermethylation-mediated silencing of the Blimp-1α promoter and hypomethylation of the Blimp-1β promoter (Zhang et al., 2015). However, the physiological functions, if any, of Blimp-1β have not been formally demonstrated.

The functions of two acidic regions located at N-terminus and C-terminus of Blimp-1 have not been fully characterized. The PR domain resembles the SET domain, which mediates histone methyltransferase activity (Dillon, Zhang, Trievel, & Cheng, 2005). However, Blimp-1 has not been shown to have histone methyltransferase activity. The proline-rich domain interacts with several proteins involved in epigenetic regulation, including HDAC1/2, Groucho family proteins, and lysine-specific demethylase-1 (Ren, Chee, Kim, & Maniatis, 1999; Su et al., 2009; Yu, Angelin-Duclos, Greenwood, Liao, & Calame, 2000). Accordingly, deletion of this proline-rich region substantively affects the function of Blimp-1 in controlling plasma-cell differentiation as well as in promoting apoptosis upon its enforced expression in immature B cells (Messika et al., 1998; Su et al., 2009). By interacting with the zinc-finger domain of Blimp-1, the histone H3 lysine methyltransferase G9a assists Blimp-1-mediated repression of *IFN-β* in the osteosarcoma cell line U2OS (Gyory, Wu, Fejer, Seto, & Wright, 2004). The interaction between histone modifiers and Blimp-1 results in a change in status of histone modifications and gene expression. For example, recruitment of HDAC1/2 to endogenous Blimp-1 target sites enables the removal of acetyl groups from histones and reduces gene expression (Yu et al., 2000). G9a methylates H3 at lysine 9, which is a repressive mark in epigenetic regulation of transcription. Moreover, lysine-specific demethylase-1 can remove mono- or dimethyl groups on H3 lysine 4, which is generally considered an active histone mark. In mouse primordial germ cells, PRMT5, a histone arginine-specific methyltransferase that transfers a methyl group to arginine 3 of histone H2A and/or H4 tails, is functionally associated with Blimp-1 (Ancelin et al., 2006), although the precise region of Blimp-1 that directly interacts with PRMT5 has not been determined. Blimp1-Prmt5 colocalization is associated with high levels of H2A/H4 arginine 4 methylation in primordial germ cells in mouse embryos at day 8.5 (Ancelin et al., 2006). Notably, interaction between Blimp-1 and those histone modifiers appears to be cell type-specific because an interaction between Blimp-1 and Prdm5 has not been detected in plasma cells (Su et al., 2009). There are five consecutive zinc-finger domains in the C-terminal half of Blimp-1, and zinc fingers 1 and 2 confer

DNA-binding activity (Kelley et al., 1998). Two independent studies have elucidated the DNA-binding consensus motifs of Blimp-1, which are (A/C)AG(T/C)GAAAG(T/C)(G/T) (Kuo & Calame, 2004), GTGAAAGT, and G(N)GAAAGT (Doody et al., 2010).

Several studies have identified the target genes regulated by Blimp-1 in B cells and other immune cells (Fig. 2). Blimp-1 directly represses *c-Myc* to halt cell cycling, a characteristic of terminally differentiated plasma cells (Lin, Wong, & Calame, 1997). However, during Blimp-1-mediated plasma-cell differentiation, *c-Myc* repression is necessary but not sufficient (Lin, Lin, & Calame, 2000). *CIITA*, which is a master regulator of MHC class II expression, is directly suppressed by Blimp-1, thereby inhibiting the antigen-presenting function of plasma cells (Piskurich et al., 2000). Blimp-1 directly suppresses *Pax5*, thereby allowing the transition from mature B cells to plasma cells (Lin et al., 2002). Blimp-1-dependent suppression of *Pax5* is sufficient to downregulate or upregulate the expression of *CD19* or *J chain*, respectively, in plasma cells. Moreover, several other key transcription factors involved in GC functions are directly suppressed by Blimp-1. For instance, Blimp-1 directly binds to the mouse *Bcl-6* genomic locus located 5 kb upstream of the first exon, such that Bcl-6 and Blimp-1 work as antagonistic transcription factors to control the fate decision during the transition

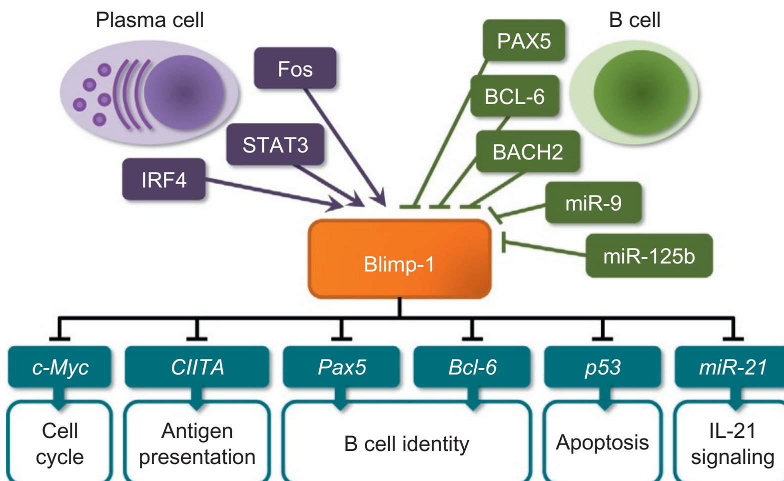


Fig. 2 Blimp-1 is a key regulator of plasma-cell differentiation. Within B-cell lineage, the transcriptional repressor, Blimp-1, suppresses target genes with functions in cell cycle, antigen presentation, B-cell identity, and apoptosis to direct plasma-cell differentiation. Blimp-1 is upregulated by Fos, STAT3, and IRF4 in plasma cells, but downregulated by PAX5, BCL-6, BACH2, miR-9, and miR-125b in B cells, particularly in GC B cells.

from GC B cells to plasma cells (Cimmino et al., 2008). Blimp-1 can directly suppress *p53* by binding to its promoter (Yan et al., 2007), suggesting a possible mechanism underlying the effects of Blimp-1 on the maintenance of long-lived plasma cells, ie, the mechanism may be partly attributable to repression of *p53*, which induces apoptosis.

Two DNA microarray studies were carried out to globally identify the genes regulated by Blimp-1 (Sciammas & Davis, 2004; Shaffer et al., 2002). Inducible expression of Blimp-1 in human mature B-cell lines was found to suppress a panel of genes that are important for BCR signaling, antigen presentation, GC reaction, and cell proliferation, whereas only a small number of genes were upregulated upon Blimp-1 expression. *SPI-B* and *ID3* were identified as Blimp-1 direct target genes (Shaffer et al., 2002). Another independent study in which Blimp-1 was expressed in murine B-cell lines showed that Blimp-1 regulates genes involved in affinity maturation, homeostasis, migration, differentiation, and antibody secretion (Sciammas & Davis, 2004). It appears that the expression of Blimp-1-dependent genes and their functions rely on the activity of specific domains of Blimp-1. For example, the PR domain of Blimp-1 is required for Ig secretion (Sciammas & Davis, 2004). Blimp-1 also regulates the expression of microRNAs in the B-cell lineage. Blimp-1 binds to the *miR-21* promoter and directly suppresses transcription of the *miR-21* mRNA (Barnes, Stephenson, Cocco, Tooze, & Doody, 2012), establishing a feedback regulatory loop that counters the effects of STAT3 on activating miR-21 induced by IL-21 signaling.

In the T-cell lineage, Blimp-1 negatively regulates the proliferation and survival of virus-specific $CD8^+$ T cells via direct suppression of *Il2ra* and *Cd27* (Shin et al., 2013). Blimp-1 also participates in limiting T-cell proliferation during an immune response by directly repressing *Il-2* and *Fos* (Martins et al., 2008). During T-cell polarization, Blimp-1 alters the production of cytokines or key transcription factors that are important for determining Th1 vs Th2 polarization. Blimp-1 binds directly to a distal regulatory region in *Ifn-γ* and to multiple sites in *Tbx21* (encoding T-bet) and *Bcl-6* (Cimmino et al., 2008). By suppressing genes critical for Th1 differentiation, Blimp-1 promotes the generation of Th2 cells. Although Blimp-1 mainly acts as a transcriptional repressor, it also can activate gene expression. For example, the IL-4/STAT4-dependent pathway induces Blimp-1 expression in Th1 cells, which in turn acts synergistically with c-Maf to induce IL-10 expression by directly binding to a region ~9 kb upstream of the transcription start site of the *Il-10* locus (Neumann et al., 2014).

4.4 Regulation of Blimp-1 Expression

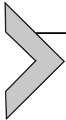
Expression of *PRDM1* is tightly regulated during plasma-cell differentiation. Several regulatory mechanisms, including transcriptional, posttranscriptional, and posttranslational, have been reported to regulate Blimp-1 expression. Aside from the aforementioned numerous transcription factors, including BCL-6, PAX5, and BACH2, that directly repress *Prdm1* transcription in mature B cells (Mora-Lopez et al., 2007; Ochiai et al., 2006; Tunyaplin et al., 2004), the expression of Blimp-1 can also be regulated by other transcription factors that act downstream of Toll-like receptor, BCR, TNFR, and cytokine signaling pathways. ERKs downstream of BCR or cytokine receptor signaling pathways induce Blimp-1 expression (Yasuda et al., 2011). Consistent with this, conditional deletion of ERK1 and ERK2 in GC B cells leads to impaired plasma-cell production following immunization with TD antigen. AP-1 binds directly to the murine *Prdm1* promoter, and CD40L/IL-4-stimulated B cells isolated from transgenic mice overexpressing c-Fos show augmented Blimp-1 expression as well as enhanced plasma-cell differentiation (Ohkubo et al., 2005). However, Fos/AP-1 is not essential for *Prdm1* activation (Ohkubo et al., 2005). Activated STAT3 acting downstream of cytokine signaling pathways also induces Blimp-1 expression. Activation of STAT3 by IL-21 as well as ectopic expression of STAT3 in human peripheral B cells induces Blimp-1 expression (Diehl et al., 2008). Although CD40L alone does not significantly affect Blimp-1 expression, IL-21 and CD40L have synergistic effects on Blimp-1 induction (Ding, Bi, Chen, Yu, & Ye, 2013). Activated STAT3 induced by CD40L treatment in human B cells outcompetes the binding of BCL-6 to *PRDM1* intron 3 and thereby induces Blimp-1 expression. The importance of STAT3 in plasma-cell differentiation is further demonstrated by B-cell-specific *Stat3* knockout mice, in which normal B-cell development, normal IgM responses, and normal GC reactions are observed, but the mice displayed profound defects in IgG response following immunization with TD antigen (Fornek et al., 2006). STAT3 also can interact with IRF4, which mediates the induction of Blimp-1 in IL-21-stimulated B cells (Kwon et al., 2009). Several binding sites for NF-κB are found in *Prdm1* (Calame, 2008; Morgan et al., 2009). Particularly, NF-κB sites have been identified upstream of the exon 1A promoter of *Prdm1*, which regulates the induction of Blimp-1 in LPS-treated B cells (Morgan et al., 2009). Mutant B cells lacking *Prdm1* exon 1A, encompassing NF-κB sites, show severe defects in the induction of Blimp-1 in response to LPS treatment and cannot generate antibody-producing plasma cells. In agreement with

this finding, stimulated B cells isolated from mice lacking *RelA*, a subunit of NF-κB, in the B-cell lineage have a severely reduced number of plasmablasts as well as dramatically impaired induction of Blimp-1 (Heise et al., 2014).

Blimp-1 expression is also regulated at the posttranscriptional level. The microRNA miR-125b binds to the 3′-untranslated region of both Blimp-1 and IRF4 mRNAs (Gururajan et al., 2010; Malumbres et al., 2009). Ectopic expression of miR-125b in LPS-stimulated B cells results in reduced production of IgM and impaired generation of $CD138^+$ plasma cells (Gururajan et al., 2010). In Hodgkin/Reed-Sternberg cells of Hodgkin lymphoma, high levels of miR-9 and let-7 are linked with the low expression of Blimp-1, whereas overexpression or inhibition of miR-9 and let-7 expression results in reduced or increased Blimp-1 expression, respectively (Nie et al., 2008). microRNA-mediated Blimp-1 expression has also been implicated in other cell lineages. For instance, Blimp-1 and let-7c reciprocally regulate each other, which confers activation of DCs; let-7c targets the 3′-untranslated region of *Prdm1* mRNA, whereas Blimp-1 directly suppresses *let-7c* expression (Kim, Gregersen, & Diamond, 2013). The expression of miR-23a and miR-125b is negatively associated with Blimp-1 expression in human DCs and plasmacytoid DCs derived from treating monocytes with IFN-α (Parlato et al., 2013). miR-23a downregulates Blimp-1, which is important for the cytotoxic function and differentiation of $CD8^+$ cytotoxic T cells (Lin et al., 2014).

Blimp-1 contains a relatively large proportion of serine (9.2% in human), threonine (5.5% in human), and tyrosine (4.1% in human) residues, and this aspect enables Blimp-1 to be potentially regulated at the posttranslational level, eg, by phosphorylation. However, the regulation of Blimp-1 expression at the posttranslational level has been only modestly studied. Small ubiquitin-like modifier (SUMO) modification of Blimp-1 has been demonstrated in two studies (Shimshon et al., 2011; Ying et al., 2012). Blimp-1 can be covalently modified by SUMO-1 but not SUMO-2 or SUMO-3. SUMOylation of Blimp-1 is mediated by the E3 ligase PIAS1. Lysine 816 in human Blimp-1 is the only SUMOylation site. More importantly, disruption of Blimp-1 SUMOylation results in defective plasma-cell differentiation, impaired transcriptional repression of target genes, and reduced interaction with HDAC2 (Ying et al., 2012). The Blimp-1 sequence contains a high percentage of PEST (proline/glutamic acid/serine/threonine) residues, and the PEST motif is associated with a short protein half-life; specifically, PEST-containing proteins are targeted for degradation by proteasomes. Indeed, human Blimp-1 has been shown to interact with

FBXO11 (Horn et al., 2014), an F-box protein that functions as the substrate-recognition component of Skp1-Cullin-F-box-E3 ubiquitin ligase complexes and is involved in the regulation of protein ubiquitination and degradation. The interaction of Blimp-1 with FBXO11 triggers Blimp-1 degradation; conversely, knockdown of FBXO11 by a short hairpin RNA results in the stabilization of Blimp-1 in MM cell lines. In DCs, Hdr1 is an E3 ubiquitin ligase for Blimp-1 and promotes its ubiquitination and degradation (Yang et al., 2014). However, the regulatory machinery that controls Blimp-1 turnover in B cells awaits full characterization.



5. PERSPECTIVES

In this review, we have discussed recent findings concerning memory B-cell fate decision, plasma-cell differentiation and maintenance, and the functional role of the key regulator, Blimp-1, in these events. However, many intricate and unsolved issues remain, including the detailed molecular mechanisms that control how memory B cells and long-lived plasma cells are selected, how long-lived plasma cells homing to the appropriate niche, and how transcriptional or signal transduction networks are integrated to support longevity. Hence, new techniques are required to facilitate analysis at the single-cell level. Such new approaches may potentiate the manipulation of memory B cells and plasma cells for boosting humoral responses in vaccination.

The current strategies for designing vaccines focus on the acquisition of high-affinity memory B cells and long-lived plasma cells. Upon reinfection, long-lived plasma cells produce neutralizing antibodies against the original pathogen; however, these antibodies are poorly reactive with variant pathogens, whereas some memory B cells with broadly neutralizing antibody specificities may undergo a reevolution upon entering GCs and undergo additional rounds of GC reactions upon reinfection with variant pathogens, which may result in the production of high-affinity variant pathogen-specific antibodies (Purtha, Tedder, Johnson, Bhattacharya, & Diamond, 2011). It is thus intriguing to further study the mechanisms that enhance the selection of broader pools of memory B cells having the ability to recognize not only specific pathogens but also variant pathogens.

In the context of Blimp-1 biology, further understanding of the molecular mechanisms, such as the role of ABF-1 in memory B cells combined with modulating the Blimp-1/ABF-1 regulatory pathway, that determine memory B-cell vs plasma-cell fate will provide valuable insight for

developing new vaccines for infectious diseases and aid in boosting immune responses following vaccination. Regulation of Blimp-1 expression is still largely unexplored. Further studies on the posttranslational modification of Blimp-1 and its relevance in the expression and function of Blimp-1 may provide an additional layer of approaches to understand the pathogenesis of plasma-cell- or GC B-cell-relevant disorders. Additionally, proteomic-based studies of Blimp-1-interacting partners in a subset of GC B cells as well as short-lived and long-lived plasma cells may provide further insight into how Blimp-1 works during the various stages of differentiation, which may provide new directions for the design of agents to control Blimp-1 expression for managing certain plasma cell-relevant diseases.

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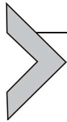

CHAPTER THREE

Deep Profiling Human T Cell Heterogeneity by Mass Cytometry

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Abstract

Advances of mass cytometry and high-dimensional single-cell data analysis have brought cellular immunological research into a new generation. By coupling these two powerful technology platforms, immunologists now have more tools to resolve the tremendous diversity of immune cell subsets, and their heterogeneous functionality. Since the first introduction of mass cytometry, many reports have been published using this novel technology to study a range of cell types. At the outset, studies of human hematopoietic stem cell and peripheral $CD8^+$ T cells using mass cytometry have shad the light of future experimental approach in interrogating immune cell phenotypic and

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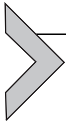
functional diversity. Here, we briefly revisit the past and present understanding of T cell heterogeneity, and the technologies that facilitate this knowledge. In addition, we review the current progress of mass cytometry and high-dimensional cytometric analysis, including the methodology, panel design, experimental procedure, and choice of computational algorithms with a special focus on their utility in exploration of human T cell immunology.



1. INTRODUCTION

Continuous and recently rapid improvements in single-cell analysis methods are allowing for exploration of cellular diversity at unprecedented depth and throughput. The development of fluorescence-based flow cytometry (Lanier et al., 1983) and the ability to probe single-cell protein expression with high throughput has been instrumental laying the foundations of modern cellular immunology (Bendall, Nolan, Roederer, & Chattopadhyay, 2012). In immunology, the use of flow cytometry is ubiquitous and is often employed as a foremost tool to identify cells of interest prior to other downstream analysis such as gene-expression profiling or other functional experiments performed in vitro or in vivo. The application of flow cytometry has been the benchmark not only for fundamental immunology but also translational biomedical research, from primary discovery to clinical diagnosis and prognosis. It offers the characterization of cell frequency, cell subsets, protein expression, and functional or proliferative assessment. More recently, high-dimensional polychromatic flow cytometry (Chattopadhyay, Gierahn, Roederer, & Love, 2014) and mass cytometry (Bandura et al., 2009; Bendall et al., 2011; Newell, Sigal, Bendall, Nolan, & Davis, 2012) allow for identification of numerous cellular subpopulations and the ability to probe relationships between expression levels of large numbers of proteins simultaneously. In this regard, recent efforts in technology development continue their push to allow better understanding of single-cell heterogeneity in terms of proteomics, transcriptomics, and immunomics (Newell & Davis, 2014).

Mass cytometry (or CyTOF, cytometry by time-of-flight) (Bandura et al., 2009) is a recently developed form of flow cytometry based on atomic mass spectrometry (Ornatsky et al., 2010; Tanner, Baranov, Ornatsky, Bandura, & George, 2013) that uses metal isotope tagged antibodies to probe cells for expression of protein antigens that are quantified on individual cells using TOF mass spectrometry. Importantly, these metal isotopes are purified

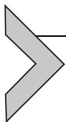
mainly from lanthanide family (with atomic numbers from 57 to 71 on periodic table) that are rarely seen in biological system (Bandura et al., 2009), making them useful as exogenous tags to probe mammalian cells. Because of the limited fluorophores and the difficulties associated with spectral compensation in flow cytometry experiments, most laboratories measure cells with no more than 10–12 parameters at a time. However, with the development of novel dyes and improved flow cytometry instrumentation, this number will continue to rise (Bendall et al., 2012). In contrast, the mass window (analog to fluorescent channel) of mass cytometry is >100 atomic mass channels with limited-to-no crosstalk. The measurements depend on the availability and quality of purified metal isotopes and the availability of appropriate chemistries for labeling probes (Tanner et al., 2013). Current practice of mass cytometry allows simultaneously measuring more than 40 different antibody parameters, permitting in-depth analysis of multiple aspects of cellular diversity of immune cells, exemplified particularly in T cell biology.

T cells are well known as key coordinators of human immune system. The roles of various types of T cells are linked to infectious disease, autoimmune disease, and cancer. Describing the phenotypes of these cells in health and disease is has been, and continues to be, a major task for biomedical immunologists. This task is particularly challenging because of the wide range of cellular factors that need to be considered simultaneously, including cell differentiation (Arens & Schoenberger, 2010; Harty & Badovinac, 2008), inhibitory/stimulatory signaling networks (Croft, 2009; Wherry & Kurachi, 2015), functional capacity (Betts et al., 2006; Newell et al., 2012), cell migration/tissue homing (Griffith, Sokol, & Luster, 2014), and antigen recognition (Newell & Davis, 2014). Study of these cellular markers is important to perceive the change of immune homeostasis upon disease perturbation.

Our understanding of adaptive immunity and categorization of types of T cell responses has been largely based on the knowledge of cellular proteins expressed by these different types of cells, such as T cell receptors (TCRs), surface markers expression, intracellular cytokines production, or intranuclear transcription factors level. In particular, these definitions of cells gave rise the operational $CD4^+$ T cell subsets (Nakayamada, Takahashi, Kanno, & O'Shea, 2012), like T_H1, T_H2, T_H17 (Harrington et al., 2005), regulatory T cells (Treg) (Josefowicz, Lu, & Rudensky, 2012), follicular T helper cells (T_{FH}) (Johnston et al., 2009), and T_H9 (Xiao et al., 2012). Conversely, lack of clear major transcription factors or dominant cytokines for segregating

subsets, human $CD8^+$ T cells have been mostly categorized using a lasting model based on their differentiation markers (eg, CD45RA and CCR7) (Sallusto, Lenig, Forster, Lipp, & Lanzavecchia, 1999). For decades, immunologists have used these markers to identify naïve, effector memory (T_{EM}), central memory (T_{CM}), and terminally differentiated (T_{EMRA}) T cells (Sallusto, Geginat, & Lanzavecchia, 2004), along with the addition of CD127 and KLRG-1 (Kaech et al., 2003). The latter was proposed by Kaech et al., which endorsed the different nomenclature as memory-precursor effector cell (MPEC) and short-lived effector cell (SLEC) (Kaech & Cui, 2012). Notably, the memory/effector subsets between these two approaches are not mutually exclusive, making it difficult to systemically identify different populations when using these four (or more, such as adding CD62L and CD28) (Gattinoni et al., 2011; Mahnke, Brodie, Sallusto, Roederer, & Lugli, 2013) markers altogether. In addition, although both systems have been widely adapted by the immunology community and proved to be informative in understanding various types of immune responses, the relationships between these two types of classifications have not been completely elucidated. As of yet, this discordance of subset terminology and their usages can make it difficult to reconcile various seemingly overlapping studies (Arsenio, Kakaradov, Metz, Yeo, & Chang, 2015; Flossdorf, Rossler, Buchholz, Busch, & Hofer, 2015).

With the advent of mass cytometry, many more markers can be integrated to systemically characterize human T cell subsets, combining markers indicative of their states of cell differentiation, memory/exhaustion, functional capacity, transcription factor expression profiles, trafficking receptor expression profile, and antigen specificity. With this type of information, it is clear that visualizing cells on two-dimension plots is insufficient and instead, a higher-dimensional perspective is needed. These methods promise a greater ability to visualize and comprehend the seemingly endless diversity of T and other lymphocyte subsets. In this review, we discuss the advancement of mass cytometry, from the technical hardware to software, and focus on their application in study of human T cell biology.



2. T CELL HETEROGENEITY: DAYS OF FUTURE PAST

After their discovery, for five decades the scientific community had seen lymphocytes as a homogeneous cell population (Murphy, 1914a). In 1967 Jacques Miller (JFAP Miller) proposed that lymphocytes were composed of T and B cells (Masopust, Vezys, Wherry, & Ahmed, 2007;

Miller & Mitchell, 1967), and received much criticism (Miller, 1999). A decade later, seminal experiments demonstrated murine Ly-1^{+} and Ly-2,3^{+} lymphocytes were functionally different (Jandinski, Cantor, Tadakuma, Peavy, & Pierce, 1976) and analogous to human CD4^{+} and CD8^{+} T cells (Reinherz & Schlossman, 1980). Unequivocal evidences also showed that the depletion of CD8α/β abrogated cell-mediated cytotoxicity (Cantor & Boyse, 1975; Shiku et al., 1975). Thus, these studies established the concept of divergent lineages of CD4^{+} and CD8^{+} T cell. Around the same time, several observations had implied the existence of distinct subsets of helper T cells (Imperiale, Faherty, Sproviero, & Zauderer, 1982; Kim, Woods, Becker-Dunn, & Bottomly, 1985; Tada, Takemori, Okumura, Nonaka, & Tokuhisa, 1978). Later, Mosmann et al. provided the first solid proof of the existence of T_H1 and T_H2 cells at the clonal level and showed their functionally distinct roles (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986). These studies were indispensible in establishing a foundation of evidence supporting the notion of T cell heterogeneity.

The rapid development of the usage of monoclonal antibodies and flow cytometry has dramatically changed our ability to look at immune cells. After its development, the widespread use of flow cytometry to investigate marker expression of T cells quickly became a standardized and required tool in immunological research. Subsequent identification of various master transcription factors and their signature cytokines has added Treg, T_H17, T_{FH}, and others to the list of CD4^{+} helper subsets in the past decade (Crotty, 2011; Josefowicz et al., 2012; Weaver, Hatton, Mangan, & Harrington, 2007). Along with other techniques, fluorescence activated cell sorting (FACS)-based experiments have enabled the exploration of the heterogeneity of T cells and their distinct functional roles, such as the reciprocal effect of Treg and T_H17 in autoimmune disease (Mucida et al., 2007). The use of antibodies to specifically probe markers of T cells and T cells subsets has therefore opened a new era of T cell heterogeneity.

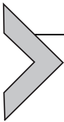
The dissimilar lineage properties of CD4^{+} and CD8^{+} T cells have earned their reputation as helper T lymphocytes and cytotoxicity T lymphocytes (CTL) during immune response, respectively. Perhaps for this reason, most immunologists view heterogeneity of these two lineages from a different perspective. In general, CD4^{+} T cells are divided into various functional subsets while CD8^{+} T cells are parted as a range of differentiation. As more CD4^{+} T cells subsets are verified, the different "helper" programs of CD4^{+} T cells seem to be delivered by various helper subsets, especially in different context of diseases. Distinctively, functional population of CD8^{+} T cells has

been often depicted as Tc1 (IFN-γ^+, TNF-α^+, and IL-2$^+$) (Woodland & Dutton, 2003) without other well defined subsets. This is possibly because most studies were only focused on a few functional markers attributed to the limited fluorescent channels in flow cytometry, or the single cytokine secretion assay such as ELISPOT. Besides their multiple functionalities (eg, ability to produce IFN-γ^+, TNF-α^+, and/or IL-2$^+$), the characterization of CD8$^+$ T cells has mainly relied on markers associated with cell differentiation as mentioned earlier. Specifically, where the loss of CD45RA suggests the activation state of cells, and the expression of CCR7 indicates the lymphoid residency/trafficking (Sallusto et al., 2004). Based on these expression profiles, Lanzavecchia and colleagues impressively discerned CD8$^+$ T cells into principle populations with distinct differentiation and effector activities based on these two markers. Furthermore, based on the nonredundancy of IL-7 signaling in T cell memory development, Kaech et al. used CD127 (IL-7Rα) and KLRG-1 to distinguish long-term memory cells and short-lived effector cells (Kaech & Cui, 2012). Others have explored the usages of these two approaches (Plumlee, Sheridan, Cicek, & Lefrancois, 2013), or expanded them by adding several other surface markers (Gattinoni et al., 2011). Unquestionably though, given the limited channels in flow cytometry experiment, the two-marker systems of subset segregations has been remarkably useful in study of human T cell. By applying one of the two-marker systems, immunologists are in favor of a clear four subsets separation that could be simply visualized and quantified using a single two-dimensional dotplot.

Although the effectiveness of these approaches for analyzing CD8$^+$ T cells responses are clear, they may also underestimate the functional heterogeneity of human T cells. Compelling evidences in recent years have proved that CD8$^+$ T cells could possess diverse functional subsets in regulating immune response instead of the simple cytotoxic ability. This includes IL-4-producing CD8$^+$ T cells (Sad, Marcotte, & Mosmann, 1995; Vukmanovic-Stejic, Vyas, Gorak-Stolinska, Noble, & Kemeny, 2000), CD8$^+$ Treg (Leavenworth, Tang, Kim, Wang, & Cantor, 2013; Smith & Kumar, 2008) cells, Qa-1 (HLA-E)-restricted CD8$^+$ T cells (Holderried, Lang, Kim, & Cantor, 2013; Kim, Verbinnen, Tang, Lu, & Cantor, 2010), and helper-like CD8$^+$ T cells (Feau, Arens, Togher, & Schoenberger, 2011; Frentsch et al., 2013). Studies also described other detailed differentiation subsets like stem-like memory T cell (T_{SCM}) (Gattinoni et al., 2011), transitional T_{EM} (Mahnke et al., 2013) or tissue-resident memory T cell (T_{RM}) (Mueller, Gebhardt, Carbone, & Heath,

2013). Second, because it takes additional markers to identify these cells of interest, only few differentiation and functional markers can be assessed simultaneously in flow cytometry. The critical relationship between certain categories of cellular property has been widely unclear, such as differentiation vs function or differentiation vs trafficking (Baaten, Cooper, Swain, & Bradley, 2013; Schmitt & Ueno, 2015).

From the differences of T cell differentiation, function, transcriptional signaling, tissue homing, and even antigen specificity, there is an unmet need to apply a more robust tool to more comprehensively assess the massive degree of T cell heterogeneity, such as mass cytometry and high-dimensional cytometric analysis.



3. MASS CYTOMETRY: EXPERIMENTAL WORKFLOW AND DATA PROCESSING

Mass cytometry is composed of cell introduction system, inductively coupled plasma mass spectrometry (ICP-MS) (Houk et al., 1980), TOF and computer that collects and processes data (Bandura et al., 2009). The methodology of mass cytometry has been described in detail elsewhere (Bendall et al., 2012; Bjornson, Nolan, & Fantl, 2013; Tanner et al., 2013). Briefly, the cells labeled with metal-tag antibodies are pushed into machine by argon gas and nebulizer to form single-cell aerosol. Together with the inductive coil and flowing argon stream, they generate high-temperature (7000 K at surface and 5500 K in the core) plasma that construct the central component, ICP. Cell is introduced into this toroidal plasma along the axis in a spray chamber heated to 200°C. The plasma further vaporizes the cells, breaking all molecular bonds to ionize each atom that have ionization capacity. Subsequently, this ionized cloud moves into the quadrupole that allows only heavier ions (>100 Da) to filter through, leaving the biological abundant atoms from cell (eg, carbon, sulfur, hydrogen, and nitrogen) behind. Filtered ions then enter into TOF and detector that configures the dual count of each metal signal, and finally processed by computer to produce data file.

3.1 Antibody Conjugation

Many laboratories use either commercial metal-conjugated antibody or crosslink the metal and antibody in-house using conjugation kits. The former offers commercial quality of metal-tag antibody, where the latter provides more flexibility on panel design. Current protocol of metal-antibody

conjugation is to use liner (X8) or dendrimer (DN3) polymer to bridge the metal and antibody (Bandura et al., 2009; Majonis et al., 2010). Each antibody could be attached by 4–5 metal-chelated polymers, of which has monomers that use diethylene triamine pentaacetic acid to preferably chelate trivalent cation (+3) elements (lanthanide family). Notably, such covalently binding is comparably as strong as streptavidin–biotin in terms of the dissociation constant (K_D) (Bjornson et al., 2013; Tanner et al., 2013). Typically, purified carrier protein-free (ie, BSA or gelatin) antibodies are used for metal conjugation as these carrier proteins can also react with polymer-crosslinkers.

3.2 Cell Staining

The experimental procedure of mass cytometry is similar to the standard fluorescence flow cytometry staining workflow with some altered steps to fit better the system of mass cytometry (Fig. 1). After cell isolation, it is recommended to perform further purification of target cells, such as T cell (CD4 or CD8) enrichment or dead cell removal. Since the cell throughput is much lower than flow cytometry, this step can greatly reduce the acquisition time of CyTOF, especially when working on large numbers of samples (ie, >20 samples and >6 million cells/sample). If cell stimulation is needed for the experiment (eg, PMA/ionomycin, anti-CD3/CD28, SEB, or peptide stimulations) (Newell et al., 2012), anti-CD107a can be added into the stimulation as reported for flow cytometry cell staining (Betts et al., 2003). In addition, some surface markers downregulate their expression under stimuli, especially chemokine receptors. To better probe T cells, a minimal amount of metal-tag antibodies against trafficking receptors can be included in the stimulation medium to increase performance of these stains (Wong et al., 2015). For instance, we observed a 10% difference in frequency of CXCR5 expression on peripheral and tonsilar $CD4^+$ T cells with or without prestain protocol (unpublished data) upon PMA/ionomycin stimulation. Importantly, such difference is affected by strong stimulation and can be prevented by this additional step (Wong et al., 2015). This may be something that is unique for mass cytometry staining as metal-tag antibodies have higher stability of antibody conjugates compared to fluorophore-tag antibodies during cell incubation.

Prior to any type of staining, a short exposure of cisplatin (Pt-195, Pt-194, or Pt-198) allows platinum (Pt) (or other reactive probe) to covalently bind to dead cells (Bendall et al., 2011; Fienberg, Simonds, Fantl,

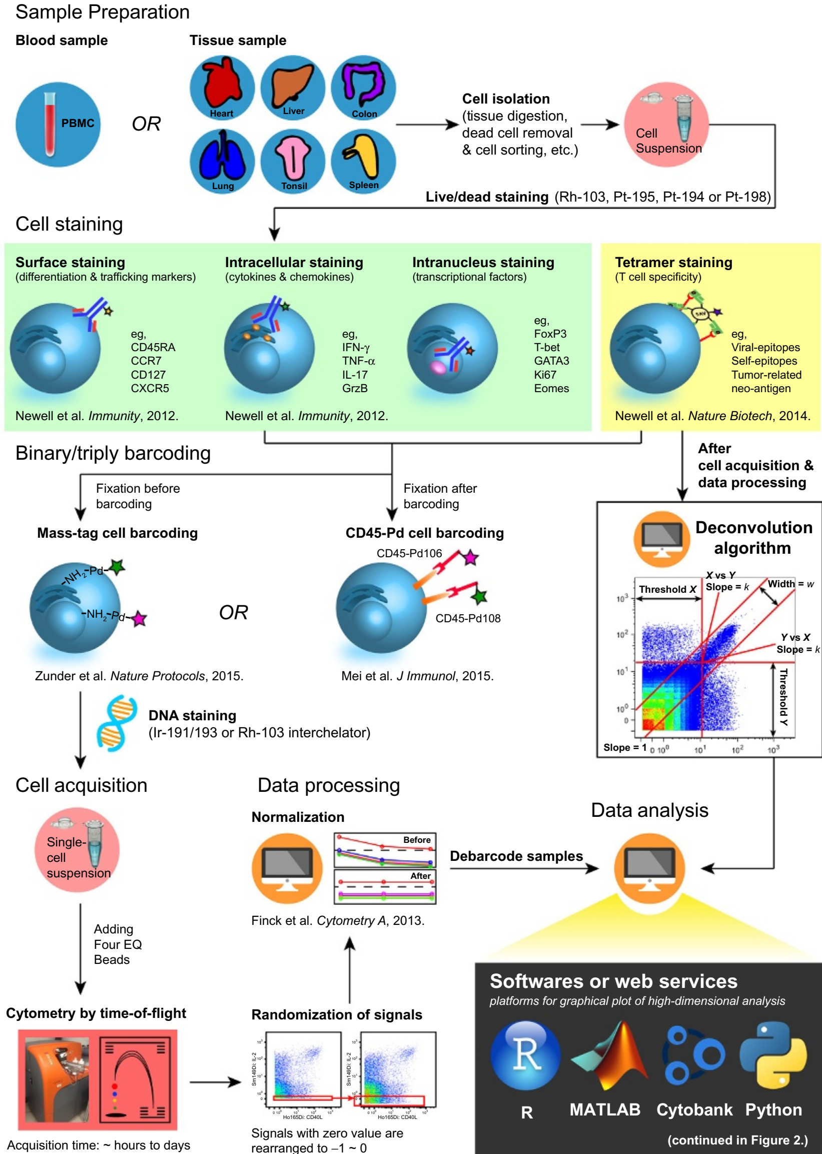


Fig. 1 Mass cytometry experiment workflow for T cell analysis. The experimental procedure for study of T cell using mass cytometry can be separated into multiple steps, including (i) sample preparation, (ii) cell staining, (iii) cell barcoding, (iv) acquisition of CyTOF, (v) data processing (randomization, normalization and cell de-barcoding), and (vi) high-dimensional data analysis. Briefly, cells are isolated from PBMCs (peripheral blood mononuclear cells) or tissue samples. After the optional enrichment step, cell (*Continued*)

Nolan, & Bodenmiller, 2012; Mei, Leipold, & Maecker, 2015). Notably, it is important to apply stringent wash to reduce the background of cisplatin. Like fluorescent antibody, most surface markers (eg, differentiation and trafficking receptors) can be sufficiently stained using metal-tag antibodies under the condition of 30 min on ice. Some laboratories have used different protocol to optimize the staining quality, such as stain pooled cells together after cell barcoding (Mei, Leipold, Schulz, Chester, & Maecker, 2015). Subsequently, cells are washed and fix with paraformaldehyde (PFA) overnight at 4°C. This resilient fixation is to prevent cell burst when resuspend cells in highly pure water upon acquisition. Permeabilization of cell could be achieved by using saponin-based commercial kit on the next day for intracellular staining (eg, cytokines, cytotoxicity molecules, and chemokines). In case of intranuclear staining for T cell transcription factor, we recommend to perform the intranuclear permeabilization soon after the surface staining ahead of overnight fixation. It is critical to carefully titrate the intranuclear antibodies for this purpose. Optionally, utilization of secondary antibody (eg, anti-FoxP3-PE followed by anti-PE-metal) to amplify the signal can also resolve better separation of positive and negative populations. In general and especially for studies intracellular signaling, the lack of cellular autofluorescence gives mass cytometry an additional advantage over fluorescence flow cytometry (Bendall et al., 2012).

3.3 Cell Barcoding and DNA Staining

Cell barcoding is highly beneficial for the efficient acquisition of good quality mass cytometry data. It provides better efficiency during acquisition by reducing time needed to wash instrumentation between samples, eliminating variations between samples, maintaining consistent quality of signal. Also, because mass cytometry does not have forward and side scatter, the light-based measurements in flow cytometry to exclude unwanted cell

Fig. 1—Cont'd suspension is stained with Cisplatin for distinguish dead cells. Principally, cells were probed with surface, intracellular, or intranuclear markers after tetramer staining (if applicable). Cells can be further barcoded by Mass-tag cell barcoding (after cell fixation), or CD45-Pd cell barcoding (before cell fixation) system. Fixed cells are stained with iridium or rhodium DNA-interchelator and resuspended in deionized water for subsequent acquisition on CyTOF. Collected data are converted into FCS file and metal signals are normalized. De-barcoded samples are loaded onto a bioinformative platform (usually in a MATLAB or R environment, or online server such as Cytobank.org) of choice for high-dimensional cytometric analysis.

doublets and debris (Zunder, Finck, et al., 2015), it is therefore important to implement cell barcoding strategy in any mass cytometry experiment. Current cell double (or triple) barcoding strategies for T cell study include Palladium-tag cell barcoding (Zunder, Finck, et al., 2015) and CD45 cell barcoding with Palladium (Mei, Leipold, Schulz, et al., 2015; Mei, Leipold, & Maecker, 2015) or other elements (Lai, Ong, Li, & Albani, 2015). The former approach uses Palladium isotopes (Pd-102, Pd-104, Pd-105, Pd-106, Pd-108, and Pd-110) to label samples after cell permeabilization. Palladium isotopes are ideal for cell barcoding because the atomic masses of these isotopes are away from the range of atomic masses used for antibody probes (139–176). The protocol and rationale have been well illustrated by Zunder et al. Alternatively, Maecker and colleagues employed six different anti-CD45 antibodies conjugated with Pd-104, Pd-106, Pd-108, Pd-110, In-113, and In-115 to barcode live human peripheral blood mononuclear cell (PBMC). This could be carried out prior surface antibody staining, which permits pooled live cells from different samples to be stained simultaneously. Hence, it reduced the antibody consumption and staining variation without the need to sacrifice any lanthanide channels for T cell phenotyping. By using 6-choose-3 or 6-choose-2, it can offer 20 or 15 unique combinations to barcode different samples, respectively. If the number of sample exceeds this limit, one can adapt multiple barcode sets and save as independent files upon cell acquisition.

Lastly, cells are probed with DNA intercalator diluted in PFA. All mass cytometry experiments to-date use either rhodium (Rh-103) or iridium (Ir-191/193) metallo-intercalators for this purpose (Ornatsky et al., 2008, 2010). Together with the cell barcoding scheme, the DNA indicators have provided practical identification of single-cell event with high level of signal intensity. Several studies have addressed the technical usage of DNA intercalators in this manner (Bandura et al., 2009; Ornatsky et al., 2008).

3.4 Cell Acquisition

After all staining procedures are completed, cells are washed, combined, and resuspended in deionized and purified water (Milli-Q). In mass cytometry, a cell introduction rate of less than 500 cells/s is critical to resolve single-cell analysis and maintain high signal sensitivity. Including ours, many laboratories have used 500,000 cells/mL (or less) in each 500 μL injection (for PBMC), which is roughly 379 cells/s. However, if experiment requires tissue samples, we recommend to perform more aggressive dilution to avoid

possible clog in the cell introduction system, especially the small capillary loop connected to nebulizer. Vigorous washes with wash buffer and 2% nitric acid are necessary before and after the acquisition of tissue samples to maintain high performance of CyTOF and signal intensity. It is also advised to separate the use of nebulizer and even the connected loop between PBMC and tissue sample. Frequent maintenance including the cleaning of sampler cone and skimmer-reducer is also important to obtain high quality data, especially for large cohort or tissue samples.

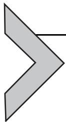
The readout of cell events for mass cytometry is not as robust as flow cytometry. The current output of CyTOF is only about 30–35% of the injected cells. Avoiding possible clog in the system during runtime is necessary. It could also differ from one-loop or two-loop system on the injection end of the CyTOF2. In our experience, the one-loop system provided with the CyTOF1 is more efficient (up to 45–48%) than the two-loop system, which may be associated with the different orientations of loops. Efficient acquisition is especially critical when investigating human antigen-specific T cells (Newell et al., 2013) (discussed later). For instance, the frequency of viral-specific T cells in human PBMC is usually less than 0.5% of total $CD8^+$ T cells. Starting with high numbers of cells and prevent cell lost during steps are consequently important, such as extremely careful when aspirating cells, flicking plate, or preventing excessive washes between staining.

3.5 Data Processing and Normalization

CyTOF collects detected TOF results and converts the configured signals into FCS file after cell acquisition (Bendall et al., 2012). Mass cytometry provides absolute quantification of metal-isotopes tagged on the cells, and cell with no metal-tag detected would be considered zero. This signal of zero can be randomized into values between −1 and 0 using a simple R-written script with flowCore package or the built-in software in CyTOF before any further data processing. In our opinion, this step provides a preferable visualization (for cells compressed on one axis in 2D plot) on FCS compatible software such as FlowJo™, although such processing does not affect further high-dimensional data analysis.

The acquisition time of CyTOF is long and laborious and last from several hours to even days depending on the sample size. If enrichment of cell is not desirable for experiment, the commitment of individual or change of multiple shifts to run the machine is needed. In addition, the strength of

signal intensity varies over time, and between machines. Signals in most channels decline gradually as the acquisition continues. In order to remove this signal variation, Finck et al. developed a normalization procedure (Finck et al., 2013) that involves the addition of four EQ™ element beads (containing Ce-140, Eu-151, Eu-153, Ho-165, and Lu-175, each bead has high number of corresponded isotope) into the cell suspension. Collected data can be loaded onto an algorithm in MATLAB to normalize the signals based on the beads. This normalization scheme makes large cohort of sample more feasible for mass cytometry. Finally, after a series of data processing, samples can be debarcoded by using bivariate Boolean gates or designated algorithm to identify specific sample for downstream high-dimensional cytometric analysis (discussed later).



4. MASS CYTOMETRY: PANEL DESIGN FOR T CELL ANALYSIS

In human studies, PBMCs are the most common sample source. Limited quantities of blood and limited access to other types of tissues make mass cytometry an invaluable solution for study of human T cells. This is particularly important when research projects require the study of longitudinal patients cohort where obtaining sufficient human samples at multiple time points is difficult. The ability of mass cytometry to acquire large in-depth information in single-cell level is hence a robust method to dissect T cell heterogeneity for limited and valuable human tissue.

4.1 Studies of T Cell Using Mass Cytometry

To date, several reports have showed the effectiveness of mass cytometry for study of T cells (Table 1) using various high-dimensional cytometric analyses (discussed later, and Fig. 2). Most publications are focused on the usage of mass cytometry in profiling T cell signaling or phenotype in various contexts. Combining mass cytometry and high-dimensional data analysis, several groups have also showed the previous elusive aspects of T cells in human viral infection, including CMV (Brodin et al., 2015; Newell et al., 2012, 2013), EBV (Newell et al., 2012, 2013), Varicella zoster virus (VZV) (Sen et al., 2014), HCV (Swadling et al., 2014), HIV (Bruggner, Bodenmiller, Dill, Tibshirani, & Nolan, 2014), influenza (Newell et al., 2012, 2013), and rotavirus (Newell et al., 2013).

Table 1 Studies of T Cells Using Mass Cytometry

References	Spieces/Tissue Types	Cell Types	Significants	Analysis Algorithm
Newell et al. *Immunity,* 2012	Human, PBMC	CD8	Differentiation continuum of human CD8 and the various functional characteristics of virus-specific T cells	PCA
Shekhar, Brodin, Davis, and Chakraborty. *PNAS,* 2014	Mouse (C57BL/6), blood	CD8	Using naïve, effector, and memory CD8 T cells subsets as an example for developing ACCENSE	ACCENSE
Newell et al. *Nat Biotechnol,* 2013	Human, PBMC	CD8	Multiplex tetramer staining using mass cytometry in investigating rotavirus-specific T cells and their distinct phenotypes	PCA
Swadling et al. *Sci Transl Med,* 2014	Human, PBMC	CD4, CD8	In vivo prime-boost vaccination induced functional HCV- specific T cells	PCA
Bruggner et al. *PNAS,* 2014	Human, PBMC	CD4, CD8	Phenotype of naïve CD4 and CD8 in HIV-infected patients.	Citrus
Mingueneau et al. *PNAS,* 2014	Mouse (C57BL/6, NOD), lymph node, and thymus	CD4	TCR signaling dynamics (ie, CD3ζ, ERK, etc.)	Hierarchical clustering
Krishnaswamy et al. *Science,* 2014	Mouse (C57BL/6)	CD4	T cell signaling network of naïve and antigen-experienced cell	DREVI, DREAMI
Fergusson et al. *Cell Reports,* 2014	Human, PBMC	CD4, CD8, γδ T cell, MAIT cell	Expression of CD161-expressing T cells and their transcriptional and functional differences	PCA

Sen et al. *Cell Reports,* 2014	Human, tonsil	CD4, CD8	Phenotypic and functional profile of tonsil T cell in VZV infection	PCA, SPADE
Wolchinsky et al. *J Immunol,* 2014	Human, cell line (T cell clone JKF6)	CD8	TCR signaling cascades, T cell anergy, Ag (MART-1_{27-35}) dependent TCR activation and inhibition	Hierarchical clustering
Mason et al. *J Immunol,* 2015	Human, PBMC	CD4, Treg	Description of the complexity phenotype of Treg	*t*-SNE, FLOCK
Spitzer et al. *Science,* 2015	Human, whole blood mouse (129S1/sv, Balb/c, C57BL/6), multiple tissues	CD4, CD8, B cell, NK	Immune cells organization from multiple tissue and their changes by times	Scaffold map
Wong et al. *Cell Reports*, 2015	Human, PBMC, and tonsil	CD4, CD8, TFH, T helper cell	Diversity of tonsilar TFH and helper T cells and cellular progression	*t*-SNE, ACCENSE, DensVM, ISOMAP
Brodin et al. *Cell,* 2015	Human, PBMC	CD4, CD8	Comparison of the immune variation between monozygotic twins and the nonheritable influences such as influenza and CMV	
Cheng, Wong, van der Maaten, and Newell. *J Immunol,* 2016	Human, PBMC	CD4, CD8	Categorical analysis of diverse CD8 T cells and Treg as an example for developing One-SENSE	*t*-SNE, One-SENSE

Abbreviation: *PBMC*, peripheral blood mononuclear cell; *Treg*, regulatory T cell; *MAIT*, mucosol-associated invariant T cell; *NK*, natural killer cell; *TFH*, follicular T helper cell; *HCV*, hepatitis C virus; *VZV*, varicella zoster virus; *CMV*, cytomegalovirus; *PCA*, principal component analysis; *ACCENSE*, automatic classification of cellular expression by nonlinear stochastic embedding; *t-SNE*, *t*-distributed stochastic neighbor embedding; *ISOMAP*, isometric feature map; *DensVM*, density-based vector machine; *Citrus*, cluster identification, characterization, and regression; *One-SENSE*, one-dimensional soli-expression by nonlinear stochastic embedding; *FLOCK*, FLOw Clustering without K.

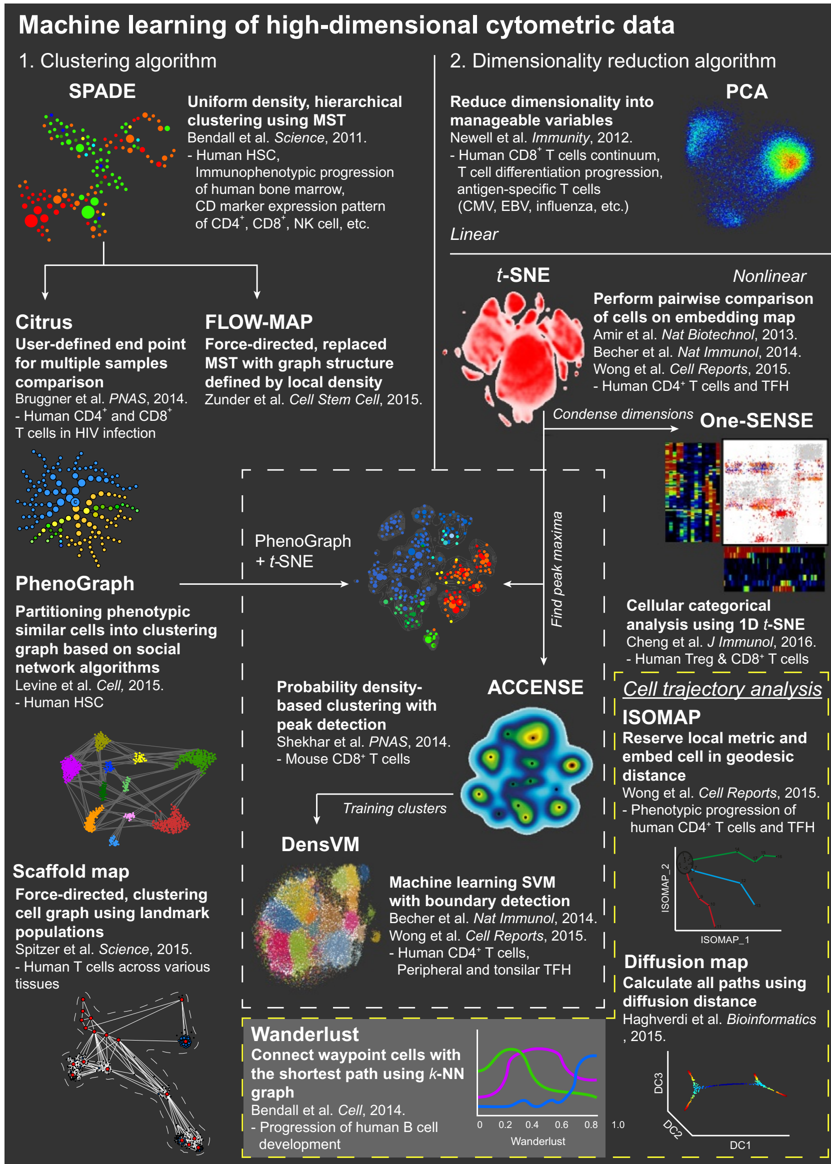


Fig. 2 Machine learning of high-dimensional cytometric data. Comparing to traditional visualization of cellular marker on bivariate dotplots, high-dimensional cytometric analysis approaches can be separated into two categories based on their algorithms. Clustering algorithm SPADE (spanning tree progression of density normalized events), a hierarchical clustering algorithm using MST (minimum spanning tree), and its derived forms including Citrus and FLOW-MAP. PhenoGraph and Scaffold map are the other two types of clustering analysis based on different rationales and algorithms. Dimensionality

(*Continued*)

4.2 Metal Signal and Panel Design

The high sensitivity and specificity of ICP-MS installment and the quadrupole filter that allows <0.0001% overlap between metal channels is what makes mass cytometry such an attractive approach (Tanner et al., 2013), providing a large number of cellular dimensions without the necessity of compensation for crosstalk between measured parameters. However, minimum crosstalk between metal channels does occur. Isotopic contamination and oxidation are the two types of cross talks in mass cytometry (Bendall et al., 2012; Tanner et al., 2013). These overlaps are relatively minor in mass cytometry experiment (compared to common fluorescence cross talks), but it should be acknowledged when designing staining panel. Of note, this is more important when working on multiplex tetramer staining where requires high mass resolution without cross talk from other channels.

Isotopic contamination crosstalk is contributed from the metal impurity as the nature of isotope purification. Most commercial enriched lanthanide isotopes are highly purified, usually less than 1% unless for isotopes of very rare natural abundance. Typically, this impurity of isotopes could leak into their neighbor channels (+1 Da and −1 Da) especially if using sources of metal that are not readily purified for mass cytometry (Bendall et al., 2012). Future correction of metal purification should overcome this type of crosstalk. Secondly, every metal isotope exhibits certain level of oxidation in argon plasma that causes oxidation crosstalk. During sample introduction, higher rates of "make-up gas" (argon) injection allows for better cell throughput and signal intensity during acquisition. However, there is a tradeoff in that higher gas-flow also leads to higher metal oxidation due to the incomplete ionization in plasma. The oxide ions (+16 Da) from their source channels are dictated by the strength of ion-oxide bond and temperature of

Fig. 2—Cont'd reduction algorithm PCA (principal component analysis) reduces dimensionality in a linear manner. Alternatively, nonlinear dimensionality reduction algorithms that are useful for high-dimensional cytometric data are *t*-SNE (*t*-distributed stochastic nonlinear embedding), and its modified algorithms ACCENSE and DensVM with the clustering ability using density-based approach. Categorical dimensions of One-SENSE (one-dimensional soli-expression by nonlinear stochastic embedding) are condensed from *t*-SNE along with the heatplots of markers expression. *t*-SNE can also be combined with PhenoGraph to better identify cell clusters. Other nonlinear dimensionality reduction algorithms used for cell phenotypic progression or trajectory analysis are ISOMAP (isometric feature mapping), Wanderlust, and a recent proposed Diffusion map that simulates cell differentiation with branched continuums.

plasma. The proper warm-up and tuning procedure of CyTOF, which includes the routinely monitoring of current, make-up gas, and oxide ratio (<3%), is important to minimize this issue.

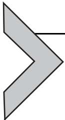
Mass window of CyTOF ranges from Y-89 to Bi-209 with lower mass channels at the two ends, while the upper mass signal starts from Eu-153, reach a typical peak signal at Tb-159 and ramp down a little around Yb-170 (Tricot et al., 2015). However, this variation of metal signal intensity is within a 2–3-fold change across the spectrum (Bendall et al., 2012), which is far more consistent than the 10–50-fold change of fluorescent intensity in flow cytometry.

There are many aspects to consider when optimizing a 40-plus panel to meet the experiment purpose, especially for T cell analysis. Based on our experience, we summarize several principles that could be helpful for T cell study using mass cytometry, and prioritize the choice of metal channel based on the different characters of markers and experiment purposes.

1. Tetramer first: If using tetramer to probe antigen-specific T cell (discussed later), build the panel around it. Leave the best metal channels for tetramer, and then put other cellular markers in rest of the channels accordingly. This is essentially important in investigating rare human antigen-specific T cells using highly multiplex combinatorial tetramer strategy.
2. Rare but important markers: Use Tb-159 or channels around it for the important but less abundant markers. In general, channels of Gd-157 to Er-167 are sufficient for this purpose (Tricot et al., 2015). It should also take into account that some markers could have different expression level on different type of T cells and tissues. For instance, PD-1 expression is weaker on human peripheral $CD8^+$ T cell without TCR stimulation.
3. Moderate markers or intranuclear molecules: Markers that usually have "smear" expression or intranuclear proteins should be included in the third priority. Use rest of the upper mass channels after tetramer and rare markers.
4. Intracellular cytokines: Most of the cytokines have robust and clear expression after stimulation, which can be strongly detected in lower mass channels ranging from Pr-141 to Nd-150. However, the concentration of metal-tag antibody should be carefully titrated to prevent isotopic contamination crosstalk, and avoid assigning a weak marker on the +1 channel of a strong cytokine channel.

5. Abundant markers: Cellular markers that usually have bimodal distribution can be labeled with less sensitive channels. Notably, markers that are coexpressed and traditionally paired up for investigating T cells, such as CD45RA vs CCR7 and CD127 vs KLRG-1, could be used on lower sensitivity channels that are far apart for better resolution without sacrificing upper mass channels.
6. Lineage markers: The markers indicate cell lineage, including CD45, CD3, CD4, and CD8, are relatively the most abundant markers on cells. They should be located in some of the weakest metal channels. However, one should also consider the minimum resolution required when gating on target cells, or paired up one "dim" channel with one moderate channel to identify target lineage cell. Markers that are lineage-specific and exclusive from each other could also be put in the adjacent "dim" channels that have less purity.
7. "Dump" markers: Markers that are least important and only for excluding unwanted cells can be put together into dim channel, leaving more metal channels to probe marker of interest.

Overall, these principles are mainly based on our experience and should be examined and varied between experiments. Importantly, to best use mass cytometry for its accessibility of investigating T cells in such depth, one should designate the panel more systemically to comprehensively probe the T cells heterogeneity or relationship between cellular properties.



5. PROBING T CELL SPECIFICITY: FROM SINGLE TO MULTIPLEX TETRAMER

Identifying antigen-specific T cells and understanding their roles during immune responses against invading pathogen have been the central component of adaptive immunity. Conventional approaches in defining such antigen-specific T cells in human differ greatly, mainly are CTL ^{51}Cr release assay, peptide-induced ELISPOT, peptide-induced intracellular cytokine staining (Doherty & Christensen, 2000; Masopust et al., 2007). Seminal work by Altman et al. showed that recombinantly produced tetramers of major histocompatibility complex (MHC) class I can provide high avidity for TCR to directly probe antigen-specific T cells (Altman et al., 1996). This allows the assessment of antigen-specific T cell phenotype ex vivo without manipulation of cells.

The Red Queen hypothesis (Van Valen, 1974a, 1974b) describes the rapidly evolving interaction between host and pathogen (Clarke et al.,

1994) that drives molecular coevolution by continuous natural selection for host adaptation (eg, immune escape). This is supported by the highly polymorphic nature of HLA (human MHC), and the enormous breadth and numbers of T cell epitopes that can be encoded by viruses (Newell & Davis, 2014). It is therefore important to broadly probe the recognition of numerous epitopes during immune response against a given virus, especially as the virus may rapidly evolve and allow for persistent infection.

The power of tetramer technology has recently been increased exponentially. The breakthrough UV-cleavable peptide-MHC exchange approach (Toebes et al., 2006) and combinatorial tetramer strategy (Hadrup et al., 2009; Newell, Klein, Yu, & Davis, 2009) have facilitated the discovery of novel antigen-specific T cells in a high-throughput manner. This unique combinatorial tetramer strategy involves what we will describe as the "Tetramer staining scheme" and the "Tetramer deconvolution scheme."

5.1 Combinatorial Tetramer Staining Scheme

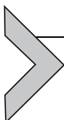
Coupled use of mass cytometry, combinatorial tetramer strategy, and computational deconvolution scheme (Fig. 1) enables the simultaneous detection of hundreds of antigen specificities (Newell et al., 2013) (unpublished data) in a single human sample with automated epitope identification. The methodological details have reported and reviewed elsewhere (Harvey & Wucherpfennig, 2013; Newell & Davis, 2014; Newell et al., 2013). The key component of combinatorial tetramer staining is the assignment of multiple streptavidin coding. It can be achieved by using a simple R-written algorithm to create the unique scrambled combination of streptavidin codings for each given tetramer. After UV-exchanging the peptide of interest, these streptavidin mixtures are used to tetramerize the assigned peptide-MHC. The numbers of unique combinations depends on the number of metal channel that are dedicated for different streptavidins. A demonstration with detailed procedures of this technique can be found elsewhere (Leong & Newell, 2015). Recently, we have successfully expanded this approach using 1001 unique codings to simultaneously probe 563 candidate $CD8^+$ T cell antigens in peripheral blood of chronically HBV (hepatitis B virus) infected patients (unpublished data).

5.2 Combinatorial Tetramer Deconvolution Scheme

Traditional identification of tetramer-positive cells relies on the expert manual gating by comparing with isotype or tetramer loaded with irrelevant

peptide. However, this can be confounded and is subjective to the researcher's expectation. In addition, rare antigen-specific T cells or T cell with low-affinity TCR are very difficult to be confidently identified on a 2D dotplot (Wooldridge et al., 2009). This is especially problematic when studying antigen-specific T cell in patients with persistent viral infection, such as HBV (Reignat et al., 2002) and HCV (Schmidt et al., 2011), where low frequencies of antigen-specific cells are expected.

Furthermore, if manual gating were to be used together with the highly multiplex tetramer scheme, it may be very difficult to verify any tetramer-positive cells in an objective manner. Instead, using several preset signal thresholds, the tetramer deconvolution algorithm objectively and automatically identifies multiple tetramer-positive populations and quantifies the different streptavidin coding on these tetramer-positive cells based on the applied combinatorial tetramer strategy (Newell et al., 2013). It provides high detection sensitivity while reducing background noise and unspecific binding of tetramer. In addition, identified tetramer-positive cells can be further self-verified by calculating the correspondence between two configurations of streptavidin coding from the same patient donor. Importantly, even the significance of rare antigen-specific T cells can be robustly distinguished and statistically quantified by this approach. It is our opinion that the computational schemes we are using offers a promising method to investigate human antigen-specific T cell in an unprecedented level.



6. DISSECTING MASS CYTOMETRY DATA: HIGH-DIMENSIONAL DATA ANALYSIS

Traditional visualization using hierarchical subgating schemes based on the marker expression on biaxial dotplots has provided a straightforward approach to analyze flow cytometry data. However, subjective manual gating is based on expert training and expectation, which could vary between researchers and laboratories. The characterization of subsets could be therefore biased. The FlowCAP (Flow Cytometry: Critical Assessment of Population Identification Methods) consortium and several groups have established various computational methods to improve the precision and accuracy of flow cytometry analysis (Aghaeepour et al., 2013). These automated methods include density-, model-, or nonparametric clustering-based methods, which have open access and are easily available using R or MATLAB source code.

Given the complexity and high dimensionality of mass cytometry data, limitations in the utility of biaxial gating approaches are immediately apparent and this has forced the adaptation and development of many high-dimensional data analysis methods (Bendall et al., 2012; Newell & Davis, 2014). However, we would like to emphasize the importance of the use of standard dotplots with mass cytometry for demonstrating the accuracy of staining and a means of validating findings in a clear and comprehensible way. Beyond this though, current platforms for high-dimensional (>10 dimensions) cytometric data analysis are mostly based on "Clustering" approaches, "Dimensionality Reduction" methods, or combinations thereof (Fig. 2). Other reports have also used conventional hierarchical clustering approach (Mingueneau et al., 2014; Wolchinsky et al., 2014), or conditional density estimation, DREVI/DREAMI (Krishnaswamy et al., 2014), to study T cell receptor activation and signaling cascades. Noteworthy, most of the analytical methods use nonparametric model (eg, kernel density estimation or k-NNs), as the probability assumption (eg, reference ellipsoid) of parametric statistics is questionable for complex cellular population in high-dimensional space.

6.1 Clustering Analysis

SPADE, Spanning Tree Progression of Density Normalized Events, was one of the first used methods for visually pleasing high-dimensional cytometric data analysis. Bendall et al. used this analytical approach to study the immune response of hematopoietic stem cells (HSCs) under drug intervention (Bendall et al., 2011). SPADE is a density normalization agglomerative clustering algorithm that visualizes the fold change of marker expression of cellular data on branches of MST (Minimum spanning tree) structure (Qiu et al., 2011). The derived method, Citrus (Bruggner et al., 2014), was improved for multiple samples analysis (>10 samples). It identifies cellular clusters that are linked to different experimental end points with biological interpretable metrics using regression analysis. Interestingly, Citrus identified a specific T cell cluster that inversely associated with AIDS-free survival risk in HIV-infected patients (Bruggner et al., 2014), and has a naïve-like ($CD27^{hi}CD28^{hi}CCR7^{hi}CD45RO^{lo}$) $CD8^{+}$ T cells phenotype in line with previous report using traditional flow cytometry analysis (Ganesan et al., 2010).

SPADE and Citrus create uniform density by downsampling cytometric data to visualize the cellular relationship in high-dimensional space. Although it has been shown to be useful, the MST structure loses single-cell

resolution and is susceptible to local variation. To overcome the pitfall, Zunder et al. replaced MST with connected graph structure between cellular clusters that based on local density, named FLOW-MAP (Zunder, Lujan, Goltsev, Wernig, & Nolan, 2015).

A robust visualization for complex cellular network should accommodate unsupervised referenced clustering and subjective definition of cellular cluster. Scaffold (single-cell analysis by fixed force- and landmark-directed) map provides such approach (Spitzer et al., 2015). It positions landmark cell population (eg, CD4, CD8, B cell, dendritic cell, and natural killer cells) and connect similar cell clusters (nodes) by borders with a length correspond to their cosine similarity using force-directed graphs. Given the tissue-specific characteristics of T cell, the authors showed a unique visualization of T cell (ie, CD4, CD8, Treg, MAIT, and γδ T cells) landscape across 10 different tissues.

Working on different concepts, Levine et al. modeled high-dimensional space of cells using a matrix of single-cell measurements that cluster the most phenotypic similar cells in set of *k*-neighborhoods, resulting a graph structure, PhenoGraph (Levine et al., 2015). Interestingly, PhenoGraph partitions the interrelated cellular graph into distinct subpopulation using social network community algorithm (Louvain Modularity) (Blondel, Guillaume, Lambiotte, & Lefebvre, 2008) and intuitively represents the phenotypic relationship between cells. PhenoGraph applies this community (ie, modules or clusters) detection method to find the maximum modularity of data network, giving dense connections between cells within clusters but dispersed connection between cells from different clusters.

6.2 Dimensionality Reduction Analysis

Dimensionality reduction method is another favorable analytical tool for high-dimensional cytometric data. It reduces the number of random variables under different purpose-driven considerations by feature extraction. Principal component analysis (PCA) has been widely used in many biological datasets and is the first of its type proposed for such purpose. PCA (Hotelling, 1933; Pearson, 1901) digests the high-dimensional cellular data into manageable surrogate variables (principal components) in a linear fashion in such a way that the first and second principal components contain the largest possible variance, and segregated major populations that resemble their relatedness. Several groups, including ours, have applied PCA in analyzing human peripheral CD8$^+$ T cells (Fergusson et al., 2014; Newell et al., 2012, 2013; Sen et al., 2014; Swadling et al., 2014), and shown a great

magnitude of T cell heterogeneity within a continuum of cell differentiation and polyfunctionality (Newell et al., 2012). Fergusson et al. used PCA and mass cytometry to reveal the functional and phenotypic diversity of CD161-expressiong $CD8^+$ T cells (Fergusson et al., 2014). Another group adapted the same approach to describe the phenotypic changes of tonsilar $CD4^+$ and $CD8^+$ T cells in VZV infection (Sen et al., 2014).

Besides delineating the T cells in global population, the definite phenotypes between different antigen-specific T cells can be conveniently distinguished and visualized under the utilization of PCA. Newell et al. showed the functional distinct CMV, EBV, and FLU (Influenza A)-specific T cells located in regions of central memory (T_{CM}), effector memory (T_{EM}), to late-stage effector cells on PCA visualization, respectively (Newell et al., 2012). We later demonstrated the unique phenotype of $Rotavirus_{VP3}$-specific $CD8^+$ T cells in intestinal epithelium, with an effector-like phenotype expressing integrin-β7 and αE (Newell et al., 2013). Furthermore, vaccine-induced HCV-specific $CD8^+$ T cells also occupied a distinct memory niche compared to FLU- and CMV-specific $CD8^+$ T cells using PCA in a human clinical trial (Swadling et al., 2014).

Although it is clear that PCA is effective in inferring the interrelationship of subsets for high-dimensional cytometric data, the resolution is limited by its nature of linear transformation. Endowing a different scope, *t*-distributed stochastic neighbor embedding (*t*-SNE) (van der Maaten & Hinton, 2008) calculates the probability distribution of pairwise distances of points (cells) in high-dimensional metric space, giving similar points high probability and dissimilar points infinitesimal probability of being chosen, and embeds the points with respect to the distances on a two-dimensional (or three) map. It has been used to visually determine immune cell subsets (Amir el et al., 2013), and worked exceptionally well in identification of rare population (Becher et al., 2014). The impressive utility of *t*-SNE analysis has been proved in uncovering high-dimensional cytometric data of $CD4^+$ T helper cells (Wong et al., 2015), Treg (Mason et al., 2015), and $CD8^+$ T cells (Cheng et al., 2016; Shekhar et al., 2014). Moreover, *t*-SNE is well capable of integrating with other machine learning methods to better interpret high-dimensional cytometric data. Levine et al. recently combined *t*-SNE with PhenoGraph (Levine et al., 2015) to acquire the different utilities of dimensionality reduction and clustering analysis (Fig. 2). McHeyzer-Williams and colleagues used *t*-SNE on fluorescence flow cytometry data to identify four different GC (germinal center) B cells subsets and further loaded these cells onto Wanderlust (discussed later) to predict their

transitional stages in GC cyclic activity (McHeyzer-Williams, Milpied, Okitsu, & McHeyzer-Williams, 2015).

t-SNE produces high resolution of cellular separation while maintaining single-cell visualization, but it requires manual gating of cell clusters. Building automation to better identify cell subsets on *t*-SNE, automatic classification of cellular expression by nonlinear stochastic embedding (ACCENSE) (Shekhar et al., 2014) computes the probability and peak of local density to automatically cluster cell subsets. ACCENSE automatically identified more than 20 different $CD8^+$ T cells subpopulations in mice blood based on 35 measured parameters. To further improve the automated cell cluster detection, we developed DensVM (density-based clustering) (Becher et al., 2014), a modified form of ACCENSE, to calculated cluster boundary based on *k*-nearest neighbor and cluster cells using machine learning SVM (support vector machine). Combining *t*-SNE and DensVM, we have identified the extremely heterogeneous $CD4^+$ T helper subsets across tonsil and blood, with six naïve T cells clusters and multiple functional effector or memory cells, including T_H1, T_H17, and T_{FH} (Wong et al., 2015). Uniquely, ACCENSE and DensVM are the only two current methods incorporate both clustering and dimensionality reduction algorithm for high-dimensional cytometric analysis (Fig. 2).

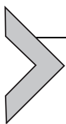
An effective reference map should enable unsupervised identification of cell with a data-driven organization that is flexible enough to accommodate different types of measurements. We have recently developed One-SENSE (One-dimensional Soli-Expression by Nonlinear Stochastic Embedding) to orchestrate the cell using *t*-SNE with purpose-driven categorical analysis of cellular marker expression (Cheng et al., 2016). It projects the cells using one-dimensional *t*-SNE analysis based on the predefined cellular category. Cells are binned and embedded defined by their categorical expression on One-SENSE map. This framework recalls the marker expressions that immerse in other high-dimensional cytometric analysis without the necessity of excessive marker annotation. This improvement provides the opportunity to unbiasedly depict all possible combinations of proteins coexpression examined in the experiment. We have used this unique approach to decipher functional $CD8^+$ T cells (with >12 different coexpressions by 15 functional parameters) and Treg heterogeneity.

One of the major goals in single-cell measurement is to understand the progression of cellular state (eg, differentiation and function). It is a great interest to model cell trajectory to resolve potential T cell heterogeneity during immune response. Isometric feature mapping (ISOMAP) (Tenenbaum,

de Silva, & Langford, 2000) preserves local metric and embeds the intrinsic geometry nonlinearly in a low-dimensional map by determining geodesic distances between cells and estimating each cell's neighbors in a given manifold. We have previously used ISOMAP to mimic the cell phenotypic progression (Becher et al., 2014; Wong et al., 2015). ISOMAP captured the path of phenotypic continuum of tonsilar and blood T_{FH} judging by the various expressions of functional (IFN-γ, TNF-α, CD40L, and IL-2) and T_{FH}-associated markers (CXCR5, PD-1, ICOS, and CD57) (Wong et al., 2015).

Belong to neither clustering nor dimensionality reduction methods, Wanderlust, another cell trajectory detection technique developed by Bendall et al. (2014) for studying the progression and regulatory pathway during B cell development. By using prechosen "early cell" and random waypoint cells, Wanderlust nonlinearly connects cells that have similar expression profile and aligns the cells into a nearest neighbor graph with shortest paths based on pseudotime estimation algorithm.

Recently, an improved method for analyzing single-cell trajectory and differentiation was proposed (Haghverdi, Buettner, & Theis, 2015). Diffusion map (Coifman et al., 2005), a dimension reduction algorithm using diffusion distance to preserve the data structure as a branched continuum. Haghverdi et al. refined the transition probability matrix to determine the diffusion distance between pair cells through all possible paths. It simulates the dynamics of cell differentiation while resisting to noise and sampling density heterogeneities. An advanced version, Destiny (Angerer et al., 2015), was introduced upon the preparation of this manuscript. Diffusion map may show a better performance in analyzing an existing HSC sequencing data, but how would it represent the T cell differentiation or functional trajectory needs to be further addressed.



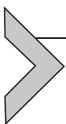
7. FUTURE PERSPECTIVES

The complexity of T cell heterogeneity is currently being explored by the conjunction of mass cytometry and high-dimensional cytometric analysis. Along with the highly multiplex combinatorial tetramer strategy, immunologists can now further delve into the specificity of various types of T cells (Newell & Davis, 2014). However, it does not demote the significance of flow cytometry. Mass cytometry cannot easily replace some important utilities of flow cytometry, such as cell proliferation (CFSE) assay and FACS-based cell sorting. It is more sensible to integrate these two methods based on the experiment purposes. To foster the maximum usage of mass

cytometry, further advancement is also needed. This includes increase the purity of metal isotopes, development of polymer for bi- and quadrivalent isotopes, and the better efficiency of cell introduction system.

Conventionally, most flow cytometry have focused on one or few types of cellular markers in single experiment because of the limited parameters. Given the multiparametric measurement of mass cytometry, we should aim for designing a more systemic panel to study T cell heterogeneity by assigning markers into different categories based on their cellular properties, such as differentiation, function, and trafficking. It can directly test the hypothesis of the critical relationship between different categorical T cell states. For instance, to evaluate the deviated cell trafficking profiles of T_{FH} in different virus infection as recently proposed (Bentebibel et al., 2013; Locci et al., 2013; Schmitt & Ueno, 2015). Or, to help resolve controversies surrounding T cell asymmetry division (Arsenio et al., 2015; Flossdorf et al., 2015) during effector and memory cell differentiation program (Arsenio et al., 2014) using mass cytometry and high-dimensional cytometric analysis. We also anticipate that mass cytometry is capable of isolating immune subsets that require many markers to identify in valuable human patient samples, such as innate lymphoid cell (ILC) (Diefenbach, Colonna, & Koyasu, 2014; Simoni et al., 2016) and myeloid-derived suppressor cell (Pallett et al., 2015).

Considering the high dimensionality on each single human T cell, the traditional bivariate dotplots might not be sufficient to reflect the T cell heterogeneity. Using functional $CD8^+$ T cells as example, Tc1 (coexpressing IFN-γ, TNF-α, and IL-2) (Makedonas & Betts, 2011; Woodland & Dutton, 2003) cells have received the most attention than other functionality, fewer have been done in addressing other heterogeneity. Moreover, hierarchical subgating strategies make it difficult to overview all the possible coexpressions. Researchers need to have preexisting knowledge to subjectively anticipate any possible coexpression. Second, the information behind the double positive population on bivariate dotplot is also missing from the visualization. These generated potential biased interpretation of T cell functionality. Alternative high-dimensional analysis approaches (eg, One-SENSE) that acquire the utility of unsupervised cell visualization with subjective experiment parameters for different analytical purposes could provide a methodological solution.



8. CONCLUDING REMARKS

In 1910s, James B. Murphy first proposed the remarkable role of lymphocytes in rejection of tissue allografts and protection against infection

(Murphy, 1914a, 1914b). However, his works were left vastly unnoticed for almost half century, until Arnold Rich and James Gowans recognized its significance (Silverstein, 2001). With the increase in accessibility of various technologies and resources in the past decades, scientists now have more capability to dissect the complex human immune cell network, especially the discrepancy of T cell role in different contexts of diseases.

Taken together, the presence of mass cytometry and high-dimensional cytometric analysis has joined the frontline of immunological and biomedical discovery, along with flow cytometry, single-cell mRNA-sequencing, and TCR sequencing. We believe this hybrid technology could substantially contribute in the exploration of human T cells from bench to bedside, such as disease prognosis, vaccine, and therapeutic design.

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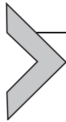

CHAPTER FOUR

Germinal Center B-Cell-Associated Nuclear Protein (GANP) Involved in RNA Metabolism for B Cell Maturation

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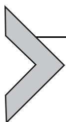
Abstract

Germinal center B-cell-associated nuclear protein (GANP) is upregulated in germinal center B cells against T-cell-dependent antigens in mice and humans. In mice, GANP depletion in B cells impairs antibody affinity maturation. Conversely, its transgenic overexpression augments the generation of high-affinity antigen-specific B cells. GANP associates with AID in the cytoplasm, shepherds AID into the nucleus, and augments its access to the rearranged immunoglobulin (Ig) variable (V) region of the genome in B cells, thereby precipitating the somatic hypermutation of V region genes. GANP is also upregulated in human $CD4^+$ T cells and is associated with APOBEC3G (A3G). GANP interacts with A3G and escorts it to the virion cores to potentiate its antiretroviral activity by inactivating HIV-1 genomic cDNA. Thus, GANP is characterized as a cofactor associated with AID/APOBEC cytidine deaminase family molecules in generating diversity of the IgV region of the genome and genetic alterations of exogenously introduced viral targets. GANP, encoded by human chromosome 21, as well as its mouse equivalent on chromosome 10, contains a region homologous to *Saccharomyces* Sac3 that was characterized as a component of the transcription/export 2 (TREX-2) complex and was predicted to be involved in RNA export and metabolism in mammalian cells. The metabolism of RNA during its maturation, from the transcription site at the chromosome within the nucleus to the cytoplasmic translation apparatus, needs to be elaborated with regard to acquired and innate immunity. In this review, we summarize the current knowledge on GANP as a component of TREX-2 in mammalian cells.



1. INTRODUCTION

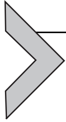
Higher organisms have developed a system of self-defense against genetic material, such as viruses and retrotransposon elements invading cells, which involves the induction of cytidine deamination by AID/APOBEC

family members and finally the generation of diverse effector molecule antibodies (Abs) for acquired immunity. The AID/APOBEC family consists of 5 members in mice and 11 members in humans (Conticello, Thomas, Petersen-Mahrt, & Neuberger, 2005). The catalysis by the members differs between RNA and single-stranded (ss) DNA molecules, although the details of their targets remain to be determined. AID is well characterized as a molecule that is essential for the diversification of immunoglobulin (Ig) variable (V) regions and class-switch recombination (CSR) in B cells. AID expression is selective to activated B cells, particularly in germinal centers (GCs) in the peripheral lymphoid organs after antigen (Ag) immunization, and targets ssDNA.

We have been studying the molecules upregulated specifically in GC B cells upon immunization with T-cell-dependent Ags (TD-Ags) by an approach using monoclonal Abs (mAbs). One of these mAbs recognizes a molecule that appears in the light zone of GCs and a study of its cDNA clone indicated that its transcripts of over 6 kb in length appeared in the light zone of GCs by in situ hybridization (Kuwahara et al., 2000). The putative protein was novel, with no known sequence similarity to mammalian molecules, and was predicted to be a 210-kDa nuclear protein-designated germinal center B-cell-associated nuclear protein (GANP). The central region of GANP was shown to be homologous to *Saccharomyces cerevisiae* Sac3, which was identified as a gene involved in the actin assembly (Bauer & Kolling, 1996). To distinguish it from its mammalian counterpart, here, we add the letter "y" in front of the yeast homologue, namely, ySac3. ySac3 was subsequently characterized as a component of the mRNA export complex in yeast, namely, the transcription/export-2 (TREX-2) complex (Fischer et al., 2002; Gallardo, Luna, Erdjument-Bromage, Tempst, & Aguilera, 2003).

GANP was shown to interact with the AID/APOBEC family of cytidine deaminases, such as AID in GC B cells and APOBEC3G (A3G) in activated human T cells, and to be involved in the targeting of AID/APOBEC family proteins to their appropriate target nucleotide sequences (Maeda et al., 2010, 2013). Since the mRNA export machinery is diverse in the cells of higher mammals and transcribes the complex structures of coding and noncoding genes assembling their exon–intron arrangements, our current knowledge of it is limited, so there is a need for comparisons with data on the homologues in lower eukaryotic cell models. GANP is involved not only in B cell IgV region diversification, but also in the escort of AID/APOBEC family cytidine deaminases, along with

the evolutionary development of self-defense. The mRNA export function of TREX-2 should be clarified as an important issue in immunological research.



2. AFFINITY MATURATION AND AB SPECIFICITY

The molecular mechanisms by which an Ab specifically recognizes a pathogen and eliminates it from the body and how diverse forms of Abs are produced have been focused on for a long time in immunology. In his selection theory, Burnet suggested that receptor–ligand specificity has a genetic basis, which was later confirmed to involve somatic-cell Ig gene rearrangements that generate enormous diversity in the specific IgV region as a primary repertoire (Tonegawa, 1983). Pauling attempted to explain the mechanism by which the diversification of receptor specificity occurs in lymphocytes after encountering an Ag as a ligand, using his "instructive theory" (Pauling, 1940). Later studies demonstrated the alteration of receptor affinities by the accumulation of somatic mutations in the IgV region of Ab molecules (McKean et al., 1984; Rudikoff, Pawlita, Pumphrey, & Heller, 1984), which was shown to be important in the affinity maturation of Abs, particularly for soluble Ags. In fact, the effectiveness of an Ab in the primary immune response is often limited and insufficient to eliminate pathogens in order to prevent various infectious diseases; it needs to be augmented in the form of affinity maturation by a vaccination. While the obtained results did not prove the validity of instruction theory, the secondary diversification of the IgV region was shown to involve the somatic hypermutation (SHM) mechanism during the immune response, which is critical for adaptive immunity.

2.1 Germinal Centers

Gray's anatomy of the human body describes this region in the lymph nodes as follows: "The nodules or follicles in the cortical portion of the gland frequently show, in their centers, areas where karyokinetic figures indicate a division of the lymph corpuses. These areas are termed germ centers." Our understanding of the physiological role of the lymphoid aggregates of lymphoid tissue was promoted by a presentation by Bob White at the first Prague meeting. Then, in 1981, Walther Flemming, who was studying cell division, more specifically pointed out a region of large lymphocytes undergoing mitosis as a GC in the follicles of secondary lymph nodes (Nieuwenhuis & Opstelten, 1984). GCs are defined as a microenvironment

that supports the proliferation and differentiation of Ag-specific B cells during the initial 3 weeks after Ag stimulation (Hollowood & Macartney, 1992; Kraal, Hardy, Gallatin, Weissman, & Butcher, 1986; Liu, Zhang, Lane, Chan, & MacLennan, 1991), undergoing CSR from the IgM to the IgG isotype in peripheral lymphoid organs (Kraal, Weissman, & Butcher, 1982), with the binding of high levels of peanut agglutinin (Rose, Booth, Habeshaw, & Robertson, 1982), particularly in Peyer's patches (Butcher et al., 1982). T cells are involved in GC formation and the generation of B cell memory (Jacobson, Caporale, & Thorbecke, 1974; Mond, Kim, & Siskind, 1974; Thorbecke, Benacerraf, & Ovary, 1963). Earlier studies had already suggested the possibility that some activated B cells enter follicles where they reencounter the relevant Ag (immune complexes) localized on the surface of follicular dendritic cells (FDCs) (Klaus, Humphrey, Kunkl, & Dongworth, 1980; Tew, Phipps, & Mandel, 1980; van Rooijen, 1972) and proliferate further without differentiating into Ab-secreting plasma cells (Coico & Thorbecke, 1985). Thus, GCs are associated with the clonal expansion of Ag-specific B cells (Jacob & Kelsoe, 1992; Jacob, Kelsoe, Rajewsky, & Weiss, 1991; Kroese, Wubbena, Seijen, & Nieuwenhuis, 1987), IgV region SHM (Berek, Berger, & Apel, 1991; Jacob & Kelsoe, 1992; Jacob et al., 1991; Kallberg, Gray, & Leanderson, 1993), selection of B cells for high-affinity Ag-binding potentialities (Liu et al., 1989), and subsequent differentiation into Ab-secreting plasma cells (Benner, Hijmans, & Haaijman, 1981; Kosco, Burton, Kapasi, Szakal, & Tew, 1989; Tew et al., 1992) or entry into the memory B cell pool (Coico, Bhogal, & Thorbecke, 1983; Klaus et al., 1980).

2.2 Selection of GC B Cells

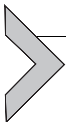
Centrocytes of GCs are sensitive to apoptosis, presumably favoring to the selection for the affinity maturation of B cell Ag receptor (BCR) by IgV region SHM. The survival of GC B cells can be achieved in vitro for a longer period by stimulation with anti-CD40 mAb (MacLennan & Gray, 1986) or CD40 ligand (CD40L) (Holder et al., 1993) plus IL-4 (Butch & Nahm, 1992; Holder, Grafton, MacDonald, Finney, & Gordon, 1995; Mangeney, Richard, Coulaud, Tursz, & Wiels, 1991). These suggest a model in which CD40L and IL-4 are located on the surface of FDCs to maintain long-term survival (Gray, Kosco, & Stockinger, 1991; Kosco, Szakal, & Tew, 1988). Distinctive FDCs, lacking the organelles of cells with active secretory and endocytic properties, but being quite different from

macrophages, are present in both the GC and the adjacent mantle region of secondary follicles (Chen, Adams, & Steinman, 1978; Chen, Frank, Adams, & Steinman, 1978).

2.3 Structure of GCs

MacLennan and coworkers described the structure of GCs with microenvironmental zones as a "dark zone" localized near the base and a "light zone" at the apex of the follicular structure (Liu, Johnson, Gordon, & MacLennan, 1992; MacLennan, 1992, 1994). The cell-cycle of B cells in the dark zone was estimated around 6 h in the form of centroblasts with the highest mitotic activity (Liu et al., 1992). The FDC network is extended in the light zone as the apical light zone with $CD23^{hi}$ FDCs and the basal light zone with $CD23^{lo}$ FDCs (Hardie, Johnson, Khan, & MacLennan, 1993). Small follicular B cells appear at the outer edge of the FDC network to form the follicular mantle, which is clearly demarcated from the central portion of the follicle. The light zone was shown to contain smaller and less closely packed lymphoid cells named centrocytes that had not proliferated in the 12 h since the last cell-cycle (Hanna, 1964).

Current real-time imaging studies have shown the dynamic migration of B cells across the two GC zones (Allen, Okada, Tang, & Cyster, 2007; Hauser et al., 2007; Schwickert et al., 2007) with similar characteristics of cell division and cell death, partially dependent on the altered expression of the chemokine CXCL13 and surface CXCR4 expression (Allen et al., 2004). GC B cells progress through the cell-cycle in both dark and light zones (Allen et al., 2007; Hauser et al., 2007; Rahman, Rao, Kalled, & Manser, 2003; Wang & Carter, 2005). The dynamic alteration in the two compartments between the light zone and the dark zone implies the individual functional properties of centrocytes and centroblasts with respect to cell size, cell-cycle progression, SHM induction, and CSR for the selection of high-affinity Ab-producing B cells during immune responses.



3. MOLECULES ASSOCIATED WITH AB AFFINITY MATURATION

A range of mutant mice produced by the gene targeting of various B-cell-associated molecules exhibited impaired GC formation: MHC class II-deficient (Cosgrove et al., 1991), human CTLA-4 Ig transgenic (Lane et al., 1994), CD28-deficient (Ferguson, Han, Kelsoe, & Thompson, 1996; Shahinian et al., 1993), B7-1/B7-2 double-deficient (Borriello

et al., 1997), CD40-deficient (Kawabe et al., 1994), CD40L-deficient (Renshaw et al., 1994; Xu et al., 1994), TNFR1-deficient (Le Hir et al., 1996), TNF-α-deficient (Pasparakis, Alexopoulou, Episkopou, & Kollias, 1996), OCA-B-deficient (Kim et al., 1996; Schubart, Rolink, Kosco-Vilbois, Botteri, & Matthias, 1996), and LT-α-deficient mice (Banks et al., 1995; De Togni et al., 1994; Matsumoto et al., 1997). Mutant mice with deficiencies in T/B cell cooperation also showed impaired affinity maturation, such as MHC class II-deficient, human CTLA-4 Ig transgenic, CD28-deficient, B7-1/B7-2-deficient, CD40-deficient, and CD40L-deficient mice. However, LT-α-deficient mice did not show a clear deficiency in affinity maturation upon immunization with TD-Ags, indicating that affinity maturation is not strictly dependent on GC formation (Matsumoto et al., 1996).

3.1 Neuberger's Model of SHM Generation by AID

After extensive studies in many laboratories attempting to identify the "hypermutator at the IgV region" enzyme, Honjo's group discovered a molecule named AID that is essential for triggering both SHM and CSR (Muramatsu et al., 1999, 2000), which was proved coincidentally using the B cells in a group of immunodeficient patients (Revy et al., 2000). Gene targeting of AID in mice caused a deficiency of IgV SHM, resulting in the impairment of affinity maturation, and CSR upon immunization with TD-Ags, but could generate the formation of larger GCs in the splenic follicles after 2 weeks of immunization; thus, IgV region SHM and affinity maturation are not necessarily associated with GC formation. The cytidine deaminase activity of AID was shown to be consistently strong in converting dC to dU in ssDNA in an in vitro deamination assay (Pham, Bransteitter, Petruska, & Goodman, 2003). Neuberger's model clearly explains the molecular mechanism after the initial cytidine deamination, creating the transition mutation from dC to dU and subsequently to dT on the target strand, accompanied by dG to dA on the opposite strand as reviewed (Di Noia & Neuberger, 2007). This nucleotide pair mismatch might be passed on to the daughter cells during DNA replication in B cells, but is often processed by uracil nucleotidyl-glycosylase, generating a basic site to be replaced by any four nucleotides (dC, dG, dA, and dT) for transversion mutations after replication or providing a fragile site at which DNA cleavage occurs and is then targeted for DNA repair by short patch repair or long fragment DNA region. In GC B cells, various error-prone DNA polymerases

generate more extensive alterations in the adjacent segments in the IgV region (Weill & Reynaud, 2008). The principle behind DNA damage repair systems involves the analogous biochemical pathways of base excision repair and nucleotide excision repair; they enable organisms to respond rapidly to massive DNA damage by copying undamaged and damaged DNA using the class of error-prone DNA polymerases (Goodman, 2002). The catalytic reaction of the enzymes in this class results in low accuracy of nucleotide incorporation, with a base substitution frequency of 10^{-1} to 10^{-3}. Error-prone DNA polymerases play critical roles in IgV region SHM in mammals in different profiles with Pol η (Rogozin, Pavlov, Bebenek, Matsuda, & Kunkel, 2001; Zeng et al., 2001), Pol ζ (Zan et al., 2001), and Pol ι (Poltoratsky et al., 2001).

3.2 B-Cell Lymphoma 6 and B Lymphocyte Maturation Protein-1

An important regulator of GC B cells is B-cell lymphoma 6 (BCL6), which is a transcriptional repressor of the Broad complex, Tramtrack and Bric-a-brac/Pox virus and zinc finger (BTB/POZ) family (Deweindt et al., 1995), the translocation of which was identified in diffuse large B cell lymphoma (Ye et al., 1993). BCL6-deficient mice show normal B cell development, but cannot form mature GCs and lack affinity maturation upon immunization with TD-Ags (Dent, Shaffer, Yu, Allman, & Staudt, 1997; Ye et al., 1997), and reciprocally, transgenic BCL6 overexpression increases GC formation (Cattoretti et al., 2005). The roles of BCL6 expression and signaling toward its transcription have been described elsewhere as an excellent review (Klein & Dalla-Favera, 2008). Briefly, BCR induces MAPK activation to phosphorylate BCL6, causing ubiquitin-mediated proteasome degradation (Niu, Ye, & Dalla-Favera, 1998) and an acetylation reaction to BCL6 through an unknown mechanism (Bereshchenko, Gu, & Dalla-Favera, 2002). Since BCL6 binds to the regulatory elements of various target genes at the consensus DNA sequence TTCCT(A/C)GAA (Chang, Ye, Chaganti, & Dalla-Favera, 1996; Seyfert, Allman, He, & Staudt, 1996) by interacting with multiple distinct components of repressors such as SMRT (silencing mediator for retinoid and thyroid receptor) (Ahmad et al., 2003; Dhordain et al., 1997), NcoR (nuclear-receptor corepressor) (Ghetu et al., 2008), and BcoR (BCL-6 coreceptor) (Hatzi et al., 2013; Polo et al., 2004), the repressive activity presumably covers many biological functions: (1) inhibition of B cell activation (BCL6, CD69, STAT1, TLR4, CCR6, and CD80), (2) regulation of the cell-cycle

(CDKN1A), (3) inhibition of DNA damage response (p53 and ATR), (4) inhibition of plasma cell differentiation (PRDM1 and IRF4), and (5) inhibition of memory B cell differentiation. BCL6 is also expressed in $CD4^+$ T cells and is involved in the establishment of GC T follicular helper (T_{FH}) cells by repressing the transcription factors TBX21 (T-bet), GATA3, and RORγt (Johnston et al., 2009; Liu et al., 2012; Marshall et al., 2011; Nurieva et al., 2009; Pepper, Pagan, Igyarto, Taylor, & Jenkins, 2011; Yu et al., 2009). Thus, the complex mechanistic roles of BCL6 in the differentiation of GC B cells and lymphomagenesis have remained as a focus of study (Bunting & Melnick, 2013).

A counter regulator of BCL6 is B lymphocyte maturation protein-1 (Blimp-1), which was cloned from BCL1 murine lymphoma upon differentiation to a plasma state (Turner, Mack, & Davis, 1994). The enforced expression of Blimp-1 drives mature B cells to become Ab-producing plasma cells (Angelin-Duclos, Cattoretti, Lin, & Calame, 2000; Piskurich et al., 2000; Schliephake & Schimpl, 1996). BCL6 acts as a transcriptional repressor to reduce the expression of *PRDM1* that encodes Blimp-1; this in turn suppresses the maturation of GC B cells into Ab-secreting plasma cells (Chang et al., 1996; Dent et al., 1997; Fukuda et al., 1997; Reljic, Wagner, Peakman, & Fearon, 2000; Shaffer et al., 2000) by targeting genes including *c-myc* (Eilers, 1999; Lin, Wong, & Calame, 1997), *CIITA* (Piskurich et al., 2000), *MHC class II* (Silacci, Mottet, Steimle, Reith, & Mach, 1994), and *PAX5* (Lin, Angelin-Duclos, Kuo, & Calame, 2002).

3.3 CD40–CD40L

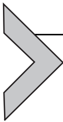
CD40 engagement activates NF-κB and STAT6 with increases of BCL6 and SWAP-70, proteins that play critical roles in GC formation (Allman et al., 1996; Dent et al., 1997; Falini et al., 1997; Onizuka et al., 1995; Qi et al., 1999; Ye et al., 1997). BCL6 physically interacts with PU.1 and is recruited to the PU.1-binding sites of Ig κ and λ 3′ enhancer regions, suggesting the complex repressor activities on various genes involved in the regulation of GC B cell maturation (Wei, Zaprazna, Wang, & Atchison, 2009). In the conventional GC model, the dark zone and light zone of GCs are thought to contain Ag-stimulated B cells with different proliferation rates. The centroblasts rapidly proliferate for clonal expansion in the dark zone by suppressing further differentiation into plasmablasts; in contrast, centrocytes are thought to be cell-cycle-arrested small B cells expressing the mature higher-affinity BCR, which will be selected by the FDC network. Although

a recent in vitro vital imaging study demonstrated the periodic transition and cell-cycle progression by cycling between the centroblast and centrocyte stages (De Silva & Klein, 2015), the notion of these distinct subpopulations might be still implicative and would be an issue for the molecular analysis. The genes with increased transcription in centroblasts from naïve B cells are: (1) involved in DNA replication (eg, PCNA), (2) encode cyclins and various CDCs, (3) control G1/S and G2/M transitions (eg, DP-1, GADD45), and (4) encode mitotic checkpoint kinases (eg, BUB1, Mad3L) and other mitotic products that are components of the centrosome, spindle, and kinetochore. Interestingly, c-myc expression was found to be lower in centroblasts than in centrocytes (Klein et al., 2003). In centroblasts, there was the upregulation of genes associated with DNA repair, such as *PMS2*, *MLH1*, *MSH6*, and *OGG1*, and with homologous recombination, such as *BRCA-1*, *Rad2*, *Exo1*, and *DNA ligase 1*. Similarly, in human follicular lymphoma cells, there was upregulation of the genes *Bsp-1*, *Bcl-2*, *Mlk-3*, *p21CIP1*, *Hsp27*, *Hsp40*, *TNF*, *Id-2*, *c-Jun*, *Ear-2*, *YY1*, *CDK10*, *ZFK*, *IL-4Rα*, *Db1*, *IL-2Rγ*, *Xpb*, *CCN1B*, *Pax-5*, *Lyn*, *SNF2-α*, *p16INK4A*, *p120*, and *Hsf-1*, but down-regulation of *MRP14*, *MRP8*, *TDPX2*, *p55CDC*, *CD40*, *thymosin β-10*, *DBI*, and *LFA-1* (Husson et al., 2002).

3.4 Lyn

One of the Src-family tyrosine kinases, Lyn, is a key mediator of B cell activation pathways through interaction with BCR complexes (Yamanashi, Kakiuchi, Mizuguchi, Yamamoto, & Toyoshima, 1991) and CD19 (van Noesel, Lankester, van Schijndel, & van Lier, 1993), and is involved in phosphorylation of the immunoreceptor tyrosine-based activation motif in the accessory molecules, leading to initiation of the tyrosine phosphorylation cascade to Igα/Igβ (Saouaf et al., 1994), Btk, Syk, and Fyn, and subsequently to the p85 of phosphoinositide 3-kinase (PI3K) (Gauld & Cambier, 2004; Pleiman, Hertz, & Cambier, 1994; Xu, Beavitt, Harder, Hibbs, & Tarlinton, 2002). In Lyn-deficient ($Lyn^{-/-}$) mice, the generation of IgV region SHM and affinity maturation was mildly impaired upon immunization with a hapten, 4-hydroxy-3-nitrophenylacetyl (NP)-chicken γ-globulin (CGG), indicating that the Lyn signal is not an absolute requirement for the generation of IgV SHM. Nevertheless, upon comparing $Lyn^{-/-}$ chicken DT40 B cells with the wild-type (wt) DT40, the former showed changes of the expression of 45 genes, and the significant decreased genes in their expression were further selected, including those involved in BCR signaling (*CD74, CD22, Igα H chain, CD79b*), proliferation (*GANP,*

tumor-associated MAGE-like, nucleoside diphosphate kinase, stathmin, prothymosin, and transglutaminase), control of transcription (*EF1α, cleavage and polyadenylation-specific factor, CEBP, TCF1, OBF-1, ICS BP*), immunity and immune response (*NF-κB*), and cytoskeletal organization (*γ-actin, transglutaminase, chaperonin CCTd, stathmin*) (Mirnics et al., 2004). The BCR cross-linking signal mediates Lyn activation, leading to STAT activation through a Jak-independent mechanism (Wang, Kurosaki, & Corey, 2007), but Lyn also promotes the activation of PU.1, presumably resulting in the upregulation of PU.1-dependent molecules in GC B cells (Mirnics et al., 2004).



4. GERMINAL CENTER B-CELL-ASSOCIATED NUCLEAR PROTEIN

4.1 Expression of GANP in GCs

As described earlier, various approaches have been applied in numerous laboratories to identify the molecules expressed in the GC area in mice and humans. A mAb prepared by the immunization of mouse B cell lymphoma recognized a protein expressed at a higher level in the GC area of the spleen after immunization with TD-Ag, sheep red blood cell (SRBC) (Kuwahara et al., 2000). Although the protein is ubiquitously expressed in many kinds of somatic cell, similarly to BCL6, immunohistochemical analysis clearly demonstrated its upregulation in GCs. A cDNA clone isolated by mAb screening using the λgt-11 phage cDNA library was shown to encode a 210-kDa nuclear protein. In situ hybridization revealed its marked upregulation in the GC area of both mouse spleen and human tonsil; thus, the protein was designated as GANP given its upregulation in immune cells. The transcription of *ganp* and expression of GANP protein were found to be augmented after 48 h of stimulation with anti-IgM Ab and CD40 cross-linking in B cells in vitro, and the phosphorylation at Ser502 (pSer502) was augmented by stimulation with CD40 cross-linking in vitro and in GC B cells in vivo, particularly in the apical region of the mouse GC area (Kuwahara et al., 2001). Promoter analysis of the mouse *ganp* gene also showed the presence of a PU.1 consensus binding site at a position 134 bp upstream of the transcription start site (EL-Gazzar et al., 2001), suggesting that the signaling to activate PU.1-mediated transcription is associated with GANP function in B cell activation, as predicted in the Lyn-deficient DT40 B cell model (Mirnics et al., 2004).

4.2 GANP and AID

GC formation was shown to occur in the absence of T cells in mice with transgenic BCR of the quasi-monoclonal IgV region that recognizes NP (de Vinuesa et al., 2000), and the sporadic appearance of GCs in athymic mice (Stedra & Cerny, 1994) indicated that T cell costimulatory signaling is not mandatory for GC formation. However, the Ab responses of B cells were found to be augmented by helper T cell interaction through CD40–CD40L for the proliferation of Ag-specific B cells, increased SHM and CSR, and affinity maturation. Recently, it has also been shown that GC-like B cells can be maintained for the long term in experiments with IL-2 and fibroblast feeder cells expressing CD40L and BAFF (B cell-activating factor, belonging to the tumor necrosis factor family) (Kitabatake et al., 2015; Nojima et al., 2011). This in vitro culture system can support GC B cell proliferation with the expression of the GC-associated transcription factors AID, IRF4, and BCL6, presumably for GC formation; however, it cannot support the generation of IgV region SHM for Ab affinity maturation.

AID seemed to require additional accessory molecules to induce efficient IgV region SHM, so attempts have been made to characterize AID-associated proteins in many laboratories (Table 1). GANP was shown to associate physically with AID (Maeda et al., 2010). Various kinds of GANP mutants were shown to associate similarly with AID (unpublished data) and such interaction was found to be independent of the presence or absence of DNA or RNA in immunoprecipitation experiments; thus, no specific interaction domain has been determined so far. AID possesses several acidic motifs, containing glutamic acid (E) and aspartic acid (D) residues, on the turns of the peptide chains between helices and β-sheets. GANP also has various basic motifs with arginine (R) and lysine (K) residues. We speculated that the physical interaction between them occurs through the attractive force generated by their opposite charges. AID has six negatively charged residues (D_{37}SAT, D_{67}WD_{69}LD_{71}, PCYD_{89}C, C$E_{117}D_{118}$R, E_{122}PE_{124}G, and D_{143}YFYC), and AID mutants with substitution of each of these amino acid residues showed altered association with GANP. In an AID mutant with the substitution D143A (Asp143 to Ala), the association between AID and GANP was critically impaired. These results indicate that GANP forms a complex with AID and the RNAs in cells, presumably through a charge-mediated mechanism in the presence of various accessory components.

Table 1 AID-Interacting Proteins

Category	Genes
Transcription units	RNA polymerase II
	DSIF (Spt5)
	PAF
	FACT (SSRP1, Spt16)
RNA exosome	Rrp (EXOSC)
Splicing factor	PTBP2
	SRSF1-3
Spliceosomal protein	CTNNBL1
Nucleolar protein	Nucleolin
	Nucleophosmin
RNA export	GANP
	Crm1
Regulatory kinase	Protein kinase A
	DNA-PKcs
	14-3-3
ssDNA-binding protein	RPA32
Ubiquitin ligase	RNF126
DNA glycosylase	UNG
Transcriptional regulator	KAP1/TRIM28
	HP1
Chaperon	HSP90
	Dnaja1
Posttranslation modification	eEF1A
Chromosome condensation	Cohesin
	Condensin
	Ino80

4.3 Nuclear Translocation of AID

AID contains a nuclear localization signal, but is highly acidic and is normally localized in the cytoplasm. For AID to target an Ig gene, it must be localized at least transiently in the nucleus. Many laboratories speculated that accessory molecules are required for AID shuttling between the cytoplasm and the nucleus in B cells. Alternatively, AID might enter the nucleus along with nuclear breakdown in the mitotic phase, when the nuclear envelope is temporarily dispersed. During reassembly of the nuclear envelope, a few copies of AID would remain in the nucleus and access the IgV region chromatin in a transcription-competent state. By cotransfection experiments in COS7 cells, AID(wt) fused with red fluorescent protein (DsRed) was found to be exclusively localized in the cytoplasm as reported previously (Ito et al., 2004; McBride, Barreto, Ramiro, Stavropoulos, & Nussenzweig, 2004; Pasqualucci et al., 2004; Rada, Jarvis, & Milstein, 2002), and AID(wt)-DsRed was shown to comigrate into the nucleus upon cotransfection with the fusion protein green fluorescent protein (GFP)-GANP. Mutant AID^{D143A}, however, never appeared in the nucleus and remained separately in the cells, as AID(wt)-DsRed in the cytoplasm and GFP-GANP in the nucleus. These results demonstrate that GANP is involved in the machinery that escorts AID to the nucleus.

4.4 Targeting of AID to the Rearranged IgV Region

The AID/GANP complex is recruited selectively to the site of the rearranged IgV region locus through the action of the histoneacetyltransferase (HAT) domain of GANP (Singh et al., 2013). The HAT domain (amino acids 1648–1783) has the ability to acetylate histones H1 and H3, which loosens the chromosome to form oligo- or monochromatin. The rearranged IgV region is grasped tightly at the two independent sites of the 5′-end of the V region exon (first site) and the tail end of the rearranged IgV region (second site), maintaining the basic IgV region chromosome structure that is resistant to Mung Bean Nuclease (MNase) treatment, while almost the entire IgV region is loosened for active transcription in mature B cells (Kodgire, Mukkawar, North, Poirier, & Storb, 2012). The HAT activity of GANP involves selective acetylation at the second MNase-resistant site, which relaxes the chromatin structure, allowing access of the AID/GANP complex. When HAT activity is lacking, the chromatin structure cannot be efficiently released. The initial histone acetylation is followed by the successive alteration of histone H3 at Lys 9 (H3K9) and Lys 27 (H3K27) over the entire rearranged

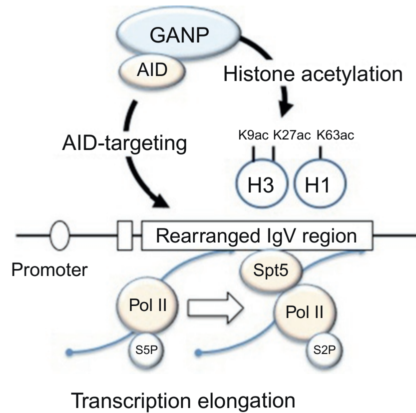


Fig. 1 Accession of the AID/GANP complex to the rearranged IgV region in B cells. The AID/GANP complex is recruited to the selective site of the rearranged IgV region gene through the action of HAT actively of GANP, acetylating H3K9, H3K27, and H1K63, consequently releasing AID to the R-loop during the active transcription with the initiation (S5P: phosphorylated at Ser5) and the elongation (S2P: phosphorylated at Ser2) competent Pol II. The AID effectively induces IgV SHM during the transcription stalling with Spt5.

IgV region locus. A chromatin immunoprecipitation (ChIP) assay revealed the access of GANP to the second MNase-resistant site in the rearranged IgV region, together with the stalling factor Spt5 (transcription elongation factor) and the insulator CTCF (CCCTC-binding factor). Moreover, GANP augments the access of RNA polymerase II (Pol II) pSer5 (as a transcription initiation phosphorylated form) and pSer2 (as a transcription elongation form). Eventually, access of the AID/GANP complex to the rearranged IgV region locus is facilitated, increasing the mutation frequency. A schematic model of the access of AID/GANP to the rearranged IgV region is shown in Fig. 1. Collectively, GANP-mediated chromatin modification promotes the recruitment of the transcription complex and positioning at the IgV region loci, which facilitates AID targeting.

4.5 Role of GANP in Ab Affinity Maturation

The expression of GANP is not restricted to the B cells in GCs; it is also expressed and plays a critical role in early embryos, as evidenced by the embryonic lethality of GANP-null mutant mice (Yoshida et al., 2007). Despite its ubiquitous expression, the upregulation of GANP protein and

its association with AID interaction in GC B cells during responses to TD-Ags suggest the significant role of GANP in immune reactions (Maeda et al., 2010; Singh et al., 2013).

4.5.1 GANP Conditional Knockout Mice

GANP-null mutant ($Ganp^{-/-}$) mice were shown to die at the embryo stage (on day 11) with their vessels displaying a fragile endothelial layer, along with massive hemorrhaging from the liver, aorta, and heart (unpublished data). $Ganp^{-/-}$ mice also displayed abnormalities in the brain, with enlarged third ventricles and a thinner cortex region. These findings indicated that GANP is essential for cell proliferation at the stage of organ formation during early development (Yoshida et al., 2007).

The first attempt to investigate the physiological function of GANP was performed by the conditional targeting of the *ganp* gene in B cells using the floxed *ganp* ($Ganp^{F/F}$) gene between exon 1 and exon 2 with *CD19-Cre* knock-in mice (Rickert, Roes, & Rajewsky, 1997). When *CD19-Cre/*$Ganp^{F/F}$ mice were born, they appeared to be normal and showed normal Ab responses, with various isotypes against T-cell-independent Ag (Kuwahara et al., 2004). These mice also showed levels of serum Abs of the IgM, IgG1, IgG2a, IgG2b, and IgG3 classes equivalent to those in the floxed control mice after immunization with TD-Ag NP-CGG under alum adjuvant immunization in an earlier inspection; however, B cells in *CD19-Cre/*$Ganp^{F/F}$ impaired the affinity maturation against NP-hapten, as measured by differential ELISA and the typical high-affinity-type W33L (Trp33 to Leu) mutation frequency of $IgV_H186.2$. *CD19-Cre/*$Ganp^{F/F}$ mice displayed a significant delay in GC formation in the spleen and a marked decrease of 4-hydroxy-5-iodo-3-nitrophenylacetyl (NIP)-binding $CD38^+IgG1^+$ B cells compared with the floxed control mice. The mutant mice also showed the increased apoptosis of GC B cells with relatively low *bcl-2* expression upon stimulation.

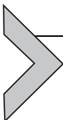
4.5.2 B Cell GANP Transgenic Mice

To further investigate the contribution of GANP expression to the affinity maturation of Abs against TD-Ags, we created transgenic mice that over-express mouse *ganp* cDNA under the control of Ig promoter and enhancer regions ($Ganp^{Tg}$ mice)(Sakaguchi et al., 2005). The $Ganp^{Tg}$ mice secrete high-affinity Abs against TD-Ags such as NP-hapten, NL41 peptide corresponding to the V3 stretch of HIV-1 gp120, and various kinds of Ags. Biacore analysis demonstrated that several mAbs established from $Ganp^{Tg}$ mice yielded 10- to 50-fold higher affinities against the NL41

peptides than those from the hybridomas of C57BL/6 mice. The effectiveness of *Ganp*^{Tg} mice was further confirmed by mAbs studies for various clinical applications. The *Ganp*^{Tg} mice were particularly effective against the epitopes of protein modification for cancer markers such as cancer-specific sugar epitope of Muc5A protein appearing in cholangiocarcinoma (Silsirivanit et al., 2011) and the pancreatic-cancer-specific modification (prolyl 4-hydroxylation) of serum α-fibrinogen (Ono et al., 2009). These results suggested that GANP is associated with the generation of high-affinity Abs against TD-Ags, even though IgV region SHM and GC formation were modestly affected by the 50% increase in GANP expression in B cells.

4.5.3 GANP in Lyn-Deficient Mice

The involvement of GANP in GC formation and the generation of IgV region SHM was further studied using Lyn-deficient mice. Lyn deficiency is associated with the impairment of GC formation (Hibbs et al., 1995; Nishizumi et al., 1995). GANP expression was shown to be regulated by Lyn-mediated signaling in DT40 B cells (Mirnics et al., 2004). Thus, the role of GANP as a Lyn-signaling downstream event was examined by breeding *Ganp*^{Tg} mouse with Lyn$^{-/-}$ mouse. *Ganp*^{Tg} Lyn$^{-/-}$ mice could not recover or affect the impairment of GC formation compared with Lyn$^{-/-}$ mice (Kuwahara et al., 2012); however, IgV region SHM was increased in GC B cells upon immunization with NP-CGG. The IgV region SHM could occur in the absence of mature GC formation when GANP expression was increased to a level equivalent to that in GC B cells. These results indicate that IgV region SHM could occur outside of the GC area in the parafollicular region with the increase of CD11c^{+} dendritic cells. Collectively, these observations provide evidence that GANP expression in GC B cells is indeed involved in the affinity maturation of Ag-specific B cells in vivo.



5. STRUCTURE OF GANP

Numerous findings that reveal the immune function of GANP have been reported, as described earlier, but the molecular mechanisms involved, besides their promoting AID targeting of the rearranged IgV region of the genome, remained unclear. To clarify the mechanistic role of GANP, it is necessary to understand the structure of GANP.

5.1 *Ganp* Gene Organization

The *ganp* (MCM3AP in human) gene encodes at least six variant transcripts and is located on human chromosome 21 (chr21: 46,235,126–46,285,394, complement), being mapped of the long arm at 21q22.3. It encodes 28 exons for the full-length functional GANP protein. FANM-V data analysis in UCSC Genome Browser on Human Dec. 2013 (GRCh37/hg38) demonstrated the sense strand transcripts starting from the 5′-UTR region as the full-length transcript. The *ganp* gene locus is surrounded by the *YBEY* (ybey metallopeptidase: putative) gene at the 5′ side and the *LSS* (lanosterol synthase: 2,3-oxidosqualene-lanosterol cyclase) gene at the 3′ side. Two additional kinds of antisense transcripts are starting from the middle of the *ganp* gene locus. Layered H3K27Ac (acetylation) analysis shows two strongly peak signals at the both sides of *ganp* genome. The middle region of *ganp* genome locus is also detected H3K27Ac, which is closely related to the transcription start site of antisense *ganp* mRNA. Interestingly, another antisense RNA (MCM3AP-AS) transcripts are also located in the *ganp* locus at the 3′ side and use the same antisense exons (exon 20, 26) of *ganp* gene. The ChIP assay identified the interaction sites in the following order: *CTCF, IKA, RAD21, CTCFL, POLR2A, ZBTB2A, HMGN3, POLR2A, SIN3AK20, POXA1, POSL2, ZBTBT33, BHLHE49, TCF12, MYC, MAX, ETS1, TAF1, YY1, RELA, PLR2A, TBL1XR1, EBF1, RUNX3, ELF1, EBF1, CTCF, RAD21,* and *SIN3AK20*. The gene at the 3′ side, *LSS*, a member of the terpene cyclase/mutase family, catalyzes the first step in the biosynthesis of cholesterol, steroid hormones, and vitamin D. The *LSS* gene is thought to be regulated by p53, Pax-3, and Brachyury, but no reports describing the increased expression of *LSS* in B cells or in cancer cells have been published. The gene at the 5′ side, putative YBEY ribonuclease has 167 amino acids, but the evidence for the protein detection is still miscellaneous (Uhlen et al., 2012), although the Gene Ontology results suggest that the biological process associated with the *YBEY* gene is rRNA processing, determined using the GenesLikeMe website (http://genecards.weizmann.ac.il/v3/index.php?path=/GenesLikeMe).

5.2 A Short Form of *Ganp* Transcript

In 1998, Takei and Tsujimoto identified a protein that interacts to the human minichromosome maintenance protein (MCM) 3 by yeast two-hybrid screening (Takei & Tsujimoto, 1998). It was predicted to encode an 80-kDa protein, named as MCM3-associated protein, MAP80. They

demonstrated that MAP80 is associated with MCM3, a component of the MCM complex with DNA helicase activity. The short-form putative protein MAP80 has a region homologous to the domain of HAT molecules (Takei et al., 2001; Wickramasinghe et al., 2011). These structural protein interactions led to the prediction that GANP might be an accessory molecule for DNA replication in rapidly proliferating cells, such as Ag-driven B cells and highly proliferating malignant cells.

5.3 Structure of GANP Protein

The full-length 6-kb transcript from the same *GANP/MAP80* locus was shown to encode a 210-kDa nuclear protein in B cells that are activated and rapidly proliferating at the GCs of the peripheral lymphoid organs upon immunization with TD-Ag, SRBC (Kuwahara et al., 2000). In a subsequent study, the short-form transcript of the *GANP/MAP80* locus could not be identified sufficiently in human B cells by Northern blot analysis (Abe et al., 2000). Western blot analysis with specific Abs against GANP did not show a protein of a size corresponding to MAP80 in B cells, suggesting that the short-form MAP80 is expressed far less than the full-length GANP protein in mice and humans. Therefore, in this review, we focus on the structure of the full-length GANP protein.

5.4 FG-Rich Domain

The N-terminal domain of GANP in the region from amino acid 1 to 400 shows some differences among the mammalians. GANP has the typical FG (Phe-Gly)-rich amino acid sequence in humans, mice, rats, rabbits, cows, and sheep, in contrast to the proteins homologous to Sac3 in lower eukaryotes, which lack such structures. The family of proteins with FG-repeat regions function as components of the nuclear envelope, as shown in Fig. 2, suggesting that GANP has properties similar to those of nuclear pore complex (NPC) components.

5.5 RNA Recognition Motif

The N-terminal side region of GANP contains an RNA recognition motif (RRM). GANP is involved in the mRNA export of *shugoshin-1*, which is required for centromere cohesion and in sister-chromatid exchange (Okamoto et al., 2010). Knockdown of GANP with siRNA induces cell-cycle arrest at the G2/M-phase and causes abnormal chromosome alignment at the metaphase. PCID2, other TREX-2 components, also selectively

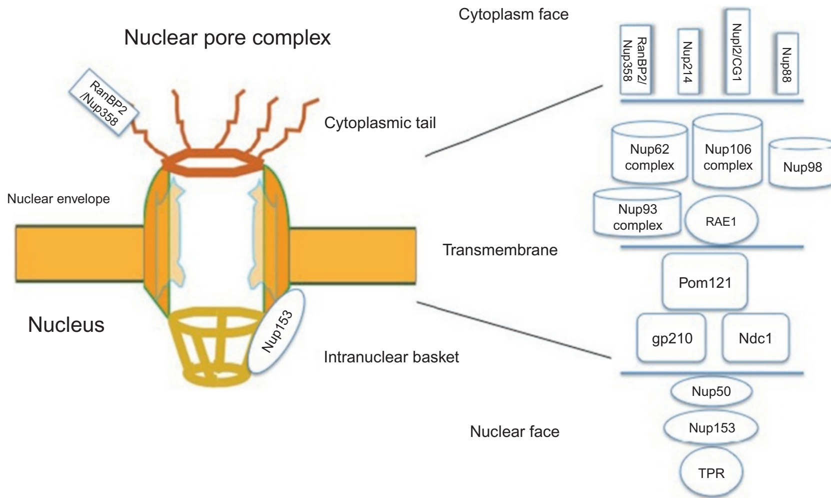


Fig. 2 Nuclear pore complex (NPC) of mammalian cells. NPC is perforating the nuclear envelope with transmembrane pipe structure composed of Nup62 complex, Nup106 complex, Nup93 complex, Nup98, RAE1, and the other components toward the cytoplasmic face, projecting the cytoplasmic tail with RanBP2/Nup358, Nup214, Nup12/CG1, and Nup88. Toward the nuclear face, the NPC involves Pom121, gp210, and Ndc1, for nuclear face with Nup50, Nup153, and TPR, creating the basket-like structure to accept the mRNPs interacting with TREX and TREX-2. The FG-rich region containing molecules play important roles for the assembly of NPC and the mRNA export.

regulates the export of *Mad2* mRNA, but not of the other cell-cycle-associated genes encoding MAD1, BUBR1, cyclin A, cyclin B1, and cyclin-dependent kinase 1 (Nakaya et al., 2010). This RRM may be contributed with Sac3-homology region.

5.6 Sac3-Homology Domain

In mice and humans, another gene homologous to *S. cerevisiae* Sac3 was identified and designated as Sac3-homology domain 1 (*shd1*) (GenBank accession number AJ131957). The Sac3-homology region of 686–910 amino acids of GANP corresponds to almost the whole of the SHD1 protein, namely, the region between amino acids 70–425 of the 425 amino acids of the mouse SHD1 protein (Khuda et al., 2004). SHD1 is necessary for cell division, especially in centrosome duplication, the spindle assembly checkpoint from metaphase to anaphase, and spindle formation. Mad2 expression was shown to be affected by a lack of SHD1, indicating the functional involvement of the Sac3-homology domain of GANP in cell-cycle

regulation through regulation of the anaphase-promoting complex-Cdc20 suppressing Pds1 activation and blocking cell division (Yu, 2002). GANP might possess a similar function in B cells.

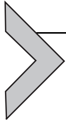
5.7 Histone Acetyltransferase Region

The C-terminal region of GANP (amino acids 1648–1783 in mouse) includes a HAT activity domain (Singh et al., 2013; Takei et al., 2001) that effectively catalyzes the acetylation of histone H1, which is different from the other HAT complex of SAGA that targets H2A/H2B and H3. Histone H1 is the fifth nucleosome-binding protein, following H2A, H2B, H3, and H4, and is positioned in doublets around a segment of which two turns of DNA are attached. A recent study also suggested the role of H1 in the response to DNA double-strand breaks (DSBs) through the interaction of ubiquitin-mediated proteolysis (Thorslund et al., 2015). H1 is known to play roles in tight chromatin folding and thus in gene regulation. It has three domains: the H1 global domain, which interacts with nucleosomal DNA, the N-terminal and C-terminal domains, which interact with the phosphate backbone of the linker DNA, and the N-terminal tails of the core histones. Acetylation alters the capacity of H1 to loosen the compact higher-order structure of the chromosome and generates the active gene-rich chromatin, and may also be involved in DNA repair after the targeting of AID to the IgV region of the genome.

5.8 MCM3-Interacting Region

Mammalian GANP is homologous to ySac3 in the central portion designated the Sac3-homology region (between amino acids 686 and 910), with similarity of 25%, but carries an additional unique region at the C-terminal end, most of which overlaps with the HAT region (Sakaguchi, Maeda, & Kuwahara, 2011). This region was identified as MAP80, a protein that interacts with MCM3 (Takei & Tsujimoto, 1998), a component of the DNA helicase complex of MCM2–7 (Tye, 1999). The MCM complex is known to be involved in genome replication by interacting with DNA Pol δ for DNA synthesis from the origin of replication center (ORC) in both directions, as the leading strand and the lagging strand, with the help of oligoribonucleotide chains creating Okazaki fragments. In eukaryotes, the ORC binds DNA replication origins and loads two MCM2–7 single hexamers through interactions with Cdc6 and Cdt1 (Evrin et al., 2009; Remus et al., 2009). The activation of the MCM2–7 complex takes place

at the S-phase via the involvement of several factors and cell-cycle-specific kinases. Initially, experimental results suggested the proximity of the functional interaction and colocalization of GANP with the MCM–DNA replication complex, but further studies failed to clarify the role of this interaction in the S-phase.



6. GANP-ASSOCIATED PROTEINS

Human and mouse GANPs have unique structures at the N-terminal and C-terminal regions compared with ySac3. Proteomic screening of human GANP-associated proteins using Abs for either its N-terminal or its C-terminal region showed unique molecules in each sample (Singh et al., 2013). The proteins that commonly appeared in both precipitates from human Ramos B cell nuclear extract were mostly involved in RNA processing and splicing, as well as the nuclear envelope, as listed in Table 2. Supporting the assertion that GANP is a component of TREX-2, it strongly

Table 2 GANP-Interacting Proteins

Techniques and Methods	Genes
2DICAL proteomics[a]	ARL6IP4,HNRNPA2B1, HNRNPC, HNRNPU, PNN, PRPF19, RBM8A, SNRPE, SFRS2B, SFRS7, SF3B3, SFRS10, DKC1, FBL, SRRM2
(Two different kinds of anti-GANP Abs)	AHCTF1, LMNB1, NUP160, NUP107, NUP85
	DDX9, DDX18, DDX21
	NSD, CIR, NPM1
	PRKDC, TOP2A
	H1, H2A, H2B, H3, H4
Yest two-hybrid screening[b] (The region of mouse GANP from 3844 to 5757 nt was used as the bait)	G5PR
Tandem-repeat proteomics[c]	MEP50
(3XFLAG TEV-CBP-GANP)	PRMT5

[a]Singh et al. (2013).
[b]Kono et al. (2002).
[c]Igarashi et al. (2009).

associated with RNA-processing components (ARL6IP4, HNRNPA2B1, HNRNPC, HNRPU, PNN, PRPF19, RBM8A, SNRPE, SNRPE, SFRS2B, SFRS7/9G8, SF3B3, SFRS10, DKC1, FBL, and SPRM2), RNA helicases (DHX9, DDX18, DDX21, DDX3, and DDX39), the nuclear envelope proteins (AHCTF1/ELYS, LMNB1, Nup62, Nup85, Nup107, Nup153, Nup160, and Nup354), chromatin regulatory proteins (NSD, CIR, NPM1), and DNA repair molecules (DNA-PKcs and Top2α), implying the unique function of GANP in RNA processing and nuclear export in B cells.

6.1 RNA Export Complex

From studies of *S. cerevisiae*, we have learned that mRNAs move from their site of transcription in chromatin toward the nuclear envelope and then through the nuclear pores into the cytoplasm (Abruzzi, Lacadie, & Rosbash, 2004; Fischer et al., 2002; Huertas & Aguilera, 2003; Jimeno, Rondon, Luna, & Aguilera, 2002; Rondon, Jimeno, Garcia-Rubio, & Aguilera, 2003). Active transcription is undertaken upon the recruitment of Pol II; the subsequently produced nascent transcripts gather together the mRNA-coating factors yTHO and yTREX and the mRNA export factors, forming a messenger ribonucleoprotein (mRNP) (Iglesias & Stutz, 2008). The initial mRNP assembles with the mRNP-coating factors and yTREX-2 complex to interact with the nuclear basket for nuclear translocation through the mRNA surveillance mechanism. After confirming the integrity of mRNPs and transportation into the cytoplasm, the mRNAs are released from the assembly by the action of yDEAD box protein 5 and yGle1 colocalized with nucleoproteins yNup42 and yNup159. All of these complexes are also composed of various subcomponents, together with the possible mammalian counterparts.

6.2 SAGA Complex and TREX-2 Complex

To achieve optimal gene activation in the chromatin, Lys123 of H2B is ubiquitinated for the *trans*-tail methylation of H3 in *S. cerevisiae* (Weake & Workman, 2008). The ySAGA (Spt-Ada-Gcn5-Acetyltransferase) chromatin-remodeling complex binds to the promoters of target genes and facilitates transcriptional activation through histone acetylation and/or deubiquitination with two distinct enzymes, yGcn5 and yUbp8 (Koutelou, Hirsch, & Dent, 2010; Rodriguez-Navarro, 2009). The ySAGA complex, demonstrated in yeast and *Drosophila* models, facilitates the local remodeling of chromatin and increases the accessibility for

elongation machinery, allowing the interaction of mRNA export factors with yTREX-2, which anchors the mature mRNPs to the NPC.

The export of mature spliced mRNAs from the nucleus to the cytoplasm is mediated by various sets of accessory factors. Typically, the conserved nuclear RNA export factor 1 (NXF1) and its cofactor p15 are involved in the nuclear export of most mRNAs, while a class of endogenous mRNAs and viral genomic RNAs use chromosome region maintenance 1 protein homologue (CRM1, exportin-1, EXO1). The NPC and the export adaptors have an FG-rich region and play important roles in the early stage of RNA biology as TREX and TREX-2 (Fig. 3). Recent studies have revealed that mRNA export in mammalian cells is mediated not only by the bulk export pathway, but also through alternative mechanisms, particularly for the molecules involved in DNA repair, gene expression, cell proliferation, cell survival, and the maintenance of cell pluripotency (Katahira, Inoue, Hurt, & Yoneda, 2009; Okamoto et al., 2010; Topisirovic et al., 2009; Wang et al., 2013; Wickramasinghe et al., 2014; Ye & Blelloch, 2014).

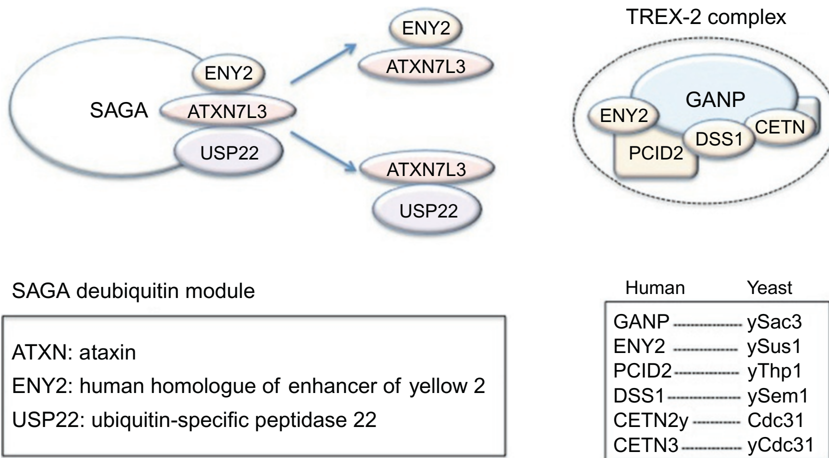


Fig. 3 Mammalian TREX-2 complex interacted with the SAGA complex. Mammalian SAGA complex is the chromatin-remodeling complex that binds to the promoters of target genes and facilitates transcriptional activation through histone acetylation and/or deubiquitination, increasing the accessibility for elongation machinery. Interaction of TREX-2 component ENY2 to ATAXN7L3 and the association of ATXN7L3 with ubiquitin-specific peptidase 22 (USP22) suggested the functional involvement of mammalian TREX-2 in the regulation of global transcription elongation based on the studies of yTREX-2 homologues. Human TREX-2 is designated as GANP–ENY2–PCID2–DSS1–CETN2/CETN3, while the physical interaction of these components has been partially determined.

Mammalian TREX-2 is an as-yet functionally undetermined complex that appears as an interface of NPC and the transcription machinery interacting next to SAGA and the THO complex. Yeast TREX-2 consists of ySac3, yThp1, ySem1, ySus1, and yCdc31, as listed in Table 3. The yTREX-2 complex has been thought to interact with the nascent transcripts after the initiation and elongation of RNA synthesis by Pol II. The processing of the primary RNA strand proceeds with splicing factors and the RNA-binding particle mRNP with a 5′-cap, 3′-poly(A) tail, and the protein coat of various export factors. The mRNP complex interacts with Mediators as a direct yTREX-2 target establishing the communication between yTREX-2 and Pol II. The yThp1 is a prototype of the mammalian PCID2 protein with a unique domain called the PCI domain that interacts with the Sac3 PCI region (amino acids 200–548) and also with ySem1 (the mammalian homologue of which is called DSS1). PCID2 plays a critical role in B cell survival and embryonic stem cell differentiation in mice (Nakaya et al., 2010; Ye et al., 2014). It associates with EID1 in the form of the CBP/p300-EID1 complex and impedes the association with MDM2, thus inhibiting the E3 ligase for Lys (K)48-linked EID1 ubiquitination for its degradation, thereby modulating the HAT activity of CBP/p300. Recent study showed that ySac3 also interacts with ySus1 and yCdc31 through the C-terminal side NPC anchor region (amino acids 723–805) (Schneider et al., 2015). According to this model, yTREX-2 is a component associated with the Mediator complex involved in mRNA transcription by interacting with

Table 3 TREX-2 Complex Components

	Mammal		Yeast
TREX-2 Components	**Gene Symbol**		
GANP	MCM3AP	Minichromosome maintenance complex component 3-associated protein	ySac3
PCID2	PCID2	PCI domain-containing protein 2	yThp1
ENY2	ENY2	Enhancer of yellow 2 homolog (*Drosophila*)	ySus1
DSS1	SHFM1	Split hand/foot malformation (ectrodactyly) Type 1	ySem1
CETN2	CETN2	Centrin, EF-hand protein, 2	yCdc31
CETN3	CETN3	Centrin, EF-hand protein, 3	yCdc31

Pol II and also plays a role in mRNA export by interacting with the NPC complex. The ySus1-mediated link of ySAGA and yTREX-2 is necessary at least for the selective transcription-coupled mRNA export (Klockner et al., 2009); however, the mechanism for mRNA export in mammals remains unclear.

6.3 Nuclear Pore

Most nascent transcripts are long precursor RNAs and only the processed functional RNAs are transported across the nuclear envelope through NPC. The nuclear envelope is composed of outer and inner nuclear membranes, both of which are spanned by the nuclear pores. The outer membrane is continuous with the endoplasmic reticulum. Transcriptional regulators cross-link inner nuclear membrane proteins and chromatin, including E2F, Pol II, and RNA splicing complex. Sites of genes in the nuclear compartments are likely to be nonrandom localized in the nuclear lamina and the developmentally repressed genes in heterochromatin (Finlan et al., 2008; Gartenberg, Neumann, Laroche, Blaszczyk, & Gasser, 2004; Guelen et al., 2008; Hediger & Gasser, 2002; Hediger, Neumann, Van Houwe, Dubrana, & Gasser, 2002; Makatsori et al., 2004; Pickersgill et al., 2006; Reddy, Zullo, Bertolino, & Singh, 2008; Taddei, Hediger, Neumann, & Gasser, 2004; Taddei et al., 2009). According to the gene gating hypothesis, the compact chromatin anchored to the nuclear membrane, but active chromatin associates with nuclear pores and allows the transport of mRNA from the nucleus to the cytoplasm (Blobel, 1985). The overall structure of the nuclear pore is conserved between yeast and mammal, although it has different sizes: 66 MDa in yeast and 120 MDa in vertebrates (Alber et al., 2007a, 2007b), involving a protein structure with eightfold symmetry, comprising the central transport channel and two rings, one toward the cytoplasmic side projecting the free loose ends of filaments and the other to the nuclear side with the connected ends forming the nuclear basket. The NPC contains multiple copies of approximately 30 different proteins named nucleoporins (Nups). The mammalian NPC is a cylindrical pipe-like structure of approximately 145 nm in diameter and 80 nm in length, in contrast to the smaller size of the yeast NPC of approximately 96 nm in diameter and 35 nm in length. The NPC is composed of eight proteins on the cytoplasmic surface, as embedded in the nuclear lamina, and a similar ring of eight on the inner surface of the nuclear membrane, positioned at the center of the NPC as a sphincter-like gate for the transportation of substances. The membrane-anchored proteins are

likely connected to secondary components of specific fold types, the β-propeller and α-solenoid, as well as FG residues separated by polar spacers of Nups of various lengths.

The NPC is known to be involved in mature mRNA metabolism in association with mRNA export by enhancing the coupling of 3′-end processing of mRNA in the mRNP (Dieppois & Stutz, 2010; Iglesias & Stutz, 2008). The C-terminal region of Pol II is strongly involved in RNA transcription and processing through association with the molecules that perform 5′-end capping, splicing, 3′-end processing, and poly-adenylation (Faza et al., 2009; Gonzalez-Aguilera et al., 2008; Pascual-Garcia et al., 2008). The interaction of SAGA–TREX-2 might support the gene–NPC link during the transcription elongation phase, which ensures high-fidelity processing of the transcripts and genomic stability during the high level transcription of specific genes in rapidly proliferating cells such as GC B cells.

6.4 Miscellaneous Proteins

6.4.1 Protein Phosphatase Subunit G5PR

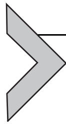
The proteins that interact with GANP were examined by yeast two-hybrid screening (Kono et al., 2002). A protein phosphatase subunit (FLJ20644, GenBank no. AK000651) was shown to bind to a truncated version of GANP containing its middle portion (from 3844 to 5757 nt). This molecule named G5PR is a novel regulatory subunit of protein phosphatase 2A (PP2A)(PPP2R3C), as a B″ subunit interacting with the catalytic subunit (PP2Ac). PP2A catalyzes a wide variety of target molecules (Anderson, Maller, Tonks, & Sturgill, 1990; Gomez & Cohen, 1991; Millward, Zolnierowicz, & Hemmings, 1999; Tamura, Simizu, & Osada, 2004). In contrast to the various kinase molecules that have unique specificity, PP2Ac is regulated to exhibit a wide spectrum of specificity with several B subunit components, such as B, B′, and B″, as well as the other subunit α4 (Inui et al., 1998). G5PR is ubiquitously expressed and associated with either PP2Ac or PP5 in various tissues including lymphoid cells. G5PR deficiency produced by conditional gene targeting in B cells was shown to be associated with the enhanced activation of JNK kinase and regulated the JNK-induced cell death signaling associated with mitochondrial membrane potential (Huq Ronny, Igarashi, & Sakaguchi, 2006; Xing, Igarashi, Wang, & Sakaguchi, 2005), leading to enhanced phosphorylation of the apoptotic BH3 protein Bim (Lei & Davis, 2003) as one of the critical targets of G5PR. The function of G5PR is rather restricted to signaling pathways involved in B cell

activation in GCs and also for T cell selection in the thymus (Xing, Wang, Igarashi, Kawamoto, & Sakaguchi, 2008). The conditional targeting of G5PR in B cells was shown to enhance BCR-mediated activation-induced cell death and markedly decrease the formation of GCs upon immunization with TD-Ag, SRBC (Xing et al., 2005). G5PR deficiency, however, did not influence the BCR-mediated activation of ERKs and NF-κB, cyclin D2 induction, or Akt activation. Conversely, transgenic G5PR overexpression in B cells with the Igμ promoter and enhancer regions, which nearly doubled G5PR expression, did not cause marked abnormality in the GC B cell reaction and without enhancing the affinity maturation against the immunized TD-Ags (Kitabatake et al., 2015). G5PR overexpression in B lineage cells, however, was shown to be associated with an increase of B1 cells in the peritoneal cavity upon aging with the development of auto-antibodies (autoAbs) of anti-ss and anti-ds DNAs spontaneously, similarly to the case in female New Zealand Black (NZB) and (NZB × New Zealand White [NZW]) F1 mice without Ag immunization. The B1 cells appeared in the GCs and interacted with the FDCs. These results suggested that G5PR is involved in B cell selection at the GCs during immune responses. The dynamism of phosphorylation and dephosphorylation through PP2A covers many reactions, so it has remained unclear how the phosphorylation state of the AID/GANP complex is affected by G5PR in immune responses. Apart from G5PR, GANP phosphorylation was detected at Ser502, which is close to the RRM, potentially serving as a primer for DNA replication associated with the MCM complex (Kuwahara et al., 2001). Ser502 is predicted to be a CDK target, suggesting that the phosphorylation reaction would occur during cell-cycle progression in rapidly proliferating GC B cells.

6.4.2 Arginine Methyltransferase PRMT5

Another approach for identifying GANP-associated proteins found the interaction of the protein arginine methyltransferase (PRMT5) using a tandem affinity purification system with 293T cells transfected with *ganp* cDNA in a vector construct under the promoter pEF-BOS with a 3 × Flag-tag and a tobacco etch virus (TEV) protease/calmodulin-binding expression construct (Puig et al., 2001). In this system, GANP interacts with PRMT5, which is associated with a 50-kDa WD (Trp and Asp) repeat protein called methylosome protein 50 (MEP50) (Friesen et al., 2002). Arginine methylation is catalyzed by nine PRMTs, mostly targeting Gly- and Arg-rich (CAR) motifs in their substrates, but the substrates did not seem to be redundant. PRMT1 and PRMT5 are asymmetric and symmetric arginine

methyltransferases, whose functions are not compatible with each other (Yang & Bedford, 2013). The primary targets of PRMT1 are H4R3, MRE11, 53BP1, and SAM68, which function in transcriptional activation, signal transduction, RNA splicing, and DNA repair. PRMT5 targets H3R8, H4R3, E2F1, p53, EGFR, and CRAF, which function in transcriptional repression, signal transduction, and miRNA pathways (Mallappa et al., 2011). PRMT5 is also associated with other molecules, such as Pol II, FCP1 phosphatase, and Blimp-1, although the details of their roles remain unclear (Amente et al., 2005; Ancelin et al., 2006). *PRMT1* transcripts are ubiquitous, but *PRMT5* was shown to be inducible by stimulation with lipopolysaccharide plus IL-4 in B cells in vitro and in GC B cells in vivo (Igarashi et al., 2009). PRMT5 also interacts with the Jak/STAT signaling pathway. Overexpression of PRMT5 causes the upregulation of STAT6-mediated gene transcription in the activation of GC B cells. GANP downregulates the phosphorylation of STAT6 by suppressing PRMT5 function. This predicted model was confirmed in vivo via evidence showing that the loss of GANP in B cells caused spontaneous hyper IgE in the serum. STAT6 activation is observed in the CSR to IgG1 and IgE, but the promoters for *Igε* and *Igγ1* transcription might have different affinities to STAT6, as reported with a 10-fold difference in the electrophoresis mobility shift assay (Mao & Stavnezer, 2001). GANP/PRMT5 is functionally involved in the regulation of B cell activation in GCs, presumably minimizing the aberrant CSR to IgE production.



7. TRANSCRIPTION-COUPLED IgV REGION SHM

7.1 R-Loop

During Ig switch region transcription, an RNA strand adheres to the template strand, but the nontemplate DNA strand remains free, resulting in R-loop formation (Daniels & Lieber, 1995a, 1995b; Reaban & Griffin, 1990; Reaban, Lebowitz, & Griffin, 1994; Roy, Yu, & Lieber, 2008). In the Ig switch region, the R-loop is initiated at a region with clusters of Gs on the nontemplate strand (Roy & Lieber, 2009) and plays a role in the physiological DNA DSBs for Ig CSR. The assembly of the nascent RNA transcripts with mature mRNP biogenesis is maintained by the appropriate formation of the multiprotein THO complex, which was originally identified in the *S. cerevisiae* (Chavez et al., 2000; Rondon, Jimeno, & Aguilera, 2010), being minimally composed of five nonessential subunits, yHpr1, yTho2, yMft1, yThp2, and yTex1 (Pena et al., 2012), allowing the recruitment of mRNA export factors. In the absence of yTHO,

DNA:RNA hybrids accumulate and generate R-loop formation (Gomez-Gonzalez et al., 2011), implying that the roles of R-loop formation can be regulated by the mRNP biogenesis TREX machinery including the yTHO complex at the hotspots of mitotic recombination and genomic instability (Garcia-Rubio et al., 2008; Gonzalez-Aguilera et al., 2008; Gonzalez-Barrera, Garcia-Rubio, & Aguilera, 2002; Gonzalez-Barrera, Prado, Verhage, Brouwer, & Aguilera, 2002; Kaneko, Chu, Shatkin, & Manley, 2007; Li & Manley, 2006).

7.2 Noncoding RNAs and Divergent Transcription Model of IgV region Locus

Global run-on sequencing detects nascent transcripts generated at a large proportion of intergenic regions with a subset emanating from transcriptional enhancers (Core, Waterfall, & Lis, 2008; Wang et al., 2011). Enhancers are *cis*-elements that can evoke transcription irrespective of orientation, either downstream or upstream of the gene (Levine, Cattoglio, & Tjian, 2014); thus, the transcripts from both orientations as the sense and antisense RNAs can be divergent. The asymmetric and enriched allocation of transcription factors in the long genomic DNA element named the super enhancer (SE) causes hyperactivation by acetylation in the form of H3K27Ac and methylation in the form of H3Kme1 and confers specialized activity of such bidirectional transcription (Creyghton et al., 2010; Loven et al., 2013; Whyte et al., 2013). AID targets such SE by on-targeting and off-targeting simultaneously (Chiarle et al., 2011; Klein et al., 2011; Liu et al., 2008; Pasqualucci et al., 2001). The convergent transcripts that arise from the antisense strand are a likely cause of DNA DSBs (Meng et al., 2014). The off-target gene elements of such SE-associated converged transcripts have been implicated in B lymphomas, including *BCL6, Myc, MIR142, CD95, Pax5*, and *BCL6* as the genes associated with GC B cell differentiation (Muschen et al., 2000; Pasqualucci, Neri, Baldini, Dalla-Favera, & Migliazza, 2000; Robbiani et al., 2009; Shen & Stavnezer, 1998; Tsai & Ho, 2008). Moreover, genome-wide analysis of the AID-targeting region clarified the selectivity in the genome sequence in the rearranged IgV region and switch region genes during the differentiation of B cells (Yeap et al., 2015).

7.3 GANP and DNA Hyperrecombination

The lack of *ySac3* causes mild alteration in the yeast cell-cycle but alters DNA repair of transcription-coupled DNA damage, showing the DNA

hyperrecombination measured by the tandem-gene reporter assay (Bauer & Kolling, 1996). ySac3 is associated with yThp1 and involved in the mRNP complex for mRNA export from the nucleus (Fischer et al., 2002). The lack of ySac3 was shown to result in DNA hyperrecombination (Gallardo et al., 2003). Similarly, GANP also resulted in DNA hyperrecombination in the reporter assay of a tandem mutant LacZ′/LacZ″ (β-galactosidase) construct in mouse embryonic fibroblast cells that were *ganp*-heterodeficient (Yoshida et al., 2007). In contrast, overexpression of GANP markedly (>10-fold) suppressed the DNA hyperrecombination that was induced by introduction of the yeast endonuclease I-*Sce*I to cleave the construct between the LacZ′ and LacZ″ regions, producing artificial DNA DSBs; however, the effect did not significantly alter the RAG-mediated Ig gene rearrangement with recombination signal sequence sites. The details of the molecular mechanism behind the inhibition of DNA hyperrecombination remain unclear. One possible explanation is a deficiency of RNA transportation because the effect of mRNA export is restricted to the genes that are actively transcribed. The suppression of DNA hyperrecombination is marked with the *Dpn*I-treated reporter DNA. The massive accumulation of nascent RNA transcripts before RNA splicing and assembly into the mRNP complex that has complete sequence complementarity to the template strand DNA causes collision stress in the supercoil winding motion, consequently generating DNA DSBs in a transcription-coupled manner.

GC B cells displayed IgV region DSBs after immunization with TD-Ags (Bross et al., 2000; Papavasiliou & Schatz, 2000; Wu et al., 2003). The association of the IgV region DSBs and the rate of AID-mediated SHM have been issues of debate, while AID might affect IgV region SHM through events before or after DSB (Bross, Muramatsu, Kinoshita, Honjo, & Jacobs, 2002; Papavasiliou & Schatz, 2002). We explored the effect of GANP on the frequency of IgV region DSBs by classical ligation-mediated PCR amplification (Kawatani et al., 2005). The frequency of $IgV_H186.2$ region DSBs was increased in GC B cells of *Ganp*^{Tg} mice upon immunization with NP-CGG. GANP was shown to play a role in suppressing DNA hyperrecombination in the LacZ′–LacZ″ tandem repeat DNA recombination assay (Yoshida et al., 2007), similarly to ySac3 and other yTREX-2 components in the yeast system. Further studies showed the role of GANP in regulation of the DNA repair pathway after treatment with the DNA-damage-inducing agents etoposide (a topoisomerase II inhibitor) and camptothecin (a topoisomerase I inhibitor). GANP suppressed the nonhomologous end-joining DNA repair pathway after the production of etoposide-induced DNA DSBs, which are

mainly repaired by Artemis-independent nonhomologous end joining (Katsube et al., 2011). In contrast, GANP augmented the repair of DNA damage induced by camptothecin, which is exclusively repaired by homologous recombination (Eid et al., 2014). GANP physically associates with DNA-PKcs, and etoposide- or AID-induced DNA damage rapidly causes the release of DNA-PKcs from the GANP complex independently of ATM or DNA-PKcs kinase activity. Thus, GANP might provide a state of less DNA-PKcs involvement at genomic regions with DNA DSBs, which presumably facilitates the choice of DNA homologous recombination pathway. GANP chooses the homologous recombination pathway that repairs the genome at high fidelity without rigorous DNA breaks, rearrangements, or chromosome translocations, promoting the maintenance of genomic integrity at the sites of DNA DSBs. In fact, in the chicken DT40 B cell clone lacking the pseudo-Vλ-light chain genes, which can be used to clearly demonstrate the frequency of AID-induced IgV region SHM in vitro, GANP overexpression markedly augmented the SHM frequency in the Vλ light chain gene and the heterodeficiency of GANP significantly reduced the SHM frequency.

7.4 Summary of Current Knowledge on GANP for B Cells

In mammalian cells, GANP exerts multiple functions by interacting with many different nuclear proteins, most likely for RNA biogenesis, from the release of chromatin for initial transcription at the locus interacted with SAGA complex and the assembly of mRNP with various factors for the efficient export of mRNA through the NPC to its presentation to the translation apparatus. At present, GANP is considered to be a component of mammalian TREX-2 interacting to the SAGA complex and the mRNPs (Fig. 4). The structure of the GANP protein seems to have undergone substantial evolutionary development by attaching to the N-terminal FG-rich region, RRM, and the HAT region interacting with the MCM complex. This evolutionary development of the molecular structure presumably occurred because of the need to adapt to (1) the diversification of RNA molecules such as coding and noncoding transcripts in mammalian cells from the simplest eukaryotic cells of *S. cerevisiae*, (2) the selective transport of transcripts, (3) the size difference of NPC for the import and export of substances, (4) retroviral threats, and (5) the development of the AID/APOBEC cytidine deaminase family, particularly in humans, as well as (6) to protect the genome from various genotoxic stresses. The GANP–AID/APOBEC interaction at the NPC might be particularly important for immune cells that are highly specialized at dealing with stresses to maintain host homeostasis.

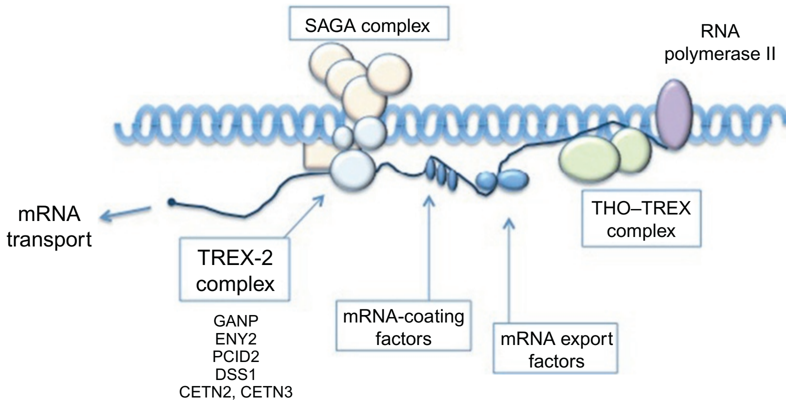
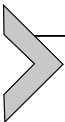


Fig. 4 A model of transcription and mRNA export in mammals. After the initial interaction of the SAGA complex, RNA Pol II is recruited to the transcription-competent chromatin, and the nascent transcript is assembled with THO–TREX complex, mRNA export factors, mRNA-coating factors including splicing factors and RNA helicases, thus creating the mRNPs. TREX-2 interacts with the SAGA complex through the component for the mRNA transport toward the NPC.



8. TREX-2 AND DISEASES

TREX-2 is a complex that is assumed to be involved in RNA metabolism from initial transcription until the cytoplasmic translation process. Adequate processing and export are now known to be critical for cellular metabolism and genomic integrity, and thus for the long life of human beings. Numerous genetic changes associated with various human diseases have now been reported, which should eventually lead to a novel paradigm regarding the etiology of human disorders associated with RNA metabolism, including the alterations of microRNAs and noncoding RNAs.

8.1 HIV-1 Infection and GANP

APOBEC3 (A, B, C, D, E, F, G, H) proteins primarily target retroviral cDNA in the cytoplasm to cause hypermutation in viral genomes. APOBEC3G (A3G) exhibits antiretroviral activity that potentially restricts HIV-1 replication in T cells. A3G is mostly degraded by the viral infectivity factor (Vif) that inhibits A3G translocation and promotes A3G degradation through core-binding factor β-mediated proteasomal degradation (Jager et al., 2012; Sheehy, Gaddis, Choi, & Malim, 2002; Zhang, Du, Evans, Yu, & Yu, 2012). However, Vif cannot completely degrade A3G and a few copies of A3G molecules that escape it are encapsidated into virions

(Browne, Allers, & Landau, 2009), presumably through the interaction of various host components and cellular factors (Didierlaurent et al., 2011; Huthoff, Autore, Gallois-Montbrun, Fraternali, & Malim, 2009; Wang, Tian, Zhang, Sarkis, & Yu, 2008). Since GANP is likely to associate with most of the APOBEC family cytidine deaminases, its role in HIV-1 infection has been studied (Maeda et al., 2013). GANP is also upregulated coordinately with A3G in human $CD4^+$ T cells that are activated with anti-CD3, anti-CD28, and IL-2 in vitro. AID and GANP directly interact with viral components through different regions: A3G to the nuclear capsid, but GANP to a more extended region covering the matrix and capsid proteins. The increased expression of GANP recruits A3G efficiently into the virion core region, facilitating the interaction of A3G and GANP with viral genomic RNAs (Maeda et al., 2013). GANP enhances A3G encapsidation into the virion core, where only two copies exist together with 7SL RNA, which instead decreases the incorporation of other cellular RNAs such as U1, U2, U4, and U5 that are snRNAs with a cap structure. As shown by an in vitro assay, GANP enhanced the rate of A3G-mediated $G \rightarrow A$ mutation by 80% and caused an increased proportion of $G \rightarrow A$ mutations, which eventually decreased the retroviral infectivity of the HIV-1 virus in a secondary infection assay. These results demonstrate that the cellular component GANP plays a role in host defense as a general accessory molecule of AID/APOBEC proteins.

8.2 Autoimmunity

GANP expression was examined in the B cells of various autoimmunity-prone mice with aging. Immunohistochemical analysis of the spleen follicular region showed high levels of GANP-positive cells in the red pulp (RP) region (parafollicular area) in aged autoimmunity-prone mice MRL/lpr, NZB and NZW F1, and BXSB mice (Fujimura et al., 2005). Other mice, BXSB and NOD, also showed $GANP^{hi}$ cells in the RP region, although the number of positive cells was lower. C57BL/6 and Balb/c mice did not display such an increase under nonimmunized conditions. The increase of $GANP^{hi}$ cells in the RP region appeared upon aging, and their frequency was correlated with the increase of serum autoantibody (autoAb) titers. $GANP^{hi}$ cells have the following expression profile: $IgM^+B220^{lo}Syndecan\text{-}1^+$, but $CR1^-$, $Thy1^-$, $GL7^-$, $CD23^-$, and PNA^-. These results might indicate that plasmablasts or plasma cells express GANP at a higher level in animals that are autoimmune. The autoAb evolution via SHM occurred outside GCs at the T-zone–RP border (William, Euler, Christensen, & Shlomchik, 2002).

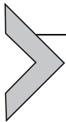
The aberrantly high GANP expression in late B cell differentiation nearly at the plasma cell stage might be pathologically associated with autoAb production. GANP might assist the diversification of the IgV region repertoire toward autoAb production in the extrafollicular RP region, where strict selection to reduce the number of autoreactive B cell clones may not be undertaken. Thus, it is important to investigate the molecular mechanism by which GANP is upregulated in B lineage cells outside of GCs and to characterize whether the GANP^{hi} cells continuously undergo IgV region SHM.

8.3 Cancer

The aberrant expression of AID has been observed not only in B lymphoma cells (Jankovic et al., 2013; Robbiani et al., 2009, 2015; Robbiani & Nussenzweig, 2013), but also in several human malignancies of hepatoma, colon carcinoma, cholangiocarcinoma, and breast cancer. Moreover, APOBEC3B and APOBEC3A are critically associated with breast cancer development (Burns et al., 2013; Nik-Zainal et al., 2014; Periyasamy et al., 2015; Taylor et al., 2013; Xuan et al., 2013). Despite the accumulated evidence on the oncogenic role of AID/APOBEC family cytidine deaminases, little is known about the function of the TREX-2 complex in cancer development, although its association with this disease has been reported (Chan-On et al., 2009; Fujimura et al., 2005; Kageshita et al., 2006; Ohta et al., 2009; Phimsen et al., 2012; Rezano et al., 2013). The lack of ySac3 is known to cause DNA hyperrecombination, but the molecular mechanism by which this is generated has yet to be elucidated. The collision theory with the triple strands involving of dsDNA and the complementary mRNA may partly explain the rigorous DNA changes, such as DNA hyperrecombination and chromosomal translocation. Recent advances in high-throughput sequencing technology have allowed the global study of gene alteration. The concept of a transcription bubble transiently forming an RNA/DNA hybrid stretch as an R-loop during active transcription has been proposed. The R-loop is the primary site for the targeting of AID cytidine deaminase that interacts with mRNA as well as DNA strands. The TREX-2 component interacting with nascent mRNA would be the component most intimately interacting with the AID-targeting loci.

Abnormal GANP expression has been observed in various highly malignant tumors. For example, brain tumor patients (101 cases of malignant glioblastoma) expressing low GANP were found to be associated with a worse prognosis. GANP^{lo} cells are highly metastatic and are linked to loss of heterozygosity on chromosome 10, epidermal growth factor receptor

gene amplification, and significantly poor prognosis compared with GANP^{hi} expression (Ohta et al., 2009). In hematopoietic tumors including myelodysplastic syndrome, acute lymphocytic leukemia, chronic lymphocytic leukemia, and Hodgkin's disease, high GANP expression was exhibited in the bone marrow samples of clinical cases (Fujimura et al., 2005). DSS1, another mammalian TREX-2 component, is known to interact with BRCA2 (Marston et al., 1999; Yang et al., 2002) and can inhibit polyubiquitination by blocking access of ubiquitin ligase to BRCA2 in yeast (Funakoshi, Li, Velichutina, Hochstrasser, & Kobayashi, 2004). In a cohort analysis of breast cancer patients, DSS1^{hi} expression was associated with a worse prognosis because of the high rate of malignancy linked to the multidrug resistance (Rezano et al., 2013), which could be explained by the resistance of tumor cells to anticancer drugs that damage DNA. These results suggest that the expression of TREX-2 is involved in the maintenance of genomic stability. The lack or impairment of TREX-2 component might be a cause of cancer in an oncogenic state.



9. PERSPECTIVES

This chapter has described the interaction of the mRNA export complex, TREX-2, in the metabolism of various cells. Advances in next-generation sequencing strategies have provided further insights regarding the global regulation of gene transcripts in mammalian cells. The diverse expression of many different transcripts needs to be processed by the cargo complex and/or the various molecules for appropriate translation and decay. The dynamic metabolism of mRNA molecules might be orchestrated so that the host genome does not become harmful or is properly repaired over the lifespan. Studying GANP as a key platform for the TREX-2 mRNA export should produce a new paradigm on regulation of the RNA metabolism of immune cells and cells at early developmental stages.

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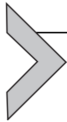

CHAPTER FIVE

Advances in PET Detection of the Antitumor T Cell Response

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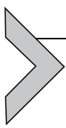
Abstract

Positron emission tomography (PET) is a powerful noninvasive imaging technique able to measure distinct biological processes in vivo by administration of a radiolabeled probe. Whole-body measurements track the probe accumulation providing a means to measure biological changes such as metabolism, cell location, or tumor burden. PET can also be applied to both preclinical and clinical studies providing three-dimensional information. For immunotherapies (in particular understanding T cell responses), PET can be utilized for spatial and longitudinal tracking of T lymphocytes. Although PET has been utilized clinically for over 30 years, the recent development of additional PET radiotracers have dramatically expanded the use of PET to detect endogenous or adoptively transferred T cells in vivo. Novel probes have identified changes in T cell quantity, location, and function. This has enabled investigators to track T cells outside of the circulation and in hematopoietic organs such as spleen, lymph nodes, and bone marrow, or within tumors. In this review, we cover advances in PET detection of the antitumor T cell response and areas of focus for future studies.



1. INTRODUCTION

1.1 T Cells as a Cancer Therapy

For decades apparent cases of sporadic tumor regression and elimination have been documented in mice and humans. These studies formed the foundation of the concept that tumors could be immunogenic and recognized by the immune system (Rosenberg, 2001). In tumors such as melanoma and sarcoma, this has been attributed to antitumor T cells (Rosenberg & Restifo, 2015). Tumor clearance occurs when the immune system recognizes the tumor via T cells reactive to immunogenic epitopes. A rapid expansion and infiltration of T cells into tumors occur, resulting in tumor reduction and/or removal. These rare but encouraging results have led investigators to develop additional immunotherapies that aid in tumor

cell recognition by T cells (Rosenberg, 2014; Sharma & Allison, 2015b). Immunotherapies can be broadly grouped into three major categories: (1) antibodies and cytokines, (2) vaccines, and (3) cell-based therapies. Each therapy aims to activate, prime, expand, or increase tumor recognition by T cells through different mechanisms.

Early work focused on high-dose cytokine therapy (interleukin-2, IL-2) to expand all T cells in vivo (Rosenberg, 2014). In clinical applications as a monotherapy, high-dose IL-2 had response rates of approximately 23% in renal cell carcinoma (Rosenberg, 2014). The successful but limited efficacy led to the development of alternative and complementary therapies (Sharma & Allison, 2015a). The next generation of immunotherapies in development are more T cell-specific antibodies that block checkpoint inhibition (current: anti-CTLA4, anti-PD1, anti-PD-L1; in trials: anti-TIM3, anti-LAG3) or act as agonists (anti-41BB, anti-OX40) (Hamid et al., 2013; Ribas, 2012; Sharma & Allison, 2015b). The newly approved anti-PD1 or anti-PD-L1 antibodies directly target T cell inhibition in vivo and have demonstrated clinical response rates of up to 52% as a monotherapy in patients receiving the highest dose (Hamid et al., 2013). In parallel with this influx of new antitumor immunotherapies, there is a pressing need for methods that can monitor systemic changes in endogenous T cells (see Sections 3 and 5).

In the case of cell-based immunotherapies including vaccines or adoptive cell therapy (ACT) with tumor-infiltrating lymphocytes (TILs) or engineered T cells (T cell receptor—TCR, or chimeric antigen receptor—CAR), robust methods are needed to monitor these cells specifically posttransplant. Although cell-based therapies are highly efficacious, they can have unforeseen mortality due to on-target/off-tumor effects (Bendle et al., 2010). In one instance, a patient receiving an anti-HER2 CAR therapy died due to low Her2 expression within the lungs (Morgan et al., 2010). Methods described in Sections 2 and 4 address ways that positron-emission tomography (PET) can monitor adoptively transferred cells.

With the increased development and utilization of immunotherapies for treating cancer, it is critical to be able to identify the antitumor T cell response and off-target effects. Advances in imaging will provide a complementary tool for clinicians and researchers to understand how newly developed therapies work systemically.

1.2 Current Methods Used to Track Antitumor T Cell Response

Conventional methods used to monitor the immune system can be limited and biased. T cell responses are monitored most often through peripheral

blood analysis and biopsy when appropriate. Blood measurements are the easiest and most robust method, providing information on cytokines, cell subsets, total cell quantity, and an easy method to track T cells in the periphery. However, blood sampling is limited by an inability to assess the T cell composition in alternative organs and tissues. Occasionally, a biopsy can be collected to allow for intratumoral (or alternative organ) examination. The advantage of biopsied tissue includes high spatial resolution (in 2D) to determine T cell location within the tumor. The drawback to biopsies includes the invasive procedure, inherent sampling error from heterogeneous tumor immune microenvironment, and being limited to a single static time point. Furthermore, following fixation and further processing, functional information can be lost. Together these methods provide information on the state of the immune system at one time point and are limited in evaluating the immune system across the whole body. This poses a clinical challenge for current cancer immunotherapies. Success of therapies frequently depends on the expansion and infiltration of antitumor cells, but there are currently limited methods to track this process. In some instances, an additional limitation is the inability to detect the on-target/off-tumor cellular cytotoxicity of the infused therapeutic cell product prior to complications, or to determine the quantity of successful tumor-infiltrating cells without biopsy (Park, Rosenberg, & Morgan, 2011). Therefore, a noninvasive, whole-body imaging technique to monitor immune cell function can complement and improve the current sampling methods (Hildebrandt & Gambhir, 2004; Kircher, Gambhir, & Grimm, 2011; Wolchok et al., 2009).

Imaging technologies providing anatomical information such as X-ray, computed tomography (CT), and magnetic resonance imaging (MRI) are used routinely as diagnostics but have had limited applications in tracking T cells specifically. The assessment of immunotherapeutic response using anatomical imaging and response evaluation criteria in solid tumors relies on the reduction of tumor volume, although there are known flaws in these methods (Wolchok et al., 2009). To date, most clinical imaging of immune responses has been based on either PET or single-photon emission computed tomography (SPECT) (Hildebrandt & Gambhir, 2004; Kircher et al., 2011). Most preclinical studies have utilized alternative imaging strategies that are restricted to small animals such as two-photon microscopy, fluorescent, and bioluminescent imaging or have adapted clinical modalities (SPECT, PET, CT, and MRI) as methods for measuring changes in the immune system (Cherry & Gambhir, 2001; Hildebrandt & Gambhir, 2004). Each technology provides unique information with inherent

advantages and disadvantages, but this review will focus on PET imaging of the antitumor T cell response. One advantage of PET is that technologies developed preclinically can be applied directly to clinical problems, allowing for imaging agents to transition quickly from bench-top to bedside (Phelps, 2000).

1.3 Utility of PET Imaging for Tracking and Analyzing T Cell Populations

T cell development and maintenance are complicated with cells residing in almost all organs (reviewed in Farber, Yudanin, & Restifo, 2014). Simplified, T cells are differentiated, activated, and exert effector function in different tissues with the possibility of homing or residing in additional locations. T cell precursors (pro-T cells) arise in the bone marrow, and then home to the thymus to undergo thymic selection prior to cells entering the periphery. Naïve T cells then need to be activated by TCR engagement with peptide-loaded major histocompatibility (MHC) and costimulation from antigen presenting cells. This activation typically occurs within secondary lymphoid tissues such as lymph nodes. Proinflammatory cytokines then signal for T cells to traffic to the tumor for effector functions. After the resolution of the malignancy (or infection), a small fraction of the effector cells will become memory T cells capable of expansion upon future antigen exposure (Belardelli & Ferrantini, 2002; Farber et al., 2014). Due to this complex development process involving orchestrated trafficking and localization of T cells, whole-body noninvasive imaging can address difficult biological questions that cannot be answered using traditional blood or biopsy samples. PET imaging provides a partial solution to this problem by providing the ability to track populations of cells in vivo by adjusting the probe of interest (Phelps, 2000). Subclasses of PET include immuno-PET, prelabeling with radionuclides, reporter gene strategies, and small molecule probes. Each technology is described in detail with current applications below. By adapting one or more PET probes, serial scans can provide longitudinal information or be applied to investigate alternative processes, all with quantitative noninvasive whole-body detection.

1.4 PET Principles

PET scanners detect the location of positron-emitting probes in vivo and produce a three-dimensional (3D) image (reviewed in Phelps, 2000). As the probe decays, positrons from the radionuclide annihilate with an

electron in nearby tissue and emit two antiparallel 511 keV high-energy photons. PET technology employs detectors that identify coincident emissions and use the information derived from the lines of response to reconstruct a quantitative 3D activity map. Preclinical microPET scanners typically have spatial resolutions of 1–2 mm, whereas clinical scanners have a resolution of approximately 1 cm, which is sufficient to detect lymph nodes in both instances (Cherry & Gambhir, 2001; Gambhir, 2002). For immunology or T cell studies evaluating alterations in lymph nodes, a coregistered PET/CT scan can be most accurate for determining size, location, and probe accumulation. Dense tissue, such as bone, does not scatter the high-energy photons in small animal imaging; however, attenuation correction is required for accurate quantification of PET images in the clinical setting. Other physiological differences between mice and men may change how probes are distributed in vivo. As discussed below, different metabolic rates, physiological differences in the half-lives of antibodies, or changes in metabolite serum concentrations can all effect how probes are distributed and accumulated in vivo.

2. EX VIVO CELL LABELING FOR TRACKING IMMUNE CELLS IN VIVO

Ex vivo cell labeling is a process in which cells of interest are harvested, incubated with the radiotracer of choice for intracellular uptake and retention, and then infused back into the patient for subsequent noninvasive detection (Fig. 1). Generally, the radiotracers are taken up by cells via passive

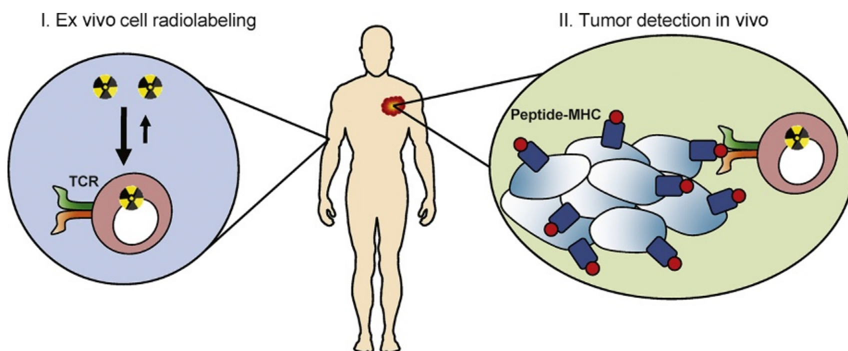


Fig. 1 Ex vivo radiolabeling of cells. In this example, cytotoxic lymphocytes transduced with a TCR specific for a tumor antigen are radiolabeled and are reintroduced for cell tracking to antigen-positive tumors. This provides information regarding both successful tumor targeting and the presence of the tumor antigen.

diffusion due to the use of lipophilic chelators and are retained by binding to intracellular proteins. There are many aspects of ex vivo cell labeling that need to be optimized/determined to establish a reproducible radiolabeling procedure that does not cause toxicity for the cells of interest prior to imaging experiments, such as incubation time, radioactivity concentration per cell, retention of radioactivity over time, viability, radiotoxicity, DNA damage, and altered phenotype or activation state. The significant drawbacks of this approach are the restricted longitudinal imaging due to radiotracer half-life, radiotracer dilution due to cell proliferation or cell death, and the small amount of radioactivity that can accumulate in each cell. In the context of radiolabeled T cells, strict attention needs to be made on cellular activation and cell death due to established radiosensitivity of T lymphocytes. Although this is a relatively routine procedure in nuclear medicine, it is nonetheless a labor-intensive process.

Since the 1970s, clinical nuclear medicine departments have routinely used planar scintigraphy in combination with ex vivo radiolabeling of leukocytes with gamma-emitting radionuclides, using ^{111}In-oxine and ^{99m}Tc-hexamethylpropyleneamine oxime (^{99m}Tc-HMPAO), to detect sites of inflammation or infection (Hughes, 2003; Peters, 1994). The advent of SPECT/CT has enhanced the diagnostic capability of gamma radionuclide-labeled leukocytes in the clinic (Djekidel, Brown, & Piert, 2011). These methods are still used clinically because the detection of locations of infection can be critical for the clinical management of patients. However, certain clinical applications requiring the detection of small numbers of cells in small volumes have created the need for PET cell-labeling methods due to enhanced sensitivity, quantification, and resolution of clinical PET scanners (Rahmim & Zaidi, 2008). Human leukocyte labeling with [^{18}F]-2-deoxy-2-fluoro-D-glucose ([^{18}F]-FDG) suffers from rapid efflux, low radiolabeling efficiency of cells with low glucose metabolism, and the relatively short physical half-life of ^{18}F (110 m) (Bhargava, Gupta, Nichols, & Palestro, 2009; Forstrom, Mullan, Hung, Lowe, & Thorson, 2000). In one study, investigators found that activated human T cells radiolabeled with [^{18}F]-FDG suffered from radiotracer efflux, decreased proliferation, and reduced cytotoxic activity (Botti et al., 1997). Recently, the use of [^{18}F]-4-fluorobenzamido-*N*-ethylamino-maleimide ([^{18}F]-FBEM) for conjugate ^{18}F to cell surface thiols did not reduce T lymphocyte viability and demonstrated homing of T lymphocytes to the spleen at 90 min postinjection (Lacroix et al., 2013). The longer-lived radionuclide ^{64}Cu (12.7 h) allows tracking of cells over a longer period of

time. In animals, ^{64}Cu-pyruvaldehyde-*bis*(N^4-methylthiosemicarbazone) (^{64}Cu-PTSM)-labeled leukocytes were shown to migrate to the spleens of mice as detected by PET (Fig. 2) (Adonai et al., 2002). However, the ^{64}Cu cell-labeling tracer suffered from rapid efflux resulting in nonspecific liver uptake (Adonai et al., 2002). Recently, ^{89}Zr (3.2 days) was used to radiolabel various cell types, including human leukocytes, with higher intracellular retention than the typical ^{111}In radiolabeling approach allowing for cell tracking at 7 days postinjection (Charoenphun et al., 2015).

The noninvasive imaging of T cell trafficking in vivo in tumor models could provide information pertaining to the ability of targeted cells to migrate to regions of interest, such as antigen expressing tumors in

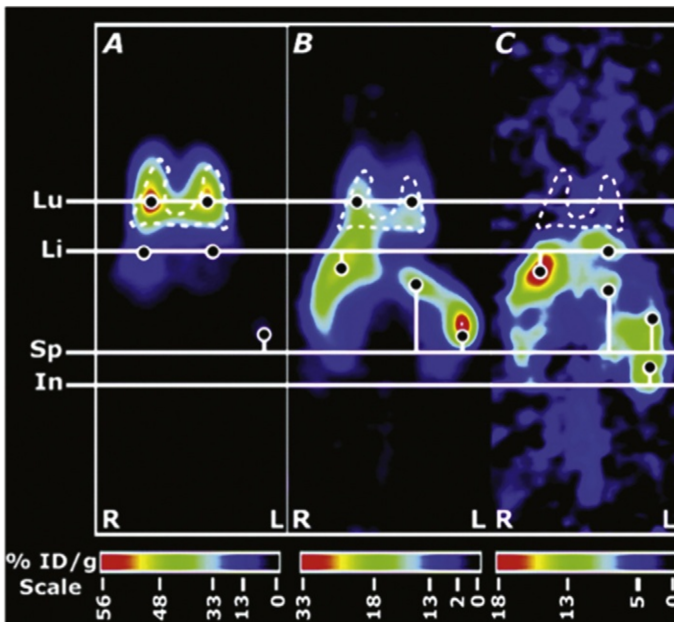


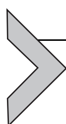
Fig. 2 ^{64}Cu-PTSM-labeled lymphocytes. Mice were injected intravenously with lymphocytes radiolabeled ex vivo with ^{64}Cu-PTSM and images were acquired at 0.12 h (A), 3.12 h (B), and 18.9 h (C) postinjection. PET images show that the adoptively transferred lymphocytes initially traffic through the lungs and then accumulate in the spleen and liver. *Lu*, lungs; *Li*, liver; *Sp*, spleen; *In*, intestine. *Adapted from Adonai, N., Nguyen, K. N., Walsh, J., Iyer, M., Toyokuni, T., Phelps, M. E., et al. (2002). Ex vivo cell labeling with 64Cu-pyruvaldehyde-bis(N4-methylthiosemicarbazone) for imaging cell trafficking in mice with positron-emission tomography.* Proceedings of the National Academy of Sciences of the United States of America, *99, 3030, and reprinted with permission from* PNAS. *Copyright (2002) National Academy of Sciences, USA.*

the context of TCR- or CAR-engineered T cells. Initial preclinical studies tracking cytotoxic lymphocytes to tumors were performed with ^{111}In-SPECT. Pittet et al. used ^{111}In-oxine-radiolabeled tumor-specific murine cytotoxic lymphocytes to track T cell migration, demonstrating that lymphodepletion before adoptive cell transfer enhanced both intratumoral migration of T cells and antitumor efficacy (Pittet et al., 2007). More recently, ^{111}In-tropoline-labeled human CAR T cells were injected by alternate routes into tumor-free or tumor-bearing immune-incompetent mice to monitor CAR T cell trafficking (Parente-Pereira et al., 2011).

Only recently has PET been used to track $CD8^+$ T cells to tumors. In one example, activated ovalbumin (OVA)-specific $CD8^+$ T cells (OT-I) were efficiently radiolabeled with ^{89}Zr-oxine, demonstrating efficient cellular retention of ^{89}Zr. Importantly, no effects on OT-I proliferation and activation as monitored by expression of CD69, CD25, and CD44 as well as production of IFN-γ and IL-2 were observed (Sato et al., 2015). The ^{89}Zr-labeled CD8 T cells injected into wild-type mice showed migration to the spleen and lymph nodes up to 7 days postinjection. Subsequently, ^{89}Zr-OT-I cells were injected into *RAG1* knockout mice bearing B16-OVA melanoma tumors demonstrating low tumor targeting via PET, but no antigen-negative tumors were shown as control for background and nonspecific targeting (Sato et al., 2015).

The use of PET to address T cell migration patterns was performed using ^{64}Cu-radiolabeled T_h1 $CD4^+$ T cells expressing the MHC-II-restricted TCR specific for OVA (OT-II) in the model of chicken OVA-induced airway hypersensitivity inflammation. Initially, ^{64}Cu-PTSM radiolabeled OT-II were used to monitor the different migration patterns as a result of intravenous or intraperitoneal administration (Griessinger et al., 2014). They demonstrated cell tracking for 48 h postinjection based on optimized radiolabeling conditions that reduced, but did not abolish, the harmful effects due to ^{64}Cu-radiolabeling, such as viability, apoptosis, proliferation, IFN-γ production, and DNA double-strand breaks. A second approach by the same group used a ^{64}Cu-radiolabeled antibody specific for the TCR expressed on OT-II cells to utilize TCR plasma membrane turnover for intracellular radiolabeling (Griessinger et al., 2015). This method resulted in reduced radiation-induced cellular damage, decreased radiotracer efflux compared to ^{64}Cu-PTSM, and reduced background in PET studies. This method can potentially be applied to radiolabeling $CD8^+$ cytotoxic T cells specific for tumor antigens to reduce radiation-induced cell damage.

In the future, ex vivo cell labeling of T cells with PET radiotracers could provide information on successful delivery and tumor targeting of TCR- and CAR-engineered T cells for antitumor immunotherapy. Tracking these engineered T cells to antigen-positive tumors could determine successful tumor-specific delivery of therapeutic T cells and potentially determine antigen expressing vs antigen-negative tumors in metastatic patients. However, ex vivo radiolabeling will not provide information about the presence of adoptively transferred cells at weeks posttransfer due to the limitation of radionuclide half-life. Consistent T cell handling, incubation times, radiolabeling efficiency, viability, and phenotype of T cells must be established for routine clinical use. Furthermore, it is unknown whether the limits of radioactivity per cytotoxic T cell will be sufficient for PET detection of low T cell numbers migrating to tumors in patients.



3. SMALL MOLECULE METABOLITE PROBES FOR TRACKING T CELLS

3.1 Introduction of Radiolabeled Metabolites

Small molecules and metabolites can be modified or directly radiolabeled to mimic their native compounds and used to track metabolic processes in vivo. This category of PET probes is the most widely used accounting for almost all clinical scans. Examples of probes include analogs of sugars, nucleosides, amino acids, small molecule drugs, hormones, or neurotransmitters (Serdons, Verbruggen, & Bormans, 2009). Once injected, the probe distribution and accumulation demonstrate where an increased consumption of the metabolite is in comparison to other tissues within the body.

When imaging with small molecule probes the time from injection to scanning is usually short in comparison to other PET probes (ranging from minutes to only a few hours after infusion) since most of these tracers exhibit rapid blood clearance. The fast metabolism of most small molecule probes also favors the use of short and intermediate half-life radioisotopes. In most cases, ^{18}F is utilized due to its intermediate half-life (110 min) providing enough time for radiochemical synthesis, transport, and imaging (Serdons et al., 2009). ^{18}F chemistry frequently replaces a hydrogen or hydroxyl group with the ^{18}F isotope to allow for a metabolite mimetic. In some instances, a Cl or F atom is initially present and can be directly replaced by the radioisotope. In cases where ^{18}F chemistry cannot be applied other radioisotopes such as ^{11}C or in rare cases ^{15}O and ^{13}N can be used (Cherry & Gambhir, 2001; Serdons et al., 2009). ^{11}C has a 20 min half-life requiring high starting

^{11}C activity in order to complete synthesis and purification with enough activity remaining for imaging due to the rapid decay.

Small molecule probes are also radiolabeled at a high specific activity to allow for the total probe administered to be only pico- to femtomole per gram of tissue (Cherry & Gambhir, 2001; Phelps, 2000). Due to the extremely low serum concentration, most probes function with no biological or pharmacological effect in vivo (Cherry & Gambhir, 2001; Massoud & Gambhir, 2003). The remaining probe not accumulated by the target tissue is then excreted in most cases through renal filtration or hepatic clearance. The total radiation exposure from metabolic probes is usually highest in the kidneys and bladder due to the rapid excretion. Total exposure in these organs is typically used for determining the radiation limit (dose-limiting organ) and is not dependent on concentrations of the substrate (Gambhir, 2002). To date, thousands of unique PET probes have been made (The Radiosynthesis Database of PET Probes [RaDaP]). We will cover key studies that have applied metabolic probes to address T cell imaging using PET.

To measure T cell activity and track lymphocyte function, metabolites that are specifically retained by T cells but not by other cell types are being developed. Each probe measures a specific metabolic process, but due to metabolism being transient and dependent on cell location and environment, not all probes will work for all stages of T cell development or activation (Fox, Hammerman, & Thompson, 2005; Radu et al., 2008). The major limitations in metabolic and small molecule probes are the risk of accumulation in non-T cells, difficulty in synthesizing certain probes, and the fact that not all T cells will have the same metabolic demands (depending on state, location, etc.). However, identifying probes that monitor relevant changes in T cell metabolism in response to infection, cancer, or autoimmunity can be the basis of novel methods to assess appropriate immune responses.

3.2 Measuring Enhanced Glucose Accumulation by [^{18}F]-FDG

The most widely used PET tracer is a fluorinated glucose analog [^{18}F]-FDG. [^{18}F]-FDG measures the glycolytic flux by being transported into cells and phosphorylation by hexokinase into [^{18}F]-FDG-6-PO_4. The phosphorylation traps the radiolabeled probe intracellularly and the position of the ^{18}F prevents further metabolism. Under normal physiological conditions, high [^{18}F]-FDG accumulation is seen in metabolically active organs such as the brain and heart (Phelps, 2000). In preclinical models of arthritis and

multiple sclerosis, [^{18}F]-FDG has been used to monitor the severity of immune lesions which have been attributed to T cells (Irmler et al., 2010; Matsui et al., 2009; Radu, Shu, Shelly, Phelps, & Witte, 2007). In clinical models, [^{18}F]-FDG accumulation has been attributed to multiple benign conditions including inflammation (Bakheet & Powe, 1998; Beckers et al., 2004). Although enhanced [^{18}F]-FDG accumulation is observed in T cell responses, it has also been demonstrated that on a per cell basis, innate immune cells will have higher glucose consumption (Nair-Gill et al., 2010). In addition, most tumors are highly glycolytic and [^{18}F]-FDG is used as a primary clinical measure for tumor detection and staging, making it impossible to distinguish tumor signal from T cell signal (Gambhir, 2002) (Fig. 3). For this reason, clinicians and investigators have looked for alternative PET probes for tracking the antitumor T cell response (Escuin-Ordinas et al., 2013).

3.3 Measuring Cell Proliferation by 3′-Deoxy-3′-[^{18}F] Fluorothymidine Accumulation

3′-Deoxy-3′-[^{18}F]fluorothymidine ([^{18}F]-FLT) is radiolabeled thymidine that is a substrate for thymidine kinase 1 (TK1). TK1 has maximal activity in dividing cells and peak expression is observed during S phase. Therefore, the accumulation of [^{18}F]-FLT is used as a marker for cell proliferation in vivo (Barthel et al., 2003; Wagner et al., 2003). In healthy dogs, signal was observed in the marrow indicating a site of high cellular proliferation (Wagner et al., 2003). Preclinical scans of canines with lymphoma then demonstrated a high signal accumulation within malignant lymph nodes

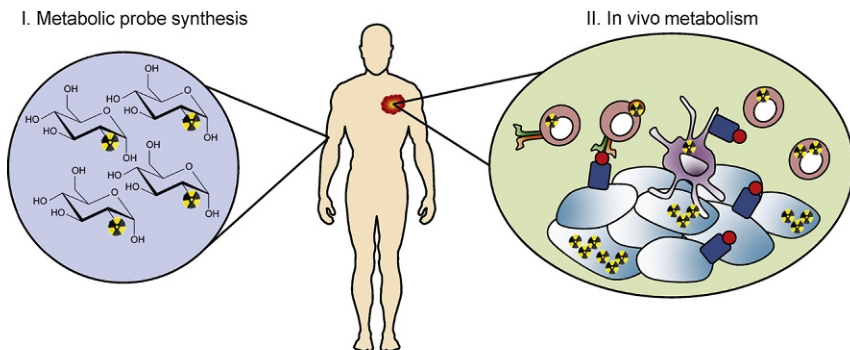


Fig. 3 Metabolic imaging of cells. In this example, a radiolabeled glucose mimetic ([^{18}F]-FDG) is directly given to patients. Cells with high glycolytic activity, including malignant cells and immune cells, will accumulate the probe.

identifying highly proliferative lymphoma cells. Transition into preclinical rodent studies with [^{18}F]-FLT have been limited due to high serum thymidine in mice and rats (approximately 100× higher than humans), limiting the efficacy of [^{18}F]-FLT scans in these animals. For example, the average [^{18}F]-FLT dose in humans is administered with endogenous thymidine at greater than 150-fold excess. The high amount of free thymidine in humans and even greater amounts in rodents has limited the sensitivity by blocking signal accumulation and retention. In clinical applications [^{18}F]-FLT demonstrated an increased signal in lymph nodes and spleen of metastatic melanoma patients being treated with anti-CTLA4 (Ribas et al., 2010). Results from this study are complicated, and cannot rule out T cell proliferation from other causes but trend toward an increase signal in spleen after anti-CTLA4 therapy. Similar to [^{18}F]-FDG, deciphering [^{18}F]-FLT signal within the tumor from immune cell infiltrates vs tumor cells is challenging and instead only measurements of hematopoietic tissues can be made (Ribas et al., 2010; Shields et al., 1998).

3.4 Nucleoside Salvage Pathway Measured by Fluorinated Analog Probes

[^{18}F]-FLT and [^{18}F]-FDG, although retained by T cells, cannot be used to distinguish between the proliferation of immune vs nonimmune cells in tumors. Recently, several groups have targeted the development of alternative nucleoside analog PET probes specific for deoxycytidine kinase (dCK) (Namavari et al., 2011; Radu et al., 2008; Schwarzenberg et al., 2011; Shu et al., 2010). dCK is the rate-limiting enzyme in the nucleoside salvage pathway and is known to have high expression in proliferating/activated lymphocytes. By targeting a metabolic pathway increased in T cells, the detection of active immune responses can be identified more accurately than with [^{18}F]-FDG or [^{18}F]-FLT alone.

dCK phosphorylates the drug gemcitabine (2′,2′-difluorodeoxycytidine, dFdC). The initial phosphorylation of dFdC traps the drug intracellularly prior to further downstream phosphorylation and incorporation into DNA. Accumulation of dFdC was the highest in activated T cells, but due to the presence of two fluorine atoms, this compound was not amenable for ^{18}F radiochemistry. Removing one of the fluorine atoms and radiolabeling the second led to the development of [^{18}F]-FAC (1-(2′-deoxy-2′-[^{18}F]fluoroarabinofuranosyl) cytosine) (Radu et al., 2008). [^{18}F]-FAC had strong retention in the spleen, thymus, and bone marrow of wild-type mice. A reduction in signal was observed when animals were

treated with the systemic immunosuppressive drug dexamethasone, which is toxic to lymphocytes (Radu et al., 2008). In a genetic knockout of dCK, a complete loss of [^{18}F]-FAC signal in all lymphoid organs was seen, with a dramatic reduction in the total number of mature T and B cells (Austin et al., 2012; Toy et al., 2010). Together these studies demonstrate the specificity of [^{18}F]-FAC for lymphoid tissues and the requirement of dCK for normal lymphopoiesis.

In models of immune cell expansion such as viral-induced tumor or autoimmunity, an increased [^{18}F]-FAC accumulation was seen in the thymus, lymph nodes, and spleen (Fig. 4) (Nair-Gill et al., 2010; Radu et al., 2008). Analysis of isolated immune cells identified that activated effector CD8 T cells from the draining lymph node had the highest accumulation of [^{18}F]-FAC in comparison to other immune cells (myeloid, B cells). The authors found that the accumulation correlated with proliferation, and during this immune insult, the activated CD8 T cells had the highest percentage

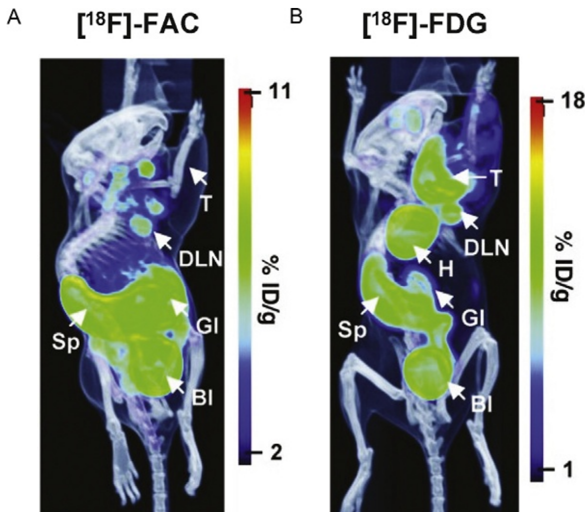


Fig. 4 [^{18}F]-FDG and [^{18}F]-FAC metabolic imaging of the antitumor response in mice. Immunocompetent C57BL/6 mice were injected intramuscularly with the MSV/MuLV retrovirus that causes a sarcoma. Mice were then imaged at the peak immune response with [^{18}F]-FAC on day 13 (A) and [^{18}F]-FDG on day 14 (B) to track the antitumor response. *Br*, brain; *H*, heart; *K*, kidney; *Bl*, bladder; *T*, tumor; *Sp*, spleen; *GI*, gastrointestinal tract; *Thy*, thymus. *Adapted from Nair-Gill, E., Wiltzius, S. M., Wei, X. X., Cheng, D., Riedinger, M., Radu, C. G., et al. (2010). PET probes for distinct metabolic pathways have different cell specificities during immune responses in mice.* The Journal of Clinical Investigation, 120, *2005, and reprinted with permission from* The Journal of Clinical Investigation.

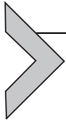
of replicating cells. However, tumor-infiltrating T cells had lower accumulation, which may be due to a reduction in the proliferation/cell-cycling rates (Nair-Gill et al., 2010). [^{18}F]-FAC is more selective for lymphocytes than other metabolic probes but has limited capacity in detecting tumor-infiltrating T cells, identifying the need to develop additional T cell-specific probes. In addition, it was observed that [^{18}F]-FAC can accumulate in tumors that are sensitive to dFdC making it difficult to distinguish tumor from T cells in vivo (Braas et al., 2012; Laing, Nair-Gill, Witte, & Radu, 2010; Laing et al., 2009; Lee, Campbell, Satyamurthy, Czernin, & Radu, 2012).

New, alternative nucleoside probes more specific for T cells are currently being developed. In a preliminary study, a radiolabeled AraG (2′-deoxy-2′-[^{18}F]fluoro-9-β-D-arabinofuranosylguanine ([^{18}F]-F-AraG)) was synthesized and incubated with primary, activated, or lymphoblastic T cells (Namavari et al., 2011). Accumulation of [^{18}F]-F-AraG was increased in activated T cells and in a leukemic cell line. Although animal studies using [^{18}F]-F-AraG are limited, the promising preliminary data suggest that [^{18}F]-F-AraG may be useful in detecting T cells or T cell malignancies in vivo. Furthermore, in vivo and ex vivo validation will determine whether [^{18}F]-F-AraG is specific for T cells or has high retention in alternate organs. Other nucleoside analogs have demonstrated signal accumulation within the myocardium that is believed to be from the mitochondrial nucleoside kinases (Campbell et al., 2012).

3.5 Current Limitations and Speculation on Alternative Probes or Pathways to Target T Cells

Results from the studies described above are encouraging but still require additional probe development for understanding and detecting tumor-infiltrating T cells. One difficulty in designing T cell probes is the change in metabolism between T cells in lymph nodes and T cells located intratumorally as observed by Nair-Gill et al. (2010). In order to identify which metabolites are necessary for tumor-infiltrating T cells, sophisticated metabolic studies will need to be done to tease out which metabolites are scavenged and taken in by the TILs vs those that are present prior to infiltration. An additional challenge is finding a metabolite highly enriched in TILs but not utilized by the tumor. Although challenging, the successful development of a unique metabolic probe for TILs would be broadly useful. In an ideal case, the probe would be radiolabeled with ^{18}F or ^{11}C for rapid

imaging. Due to the fast decay, it would also be possible for patients to have a follow-up [^{18}F]-FDG scan to track total tumor burden either the same or the following day.



4. PET REPORTER IMAGING FOR TRACKING ENGINEERED CELLS IN VIVO

Engineering cells to express reporter genes is a common practice in preclinical research. Most often, investigators will engineer cells to include a fluorescent or bioluminescent reporter, which are restricted to preclinical applications due to the depth of penetration limits of optical detection methods (Acton & Zhou, 2005; Herschman, 2004; Kircher et al., 2011). Similarly, cells can be engineered to express a PET reporter gene that can be directly translated into clinical use. To date, one PET reporter gene has been translated into clinical use (NCI clinical trial, NCT01082926) (Yaghoubi et al., 2009).

In PET, preclinical applications of reporters have been studied since 1996 with many studies investigating lymphocyte tracking after immunotherapies (Herschman, 2004; Tjuvajev et al., 1996). Reporter genes are ideal for therapies that need to monitor the fate of transplanted immune cells (Herschman, 2004). The exogenous protein is expressed in the cells transduced with the PET reporter and is then monitored for location, quantity, and distribution by the corresponding PET probe. Studies have tracked total hematopoietic cells, engineered T cells, dendritic cells, and other phagocytic cells in vivo but we will focus on the applications of PET reporter genes only in T cell biology for antitumor responses (Herschman, 2004; Tjuvajev et al., 1996). The major limitations in reporter imaging are the need to genetically manipulate cells ex vivo and the risk of immunogenicity from ectopic expression of a foreign protein.

4.1 Advantages and Subclasses of PET Reporter Genes

PET reporters fall generally into three distinct categories: transporter, membrane bound, or enzymatic (Herschman, 2004) (Fig. 5). The corresponding probes are therefore designed to minimize off-target signal or signal from nonengineered T cells. Investigators are then able to detect cells expressing the reporter gene in vivo by imaging with the corresponding reporter probe (Fig. 6). Stable expression of a PET reporter also overcomes some of the variability of imaging using metabolic PET probes allowing for more

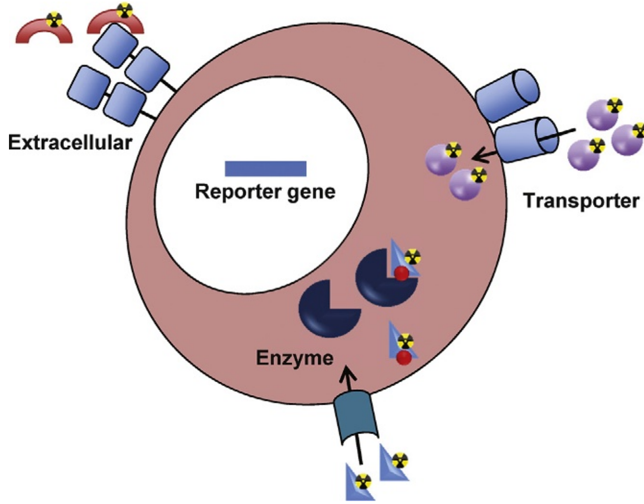


Fig. 5 Types of PET reporter genes. PET reporter genes are one of three categories: extracellular, transporter, or enzyme. Extracellular reporters can include unique transmembrane proteins that can be targeted with small molecules or engineered antibodies/proteins. Transporter reporters bring probes in intracellularly and allow for accumulation. Enzyme, or kinase, reporters modify or phosphorylate probes to allow for intracellular accumulation.

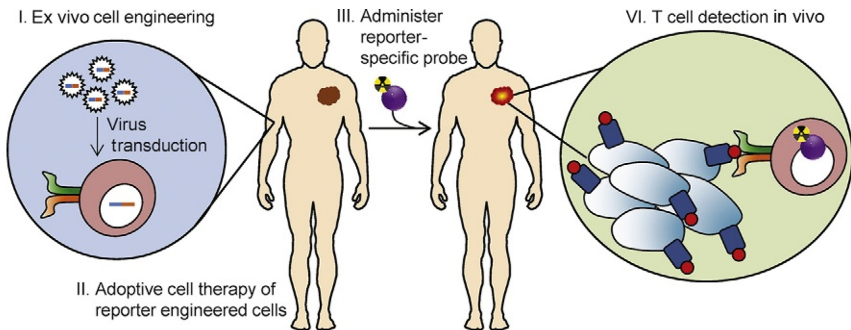


Fig. 6 Reporter gene imaging of adoptive cell therapy. Isolated T cells are engineered to express a PET reporter gene (and TCR or CAR). Cells are then adoptively transferred into recipients. At later time points, reporter-labeled cells are detected by injection of the reporter-specific probe.

consistent detection regardless of tissue location. This is important for sequential imaging that can occur days to months after the initial transplant. Extracellular or membrane-bound reporters allow for direct targeting by the radiolabeled probe and are limited based on total surface expression.

Membrane transporters improve signal to noise by actively transporting their probe intracellularly leading to an accumulation. Lastly, the most common PET reporters are kinase reporters that phosphorylate-specific radiolabeled PET probes. Phosphorylated molecules are typically trapped within cells that express the reporter leading to an increase in signal compared to nonreporter cells (Herschman, 2004). These kinase PET reporter genes (and in theory membrane bound) have also been demonstrated as effective suicide genes allowing for selective cell elimination if needed. For antitumor T cells, the ability to monitor subsets of cells (ie, only the engineered T cells expressing a CAR, TCR, or transduced TILs) and remove them in the case of adverse events by suicide gene action is advantageous in both clinical and preclinical applications.

4.2 Extracellular PET Reporters

Receptor-based PET reporter genes have been developed, but their applications to tracking antitumor T cell imaging have not been tested. A truncated recombinant carcinoembryonic antigen (CEA) was tested as a potential PET reporter gene by expression in the Jurkat T cell leukemia cell line (Kenanova et al., 2009). Imaging using an engineered anti-CEA antibody fragment (minibody; see later) detected CEA-positive Jurkat cells, thereby demonstrating the potential applications of receptor or extracellular PET reporter genes (Barat, Kenanova, Olafsen, & Wu, 2011; Kenanova et al., 2009). The type 2 human somatostatin receptor (hSSTr2) was originally targeted as a highly expressed target on neuroendocrine tumors, with SPECT and PET agents developed for diagnosis and staging (Gabriel et al., 2007). Outside of these tumors, expression of hSSTr2 is low throughout the body making this a suitable PET reporter gene. Several studies have demonstrated longitudinal reporter imaging with [^{68}Ga]-DOTATOC in mouse models of xenografts and AAV virus (Zhang et al., 2011; Zinn & Chaudhuri, 2002).

Recently, investigators have made decoy or truncated receptors for tracking T cells by flow cytometry or for use as a suicide gene. These truncated receptors include CD34 (Qasim et al., 2007; Zhan et al., 2013), CD20 (Philip et al., 2014), epidermal growth factor (EGFR) (Mardiros et al., 2013; Wang et al., 2011), and low-affinity nerve growth factor receptor (CD271) (Bonini et al., 2003; Thomis et al., 2001). In each instance, the signaling domains have been removed making the truncated protein nonfunctional. In some cases, the intracellular portion has been fused to a secondary suicide

gene (Thomis et al., 2001; Zhan et al., 2013). Normal T cells also lack expression of these receptors allowing engineered cells to be distinguished from other subsets by flow cytometry using a fluorescently labeled antibody against the receptor. Investigators have also tested antibody therapy in vivo as a method to selectively eliminate reporter-labeled cells (Wang et al., 2011). In theory, these same antibodies clones used for suicide gene function or flow cytometry could be radiolabeled and used for an in vivo PET reporter scan. Additional information on radiolabeling antibodies is described in Section 5.

4.3 Transporter PET Reporter

Adoptive T cells reactive to Epstein–Barr virus (EBV) were engineered to express the human norepinephrine transporter (hNET) as a PET reporter gene and detected by [^{124}I]-metaiodobenzylguanidine ([^{124}I]-MIBG) (Doubrovin et al., 2007). Initially, T cells were injected intratumorally and imaged after 4 h. hNET was able to detect as little as 10^4 cells per tumor in the microPET scan. Investigators then tracked the long-term tumor infiltration when EBV reactive CD8 T cells expressing the hNET PET reporter were transplanted intravenously. On days 1, 8, and 28, animals were scanned showing a progressive increase in signal from the TILs. Although hNET was capable of detecting a small number of engineered T cells, when compared to enzymatic PET reporters hNET was determined to be less sensitive (Doubrovin et al., 2007).

The sodium iodine symporter (NIS) is an alternative transporter expressed endogenously in the thyroid and stomach. Ectopic expression of NIS can allow reporter gene imaging by administration of iodine radionuclides (^{124}I, ^{131}I) for PET or alternative isotopes for SPECT (Penheiter, Russell, & Carlson, 2012). In preclinical studies, the expression of NIS was detected by ^{124}I in cancer cells infected with adenovirus expressing NIS (Groot-Wassink et al., 2004). One advantage to NIS is the high specificity demonstrated in vivo without needing to synthesize or radiolabel a reporter-specific probe or by utilizing ^{18}F probes that have a short half-life (Jauregui-Osoro et al., 2010).

4.4 Enzymatic PET Reporters

Kinase PET reporter genes have been the most widely used in tracking antitumor T cells. One reason for utilizing a kinase PET reporter is that even with low expression, the signal is amplified due to the enzymatic turnover

rate of the reporter (Herschman, 2004). The probe is administered systemically and cells must actively transport the probe intracellularly. Once the probe is intracellular, the reporter enzyme can phosphorylate and trap the modified probe intracellularly. Continued phosphorylation of free probe leads to an accumulation in signal allowing for detection of reporter-labeled cells. In cells lacking the reporter, the probe can freely efflux, reducing nonspecific signal. Therefore, the largest limitation of enzymatic PET reporter genes is that the probe must be delivered into cells by endogenous transporters that are not coregulated by the PET reporter gene and whose activity may vary depending on cell state or location (Acton & Zhou, 2005; Gambhir et al., 2000; McCracken et al., 2013; Pastor-Anglada et al., 2001; Radu et al., 2007; Shu et al., 2010; Tjuvajev et al., 1996).

4.4.1 Herpes Simplex Virus Type 1 Thymidine Kinase Reporters in Preclinical Models

The first enzymatic PET reporter gene described was herpes simplex virus type 1 thymidine kinase (HSV-TK). Initially, HSV-TK was demonstrated as a suicide gene with selective elimination of expressing cells by acycloguanosine compounds such as ganciclovir (GCV) (Tiberghien et al., 1994). HSV-TK was then used as a reporter in SPECT imaging with a radiolabeled 5-iodo-2′-fluoro-2′deoxy-1-beta-D-arabinofuranosyluracil (FIAU) demonstrating the possibility of engineered reporters and probes for noninvasive imaging (Tjuvajev et al., 1995). To increase resolution and sensitivity investigators then designed and radiolabeled PET probes for HSV-TK to be used as a PET reporter with ^{124}I and ^{18}F (Gambhir et al., 1998; Tjuvajev et al., 1998). In each case, a radiolabeled acycloguanosine-like-compound was able to visualize HSV-TK expression in mice.

In 2002, a mutant form of HSV-TK with improved V_{max}/K_m for GCV (sr39TK, described by Gambhir et al., 2000) was used to monitor tumor burden in mice transplanted with sr39TK expressing leukemic cells (Le et al., 2002). This study was the first demonstration of the feasibility for PET reporter imaging of HSV-TK as a tool to monitor the location of systemic immune cells not located as a subcutaneous graft. HSV-TK was then applied to monitor the migration of EBV reactive human T cells into tumors expressing EBV antigens in mice (Koehne et al., 2003). Signal was observed in the spleen and in the EBV+ HLA-matched tumors with peak signal seen at 48 h. Infused cells could be monitored by PET imaging for up to 15 days posttransplant. In contrast, cells that were prelabeled

with the probe [^{124}I]-FIAU (a radiolabeled antiviral nucleoside analog fialuridine-2′-deoxy-2′-[^{18}F]fluoro-5-iodo-1-β-D-arabinofuranosyluracil) (half-life 4.18 days) in vitro maintained signal up to 8 days postinfusion (Koehne et al., 2003). This encouraging longitudinal reporter imaging study demonstrated that both prelabel and in vivo probe administration can be used to track T cells homing intratumor in mice.

Most recent studies with HSV-TK have shifted away from the [^{124}I]-FIAU probe and instead utilize a penciclovir analog, 9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl) guanine ([^{18}F]-FHBG). These studies have successfully tracked the location and infiltration of tumor reactive T cells in mouse models of immunotherapy. These have included engineered TCRs, CARs, and tumor reactive T cells from viral-induced sarcomas (Dobrenkov et al., 2008; Dubey et al., 2003; Koya et al., 2010; Shu et al., 2005, 2009; Vatakis et al., 2011). Animals that received a bone marrow transplant with cells engineered to express HSV-TK were challenged with a viral-induced sarcoma and this allowed for the detection of a primary antitumor T cell response in vivo (Shu et al., 2005). The dual functionality and efficacy of sr39TK as a reporter and suicide gene were recently validated in a humanized mouse model (Gschweng et al., 2014; Vatakis et al., 2011). Human hematopoietic stem cells (HSCs) were transduced to express a TCR and sr39TK. Engraftment and T cell development were tracked by [^{18}F]-FHBG signal. To ablate the engineered cells, therapeutic doses of GCV were given. Efficacy was determined by follow-up [^{18}F]-FHBG scans that displayed little to no signal in ablated animals (Gschweng et al., 2014).

By incorporating HSV-TK, reporter T cells are detected with a 3D image of the location of engineered cells. This has enabled investigators to decipher tumor draining lymph nodes signal from tumor signal because of the improved resolution of PET in comparison to alternative imaging modalities.

4.4.2 Clinical Applications with HSV-TK PET Reporters

HSV-TK was first given to patients clinically when expanded lymphocytes used to treat graft vs leukemia were engineered to express HSV-TK as a suicide gene. Expression of HSV-TK was immunogenic in patients with CD8 T cells against the HSV-TK gene (Berger, Flowers, Warren, & Riddell, 2006; Traversari et al., 2007). Immune responses were rapid and most likely due to memory T cells. In contrast, no preclinical studies have reported immunogenicity from HSV-TK in mice. The lack of prior

exposure to HSV may prevent mice from developing immunogenicity to this PET reporter making it useful as a preclinical tool.

Although immunogenicity was observed, there are a number of positive reasons to utilize HSV-TK as a reporter gene clinically. To date, one case report has been published on the use of HSV-TK PET reporter in clinical use (Yaghoubi et al., 2009). This patient presented with glioblastoma (GBM) and was enrolled in a clinical trial of ACT with engineered T cells expressing the IL-13 zetakine and HSV-TK. [^{18}F]-FHBG scans showed a demonstrably higher signal after the infusion of engineered T cells within the tumor lesions of the brain. Due to the brain being isolated the follow-up of cells after transplant is difficult and the peripheral blood is not believed to represent the state of intracranial T cells. The difficulty and high risk of complications from biopsy in GBM also make PET reporter imaging ideal. Interestingly, the observed immunogenicity seen with previous ACT of lymphocytes expressing HSV-TK was not observed. This could be due to transplant conditions, location, or lack of HSV memory in the recipient. An additional success of this case report is the penetrance of [^{18}F]-FHBG across the blood brain barrier. Under healthy physiological conditions, [^{18}F]-FHBG does not cross (Yaghoubi et al., 2001). The success of this case report led to an expanded trial (NCT01082926) but results are yet to be published on the efficacy.

4.4.3 Human Nucleoside Kinase PET Reporters

Due to the observed clinical immunogenicity when HSV-TK lymphocytes were transplanted clinically, alternative enzymatic PET reporters have been developed. The homologous human nucleoside kinases were tested as potential alternatives to HSV-TK in preclinical PET applications. Two mutant forms of human deoxycytidine kinase (hdCK) and one mutant form of human thymidine kinase 2 have been established as PET reporters (Campbell et al., 2012; Likar et al., 2010; McCracken et al., 2013, 2015).

Expression of an hdCK mutant reporter (hdCKDM-point mutations of R104M and D133A) when probed with 2′-[^{18}F]-fluoro-2′-deoxyarabinofuranosyl-5-ethyluracil ([^{18}F]-FEAU) was able to track tumor-infiltrating CAR modified T cells in a humanized mouse model of metastatic prostate cancer (Likar et al., 2010). Human T cells were first engineered to express hdCKDM and the Pz-1 CAR that is directed to prostate-specific membrane antigen. T cells were infused into mice and imaged 6 h posttransplant. Specific antitumor T cell signal was observed in the lung tumors of animals given the hdCKDM expressing T cells.

The rapid imaging time in this study demonstrates that hdCKDM could be used in monitoring cellular adoptive immunotherapy, including tracking the location of engineered cells within metastatic lesions (Likar et al., 2010).

A separate study evaluated the expression of hdCK3mut (three point mutations within the active site) with [^{18}F]-L-FMAU to monitor tumor infiltration of immune cells in a humanized mouse model of melanoma (McCracken et al., 2015). Human CD34^{+} cells engineered to express the PET reporter and an engineered TCR was engrafted into a modified humanized "BLT" (human bone marrow, liver, thymus) mouse model. Expression of hdCK3mut allowed for detection of reporter T cells intratumorally in HLA-matched/antigen-positive tumors (Fig. 7). Importantly, weak/no signal was seen in the HLA mismatched/antigen-positive

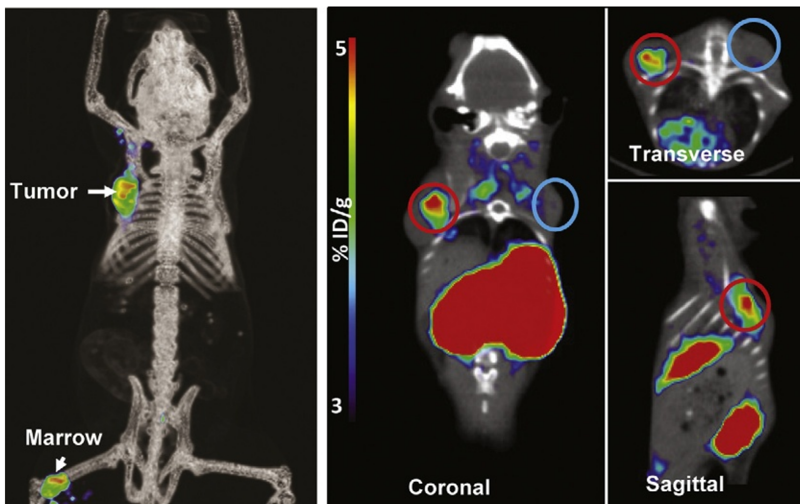


Fig. 7 hdCK3mut PET reporter gene detects engineered T cells in vivo. NSG mice were humanized with CD34^{+} human HSCs containing the F5 TCR (TCR toward MART1). HSCs develop in vivo into CD8^{+} T cells with the PET reporter and F5 TCR. Animals were sequentially transplanted with M202 (an HLA-A2.1 human melanoma cell line that is MART^{+}, *red* (*dark gray* in the print version) circle on 2D projection) or M207 (a MART + cell line with a mismatch HLA, *blue* (*gray* in the print version) circle on 2D projection). PET reporter imaging with [^{18}F]-L-FMAU demonstrates signal in bone marrow (sites of HSC engraftment) and within the tumor (*red* (*dark gray* in the print version) circle, site of lymphocyte infiltration) seen in both the 3D and 2D projections. No signal is observed within the HLA mismatched tumor (*blue* (*gray* in the print version) circle), demonstrating T cell homing into the correct HLA-matched tumor. *Adapted from McCracken, M. N., Vatakis, D. N., Dixit, D., McLaughlin, J., Zack, J. A., and Witte, O. N. (2015). Noninvasive detection of tumor-infiltrating T cells by PET reporter imaging.* The Journal of Clinical Investigation*, 125, 1815, and reprinted with permission from* The Journal of Clinical Investigation.

tumors demonstrating specific detection and homing into the correct tumor. Isolated lymphocytes from these reporter mice were then subjected to activation and stimulation with reporter-labeled cells functioning equivalent to nonreporter cells.

These studies show that hdCK-based PET reporter genes can be applied to current cell-based immunotherapies to monitor the location and longevity of transplanted engineered antitumor T cells. Examples of therapies that could benefit from reporter expression include tracking engineered T cell therapies (TCRs, CARs), or monitoring experimental GvHD. hdCK-based reporter genes have also been tested for long-term expression and normal T cell function in vivo (Likar et al., 2010; McCracken et al., 2015). Most importantly, hdCKDM and hdCK3mut are human enzymes with minimal mutations and should thus not be immunogenic if translated into clinical studies.

4.5 Future Applications and Limitations in PET Reporter Imaging

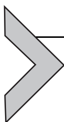
The major disadvantage of PET reporter gene imaging is the need to manipulate the cell either ex vivo or through targeted vector delivery in vivo. Furthermore, inducing expression of a foreign protein may trigger immune responses, or expression may impair function or cause malignant transformation to the reporter cells (Kircher et al., 2011; Koehne et al., 2003). In preclinical studies, no impact on T cell function was observed when kinase PET reporter genes were expressed (Koya et al., 2010; Likar et al., 2010; McCracken et al., 2015; Vatakis et al., 2011). Another disadvantage to viral gene delivery is the risk of insertional oncogenesis, vector splicing, or vector silencing (Blumenthal et al., 2007). Although these issues can arise due to the use of viral vectors, the coexpression of a PET reporter with a CAR or TCR will not increase the likelihood of these events.

Future directions of PET reporter genes include targeting T cell activation or specific subsets of cells by using lineage-specific promoters. One preclinical mouse study has tested this concept by utilizing HSV-TK expression and PET reporter imaging to detect cells in vivo only after T cell activation (Ponomarev et al., 2001). Jurkat cells (a human T-ALL cell line) were transduced with HSV-TK under the control of the TCR-dependent nuclear factor of activated T cells promoter allowing for detection in animals only when treated with anti-CD3 and anti-CD28 to activate cells. Similar inducible reporter systems may be helpful in addressing the behavior of immune cells after immunotherapies (eg, anti-PD1, DC vaccine, anti-CTLA4, or engineered T cells with TCRs or CARs) are given allowing for detection of a small subset of cells. In particular, if signal is weak or absent, or if signal is

seen outside the tumor, it might predict treatment failure or off-target activation. Lineage-induced reporters would also be useful in tracking the development/fate of lymphoid lineages including tumor-infiltrating T_{reg} cells. Prior to implementing these technologies, advances in the current lineage reporters are needed to amplify expression increasing the overall sensitivity of lineage inducible systems (Hildebrandt & Gambhir, 2004).

Improvements in the PET reporter probes nonspecific background will enable reporter genes to be applied to a greater number of tumor-infiltrating T cell tracking studies. For example, [^{18}F]-FHBG has high gastrointestinal uptake and clearance through the kidneys and bladder (Yaghoubi et al., 2001). This would make imaging within the peritoneal cavity especially near the intestines difficult. Other probes including [^{18}F]-L-FMAU have higher liver uptake in humans, potentially limiting detection of reporter cells within this organ (Campbell et al., 2012). In each instance, these are sites that could potentially harbor primary or metastatic lesions and probes with low/no background would increase the use of PET reporters. Low probe background will also increase sensitivity, or superior PET reporter activity will enable detection below the current limits for studies tracking a small number of cells (Moroz et al., 2015).

To date, the most common use of HSV-TK clinically has been as a suicide gene with the reporter gene as a secondary function. When given pharmacological doses of acycloguanosine compounds (eg, GCV), the HSV-TK expressing cells are selectively eliminated (Gschweng et al., 2014; Tiberghien et al., 1994). The truncated receptors expressed on the cell surface that are designed for selective elimination if necessary by a therapeutic antibody are predicted to also function as a dual reporter/suicide gene (Wang et al., 2011). For the new human enzymatic PET reporters being developed, the potential of utilizing the reporter as a suicide gene has been discussed (Likar et al., 2010; McCracken et al., 2015). It is anticipated that those drugs with a higher affinity for these reporters should also function as a suicide gene/drug combination. In future studies, the codevelopment of the suicide gene function will allow investigators to remove reporter cells in case of adverse events, improving the safety of engineered T cell-based therapies.



5. IN VIVO DETECTION OF IMMUNE CELLS USING RADIOLABELED PROTEINS

5.1 In Vivo Cell Labeling Using Radiolabeled Proteins

Targeting of extracellular epitopes in vivo by utilizing the high specificity of antibodies to tumor-specific antigens, a concept first introduced by Paul

Ehrlich, has long been a goal of cancer therapy to eradicate tumors. Intact IgG antibodies engage multiple proteins, including the antigen of interest determined by the variable domains and both Fc-receptors and complement by the Fc domain. Antibodies function directly by either blocking proliferative signaling networks on tumor cells (EGFR) or modulation of signaling via coinhibitory or costimulatory receptors effecting immune cell activation (CTLA-4, PD-1, and PD-L1 or 4-1BB and CD40, respectively). Antibodies also function indirectly by eliciting immune responses utilizing the antibody Fc domain engaging Fcγ receptors on immune cells (antibody-dependent cellular cytotoxicity) or complement (complement-dependent cytotoxicity). Furthermore, antibody half-life is extended by the use of Fc binding to the neonatal Fc receptor (FcRn). The utility of antibodies has been demonstrated by their therapeutic development across a variety of clinical settings, including inflammatory diseases, transplantation, infectious diseases, cardiovascular medicine, and agonistic and antagonistic cancer immunotherapy. Furthermore, the development of bispecific antibodies, fusion proteins, and antibody–drug conjugates continues to revolutionize the field due to advances in protein engineering (Carter, 2011). The clinical impact of antibody-derived therapeutics is demonstrated by the fact that there are currently over 30 FDA approved antibodies for the treatment of a variety of diseases and hundreds more are currently in clinical development (Reichert, 2010, 2015).

The clinical success of antibodies validates the delivery of antibodies to specific targets in vivo and has led to the development of antibodies and antibody fragments as molecular imaging agents (Fig. 8). In the context of oncology, antibody-based imaging has the ability to profile cell surface markers expressed on tumors in vivo, with applications in primary and metastatic tumor detection, patient staging and stratification in treatment groups, determining radioimmunotherapy dosing and receptor occupancy, and evaluation of response to therapy (Knowles & Wu, 2012). Early efforts into intact antibody imaging utilized planar scintigraphy or SPECT with several receiving regulatory approval, including OncoScint (^{111}In-satumomab pendetide), ProstaScint (^{111}In-capromab pendetide), and CEA-Scan (^{99m}Tc-arcitumomab). However, their use is very restricted due to limitations in sensitivity, quantification, and immunogenicity. With the expansion of target validation, protein and antibody engineering, antibody humanization, availability of long-lived PET radionuclides, and the use of PET in clinics across the globe, the translational potential of immuno-positron emission tomography (immuno-PET) is increasing (van Dongen & Vosjan, 2010; Wu, 2009).

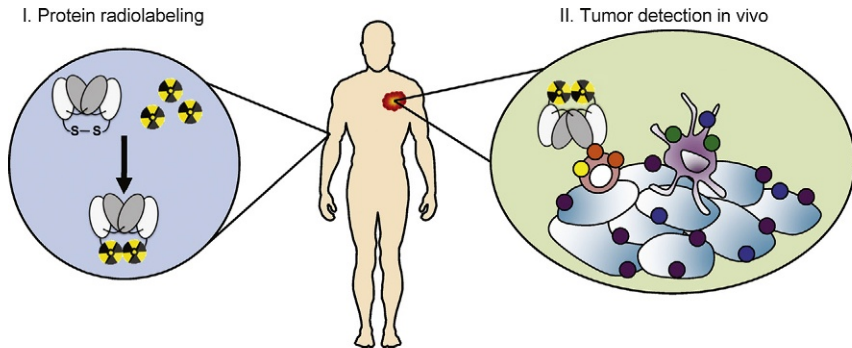


Fig. 8 In vivo targeting of endogenous antigens. In this example, a cys-diabody is radiolabeled and then injected for targeting endogenous antigens present in the tumor immune microenvironment. These antigens can be cell type specific, such as CD8 or CD4 on T cells (*orange* (*gray* in the print version)) and CD11b expressed on MDSCs (*green* (*dark gray* in the print version)), or activation specific, such as the checkpoint inhibitors CTLA-4 or PD-1 expressed on tumor-infiltrating cytotoxic T lymphocytes (*yellow* (*white* in the print version)) or PD-L1 expressed on tumor cells and/or MDSCs (*blue* (*dark gray* in the print version)). *Purple* (*dark gray* in the print version) antigens are tumor-specific antigens that can also be targeted using immuno-PET.

5.2 Radiotracer Design

The long circulation half-lives of intact antibodies that range from days to weeks requires the use of long-lived PET radionuclides, such as ^{124}I (4.2 days), ^{89}Zr (3.2 days), and, to a lesser extent, ^{64}Cu (12.7 h) (Boswell & Brechbiel, 2007; Nayak & Brechbiel, 2009; Tolmachev & Stone-Elander, 2010). Immuno-PET acquisition of intact antibodies is generally 5–7 days postinjection of the radiotracer, resulting in increased radiation dose for the patient and inconvenient patient scheduling. To enhance the target-to-background at early times postinjection, reduce radiation exposure, and make use of short-lived radionuclides, antibody engineering has allowed for the construction of antibody fragments for immuno-PET, including diabodies and minibodies (Fig. 9A) (Olafsen & Wu, 2010; Wu, 2014). The bivalent diabody (scFv dimer; ~55 kDa; terminal half-life ~2–5 h in mice) and minibody (scFv-C_H3 dimer; ~80 kDa; terminal half-life ~5–10 h) fragments allow for directed clearance through either the kidney or liver, respectively, due to the renal filtration cutoff of ~60 kDa. Although the total overall antigen targeting (as indicated by percentage injected dose per gram or %ID/g) of antibody fragments is reduced compared to the respective intact antibody, it is the high target-to-background ratio at early times postinjection that allows for the potential of same day imaging and the use of shorter-lived PET radionuclides, including ^{18}F, ^{68}Ga, and ^{61}Cu.

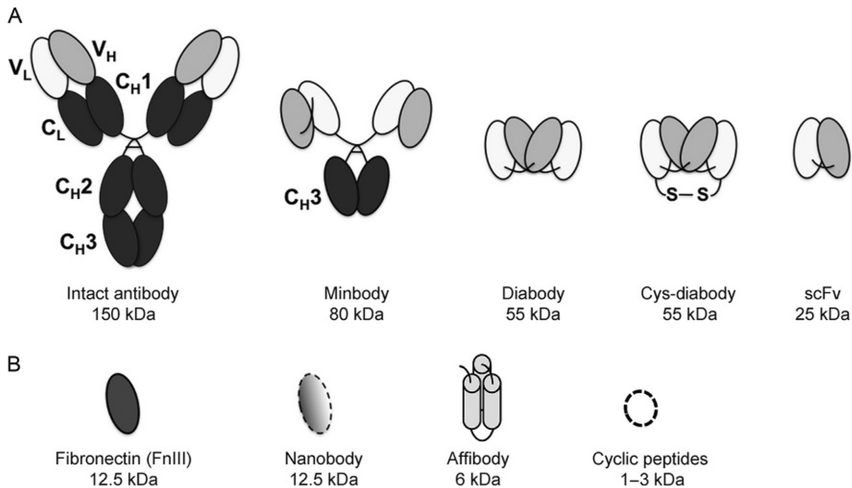


Fig. 9 Protein-based scaffolds for targeting cell surface antigens in vivo. (A) Antibodies and the respective antibody fragments engineered for enhanced immuno-PET characteristics can be radiolabeled for targeting endogenous antigens in vivo. (B) Similarly, alternate protein scaffolds that are engineered to bind antigens of interest can be engineered and radiolabeled for targeting antigens in vivo.

Simultaneously, advances in protein engineering and molecular evolution strategies have led to the construction of highly diverse libraries derived from either immunoglobulin-like structures, such as the human fibronectin type III domain and single domain antibodies, or derived from various other protein-based scaffolds, such as affibodies and cyclic peptides among multiple others (Hosse, Rothe, & Power, 2006) (Fig. 9B). These libraries allow for the selection of high affinity binders to virtually all antigens of interest. The molecular weight of these scaffolds is below the renal filtration cutoff and due to their small size have inherently fast clearance rates in vivo, further expanding the use of the shorter-lived PET radionuclides. However, as clearance rates increase, the ability to target the antigen of interest is decreased due to reduced antigen exposure, which has led to technologies that prolong the circulation time, such as PEGylation and fusion to albumin or IgG-binding domains (Kontermann, 2011). Whatever the engineered scaffold, the designed radiotracer must radiolabeled with a suitable PET radionuclide and sufficiently bind the antigen of interest for in vivo PET detection.

When generating a protein-based radiotracer, there are other variants to take into account other than radionuclide selection based on half-life for optimal probe design, such as conjugation strategy, chelator, target antigen,

antigen internalization, valency and route of clearance, among others (reviewed in Wu, 2014). Radionuclides are conjugated to an antibody directly or indirectly via the use of a bifunctional chelator (Nayak & Brechbiel, 2009; Tolmachev & Stone-Elander, 2010). Direct conjugation is performed by halogenation of radioiodine to solvent exposed tyrosine residues. This method is not suitable for radiotracers that target rapidly internalizing antigens, as dehalogenation and intracellular catabolism cause the rapid clearance of radioiodine and the radiometabolite iodotyrosine, respectively, from the target tissue. Furthermore, iodination can potentially reduce antigen-binding capacity if the halogenation occurs to critical tyrosines in the antigen-binding site. Indirect conjugation generally requires a bifunctional chelator that contains a radiometal chelator to form a complex with a radiometal and a reactive group that binds to the protein. The use of residualizing radiometals causes intracellular retention of radioactive metabolites after cellular internalization and catabolism. This results in accumulation of radioactivity in both the target cells and the organs of clearance, such as the liver and kidney.

Conjugation of bifunctional chelators has been directed to the nucleophilic ε-amino groups of solvent exposed lysine residues using *N*-hydroxysuccinimide esters or isothiocyanates, for example. This nonsite-specific conjugation approach has the ability to abolish antigen-binding capabilities if the conjugation occurs to critical lysine residues in the antigen-binding site. As the radiotracer scaffold reduces in size, the conjugation to lysines with chelators or tyrosine via direct iodination becomes more crucial as there are less available noncritical solvent exposed lysines or tyrosines available for conjugation. Nonsite-specific conjugation methods result in a distribution of conjugated products all with different chelator-to-protein ratios resulting in altered pharmacokinetics of different species in the same batch. This conjugation strategy can also result in inconsistent batch-to-batch conjugations. Therefore, site-specific conjugation methods have become increasingly important to create a single, homogenous and well-characterized radiotracer that can be consistently and efficiently produced. For example, the introduction of an engineered cysteine residue can drive the conjugation of sulfhydryl-reactive bifunctional chelators away from the antigen-binding site to reduce the probability of affecting the antigen-binding capacity of the radiotracer. In combination with the utility of small protein targeting scaffolds, the expansion of bioorthogonal (radio)chemistry has further increased the protein radiochemistry capabilities, which has become important especially when using the short-lived radionuclides as rapid radiolabeling kinetics are preferred

(Sletten & Bertozzi, 2009; Zeng, Zeglis, Lewis, & Anderson, 2013). The variety of bioconjugation techniques for protein radiochemistry is constantly evolving to establish best-in-class imaging agents.

The pharmacokinetics of radiolabeled proteins varies greatly depending on the chelator, radionuclide, conjugation strategy, and purification tag due to alterations in surface charge and conformation. For example, cys-diabodies conjugated to DOTA using different peptide linkers, radiometals, and conjugation methods greatly affects the renal and hepatic clearance (Li et al., 2002; Tavaré, Wu, et al., 2014). Similarly, studies performed with affibodies demonstrate the impact of purification tag sequence and location, chelator, radiometal, and conjugation method on tumor targeting and pharmacokinetics (Hofstrom et al., 2013; Strand et al., 2013; Tolmachev & Orlova, 2010). As low-molecular-weight proteins radiolabeled with residualizing radionuclides below the renal filtration cutoff of ∼60 kDa suffer from high renal accumulation, efforts have been made to determine the optimal radiometabolites of altered chelators that reduce radioactive renal accumulation (Uehara et al., 2014). Interestingly, the same chelator can behave very differently with the use of a different radiometal.

In summary, the protein scaffold, conjugation strategy, radionuclide, and chelator used all effect the radiotracers pharmacokinetics, and a well-characterized and optimized combination of these factors will increase radiotracer accumulation to the antigen of interest in vivo and, therefore, successful detection using PET.

5.3 Imaging Immune Cells with Radiolabeled Proteins

Intact antibodies have been used previously to detect T and B cells in patients with a range of disorders using planar scintigraphy (Malviya, Galli, Sonni, Pacilio, & Signore, 2010). Early efforts into targeting immune cells with radiolabeled antibodies resulted in the FDA approval of two radioimmunotherapies targeting CD20, ^{90}Y-ibritumomab tiuxetan (Zevalin), and ^{131}I-tositumomab (Bexxar). More recently, anti-CD20 antibodies radiolabeled with positron-emitting radionuclides were developed to establish CD20^{+} tumor burden and to determine radiotherapy dose estimates. ^{89}Zr-Zevalin was developed to predict ^{90}Y-Zevalin radioimmunothearpy doses (Perk et al., 2006; Rizvi et al., 2012). Subsequently, ^{89}Zr-rituximab was used to determine the effect of the required predose of nonradiolabeled rituximab that is standard of care for ^{90}Y-anti-CD20 radioimmunotherapy (Muylle et al., 2015). This paper highlights impaired

tumor targeting of ^{90}Y-radioimmunotherapy due to the use of a predose in patients that have already received rituximab-based therapy and calls into question the use of the predose to enhance anti-CD20 radioimmunotherapy. ^{64}Cu-rituximab has also been developed for clinical translation and PET detection of CD20 expression (Natarajan, Arksey, Iagaru, Chin, & Gambhir, 2015). Furthermore, an engineered anti-CD20 radiotracer based on the human fibronectin type III domain was developed for rapid ^{64}Cu-PET detection of B cells (Natarajan, Hackel, & Gambhir, 2013).

The ability to detect CD8^{+} cytotoxic T lymphocytes noninvasively using immuno-PET was recently demonstrated using antibody fragments targeting mouse CD8 derived from the parental depleting antibodies produced from the hybridomas 2.43 and YTS169.4.2.1 that differ in their binding to CD8α (Lyt2) (Tavaré, McCracken, et al., 2014; Tavaré et al., 2015). Anti-CD8 immuno-PET using both the 2.43 and YTS169 minibodies demonstrated in vivo specificity for CD8 expressed in the lymph nodes and spleens of antigen-positive mice (Tavaré, McCracken, et al., 2014). These engineered minibodies did not deplete CD8^{+} T cells in vivo due to the removal of the full Fc domain when engineered to minibody format. More recently, the 2.43 cys-diabody was used to detect CD8^{+} T cell repopulation over time in a model of HSC transfer (Fig. 10) (Tavaré et al., 2015). Similarly, CD4^{+} T cell repopulation was also detected using a newly developed anti-CD4 cys-diabody derived from the GK1.5 hybridoma (Fig. 10). This work demonstrated that anti-CD4 and -CD8 immuno-PET have the ability to monitor helper and cytotoxic T cell expansion and localization noninvasively and has the potential to detect enhanced T cell repopulation from optimized HSC engraftment protocols. Antigen-specific HSC therapy utilizing CAR or TCRs has become an attractive therapeutic approach for long-term immunity for multiple malignancies. Anti-CD8 immuno-PET may be able to detect CAR/TCR transduced HSC engraftment and subsequent T lymphocyte targeting to antigen-specific tumors.

The YTS169 cDb specifically detected endogenous CD8 expressed in the spleen and lymph nodes of immune competent mice using ^{89}Zr-immuno-PET (Fig. 11). ^{89}Zr-YTS169 cDb could detect increased CD8^{+} TILs in mice bearing CT26 colon carcinoma tumors treated with agonistic anti-CD137 antibody therapy (Tavaré, Escuin-Ordinas, et al., 2014). Immuno-PET of CT26 tumors in untreated mice had a peripheral ring of activity, while the tumors of anti-CD137-treated mice showed enhanced uptake throughout the tumor (Fig. 11). Anti-CD8 immuno-PET

was also used to detect tumor-infiltrating T cells in models of anti-PD-L1 immunotherapy and adoptive cell transfer (Tavaré et al., 2016).

The ability to detect tumor-infiltrating macrophages has been demonstrated with recent work utilizing nanobodies to target myeloid cell markers. Initially, an antimacrophage mannose receptor (MMR) nanobody was radiolabeled with ^{99m}Tc for SPECT detection of MMR-expressing tumor-infiltrating macrophages (Movahedi et al., 2012). Specificity for

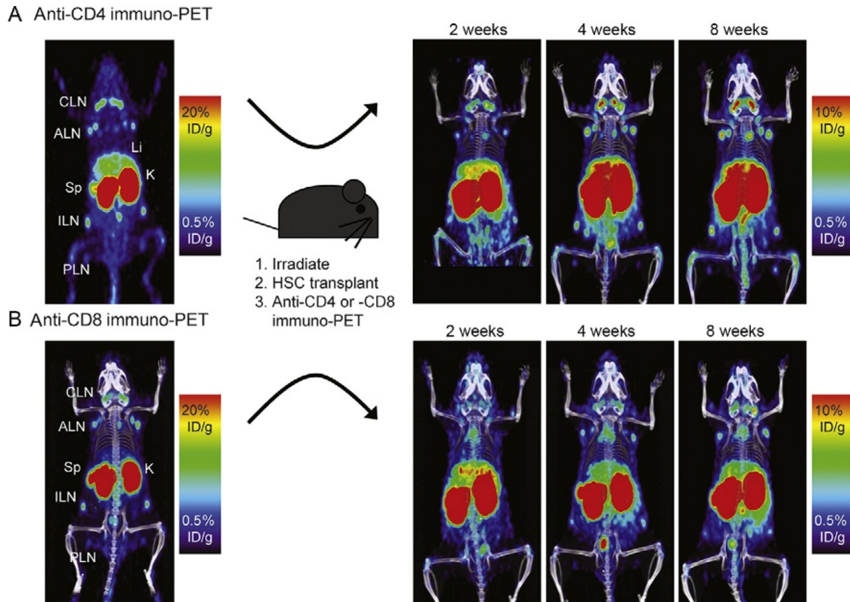


Fig. 10 Anti-CD4 and -CD8 immuno-PET detection of helper and cytotoxic T cell repopulation posthematopoietic stem cell transfer. ^{89}Zr-radiolabeled anti-CD4 cys-diabody (A) and anti-CD8 cys-diabody (B) derived from the parental depleting GK1.5 and 2.43 antibodies, respectively, injected into wild-type C57BL/6 mice (*left panel*) demonstrated specific detection of lymphoid organs and nonspecific kidney uptake due to renal clearance. In a model of hematopoietic stem cell therapy, wild-type mice were lethally irradiated prior to HSC therapy. Anti-CD4 and -CD8 immuno-PET was acquired at 2, 4, and 8 weeks after HSC therapy (*right panel*) demonstrating the detection of helper and cytotoxic T cell repopulation over time. Immuno-PET images were acquired at 22 h postinjection and presented as 25 mm maximum intensity projections. *ALN*, axillary lymph nodes; *B*, bone; *CLN*, cervical lymph nodes; *ILN*, inguinal lymph nodes; *K*, kidney; *Li*, liver; *PLN*, popliteal lymph nodes; *Sp*, spleen. *This figure was adapted from research originally published in* JNM. *Tavaré, R., McCracken, M. N., Zettlitz, K. A., Salazar, F. B., Olafsen, T., Witte, O. N., et al. (2015). Immuno-PET of murine T cell reconstitution post-adoptive stem cell transplantation using anti-CD4 and anti-CD8 Cys-diabodies.* Journal of Nuclear Medicine, 56, *1258–1264. © by the Society of Nuclear Medicine and Molecular Imaging, Inc.*

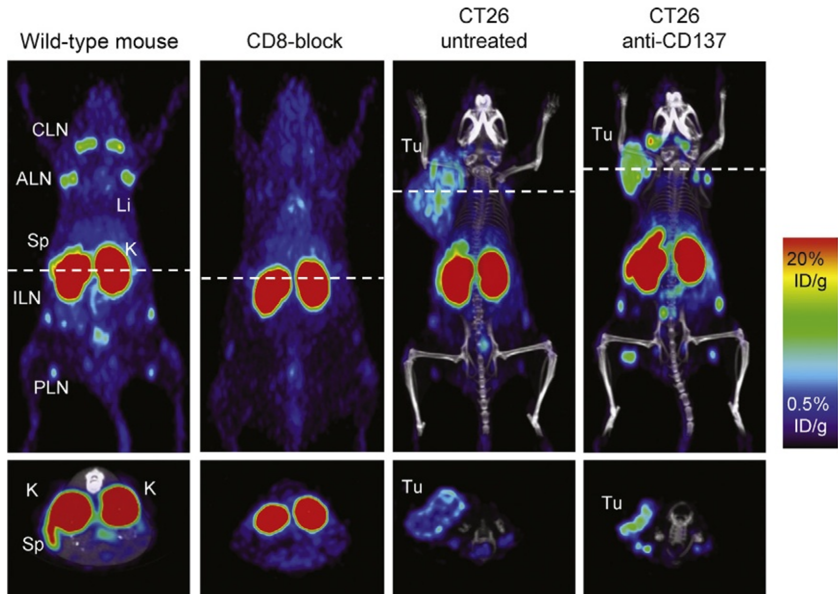


Fig. 11 Anti-CD8 immuno-PET detection of tumor-infiltrating cytotoxic $CD8^+$ T cells. ^{89}Zr-radiolabeled anti-CD8 cys-diabody derived from the parental depleting YTS169 antibody was injected into a wild-type mouse, CD8-blocked mouse (bolus coinjection of 3 mg/kg nonradiolabeled cys-diabody), Balb/c mouse bearing 15-day-old syngeneic CT26 colon carcinoma subcutaneous tumor, and Balb/c mouse bearing 15-day-old CT26 tumor treated with anti-CD137 antibody. Immuno-PET images were acquired at 22 h postinjection and presented as coronal 25 mm or transverse 2 mm maximum intensity projections. *ALN*, axillary lymph nodes; *B*, bone; *CLN*, cervical lymph nodes; *ILN*, inguinal lymph nodes; *K*, kidney; *Li*, liver; *PLN*, popliteal lymph nodes; *Sp*, spleen; *Tu*, CT26 tumor. *Figure adapted from Tavaré, R., Escuin-Ordinas, H., McCracken, M. N., Mok, S., Zettlitz, K. A., Salazar, F. B., et al. (2014). Anti-CD8 immunoPET detection of CD8+ tumor infiltrating lymphocytes. In* World molecular imaging congress, Seoul, Korea.

tumor-infiltrating macrophages was demonstrated with MMR-deficient mice that have reduced tumor-associated macrophages. More recently, this group has used ^{18}F-labeled anti-MMR nanobody to detect tumor-infiltrating macrophages with immuno-PET (Blykers et al., 2015). Nanobodies targeting MHC-II and CD11b have been radiolabeled with ^{18}F for PET detection of the myeloid-specific cell surface makers in vivo (Rashidian et al., 2015). Specificity was demonstrated in vivo using MHC-II and CD11b knockout mice.

In addition to ex vivo labeling of cells directly with a radiotracer or a reporter gene for subsequent PET detection with a reporter probe, protein

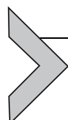
scaffolds selected to target endogenous immune cell subtypes in vivo have immense potential to grow as diagnostic and prognostic imaging agents in parallel with the cancer immunology targets being developed. PET detection of immune antigens of interest using these targeted scaffolds could provide noninvasive information relating to the tumor microenvironment, predict responders from nonresponders to immunotherapeutic treatment, and detect antitumor responses posttherapy resulting from immune altering therapies.

5.4 Future Applications and Limitations of Radiolabeled Proteins for PET

The dynamic tumor microenvironment has become a topic of great importance in the field of tumor immunology and immunotherapy (Hanahan & Weinberg, 2011; Mantovani, Allavena, Sica, & Balkwill, 2008). Specifically, the presence of various cell types, such as CD4 and CD8 TILs, regulatory T cells, macrophages, and monocyte-derived suppressor cells (MDSCs), contribute to the up- or downregulation of antitumor immune responses. The activation state of each of these cells plays a critical role in determining the outcome of the antitumor immune response. Furthermore, the ability of immunotherapies to alter the activation state provides multiple cell surface markers that are either cell type or activation state specific for the development of future protein-based targeted molecular imaging agents. These markers include cell-specific lineage markers (eg, CD8, CD11b, and CD16) and activation markers (eg, CTLA-4 and PD-1/L1). Antibody-based tracers for the immuno-PET detection of both CTLA-4 and PD-L1 have recently been reported and future development of other agents targeting the same or similar receptors are expected in the future (Heskamp et al., 2015; Higashikawa et al., 2014). The key challenge for imaging these potential biomarkers in the field of cancer immunotherapy is how their detection will guide clinical decisions. For example, molecular biomarkers that predict who will respond to or which patients have responded to a certain immunotherapy need to be clinically identified for further development of molecular imaging agents. Furthermore, these markers may have different utility in predicting or assessing antitumor immune response in different malignancies. For example, the presence of cytotoxic $CD8^+$ T cells has had mixed results when used to predict overall survival in breast, lung, ovarian, melanoma, and colorectal cancers (Gooden, de Bock, Leffers, Daemen, & Nijman, 2011; Pages et al., 2010).

Other challenges for detecting tumor-infiltrating immune cells are the presence of the antigen sink (ie, spleen and lymph nodes) and how the injected protein dose affects efficient and reproducible tumor-infiltrating immune cell targeting. The optimal protein dose will be dependent not only on the patients' treatment history but also on the type of immunotherapeutic treatment. For example, agonistic CD137 therapy causes the systemic expansion of CD8 T cells but checkpoint inhibitor therapy (anti-PD-1/L1) causes local cytotoxic T cell activation at the site of PD-1/L1 expression. Therefore, the optimal protein dose for sufficient CD8 targeting outside the antigen sink might be different due to the mechanistic differences of the two immunotherapies. Methods to establish the optimal protein dose on a patient-by-patient basis should be established to ensure reproducible tumor-infiltrating immune cell targeting. The importance of protein dose for targeting of CD20 B lymphocytes in the clinic was recently reported for radioimmunotherapy treatment of lymphoma patients (Muylle et al., 2015). The lessons learned from B cell targeting in the clinic are relevant to other immune cell targeting, as the principles of antibody-based radioimmunotherapy targeting are similar to that of immuno-PET targeting.

Future directions for immune cell targeting in the field of ACT are the use of cell surface expressed reporter probes as discussed in Section 4.2. These reporter probes can be targeted not only with an imaging probe for longitudinal cell tracking but also with an intact antibody for the removal of adoptively transferred cells if a patient has adverse reaction to the therapy. Examples of these include a human CD20 mimetic (Philip et al., 2014), the truncated human EGFR (Wang et al., 2011), and the truncated human CEA domain (Barat et al., 2011; Kenanova et al., 2009). One challenge for the clinical development of these cell surface reporter probes will be the epitope density expressed on the cell surface and if the expression is high enough to be detected via immuno-PET. In the future, methods for signal amplification for molecules that are not highly expressed should be examined. The development of advanced pretargeting strategies that enhance radiotracer uptake may help achieve this goal.



6. CONCLUDING REMARKS AND FUTURE DIRECTION OF PET IMAGING THE ANTITUMOR T CELL RESPONSE

Resolution of the PET scanner in both clinical and preclinical applications is approximately the size of lymph nodes. Being close to the limit of detection in scans can cause a partial volume effect due to spatial resolution

in some cases (Lehnert, Gregoire, Reilhac, & Meikle, 2012). This is something for researchers and clinicians to be aware of and account for in their analysis of the data. Although these resolution parameters may not be improved, the overall sensitivity of the instrument may help in obtaining a scan with less total activity (Herrmann et al., 2013). New microPET scanners are significantly more sensitive allowing for reduced probe administration. If these improvements are translated into clinical scanners this will provide a safer and lower dose per patient potentially increasing the number of scans each patient can receive. Another current limitation in PET is the ability to synthesize clinical grade probes. As the popularity and versatility of PET increases we anticipate that a larger number of hospitals and clinicians will setup a means to self-produce or order in the necessary PET probes through off-site radiopharmacies.

PET is a powerful imaging technology that has immense applications. To date, preclinical applications have shown encouraging data and ways that PET imaging can improve how we monitor immunotherapies. As investigators continue to make improvements in protein engineering, probes, reporter genes, and scanners, the applicability of PET imaging to track the antitumor T cell response will move from a research technology to a primary method for monitoring T cell efficacy. These combinatorial imaging strategies should greatly enhance our understanding of the antitumor T cell response as well as the overall immune status of the tumor microenvironment and whole-body providing previously unattainable information.

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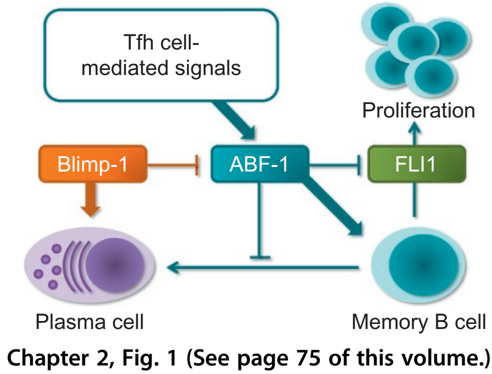

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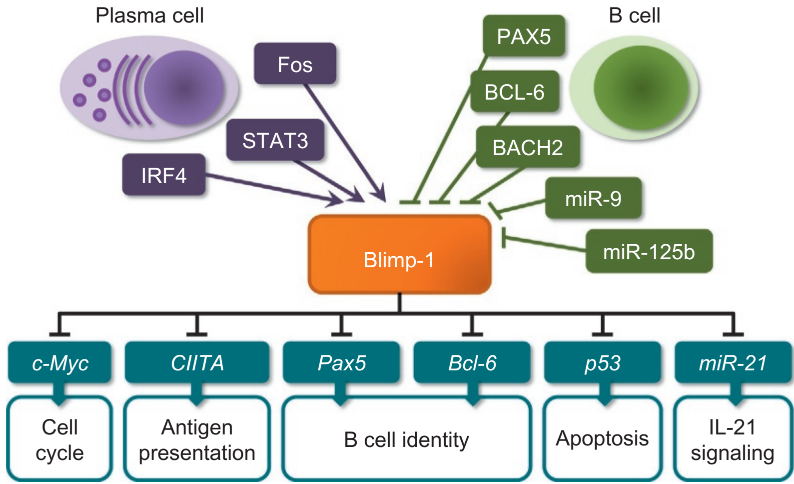

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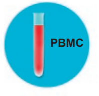
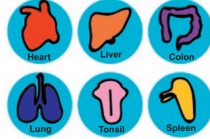
Sample Preparation

Blood sample

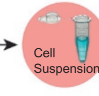


OR

Tissue sample



Cell isolation
(tissue digestion, dead cell removal & cell sorting, etc.)



Live/dead staining (Rh-103, Pt-195, Pt-194 or Pt-198)

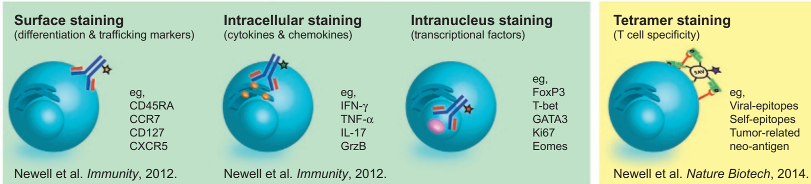
Cell staining

Surface staining
(differentiation & trafficking markers)

Intracellular staining
(cytokines & chemokines)

Intranucleus staining
(transcriptional factors)

Tetramer staining
(T cell specificity)



eg, CD45RA CCR7 CD127 CXCR5

eg, IFN-γ TNF-α IL-17 GrzB

eg, FoxP3 T-bet GATA3 Ki67 Eomes

eg, Viral-epitopes Self-epitopes Tumor-related neo-antigen

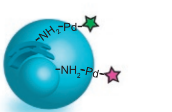
Newell et al. *Immunity*, 2012. Newell et al. *Immunity*, 2012. Newell et al. *Nature Biotech*, 2014.

Binary/triply barcoding

Fixation before barcoding

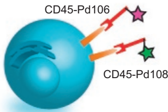
Fixation after barcoding

Mass-tag cell barcoding



OR

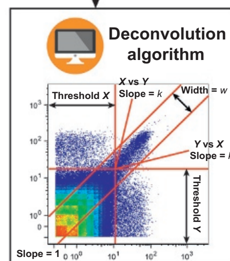
CD45-Pd cell barcoding



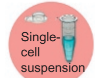
Zunder et al. *Nature Protocols*, 2015. Mei et al. *J Immunol*, 2015.

DNA staining
(Ir-191/193 or Rh-103 interchelator)

After cell acquisition & data processing



Cell acquisition



Adding Four EQ Beads

Cytometry by time-of-flight

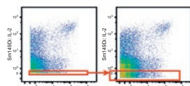
Acquisition time: ~ hours to days

Data processing

Normalization

Finck et al. *Cytometry A*, 2013.

Randomization of signals



Signals with zero value are rearranged to -1 ~ 0

Debarcode samples

Data analysis

Softwares or web services
platforms for graphical plot of high-dimensional analysis

(continued in Figure 2.)

Chapter 3, Fig. 1 (See page 109 of this volume.)

Machine learning of high-dimensional cytometric data

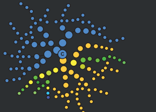
1. Clustering algorithm

SPADE

Uniform density, hierarchical clustering using MST
Bendall et al. *Science*, 2011.
- Human HSC, Immunophenotypic progression of human bone marrow, CD marker expression pattern of CD4^{+}, CD8^{+}, NK cell, etc.

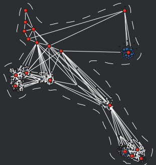
Citrus
User-defined end point for multiple samples comparison
Bruggner et al. *PNAS*, 2014.
- Human CD4^{+} and CD8^{+} T cells in HIV infection

FLOW-MAP
Force-directed, replaced MST with graph structure defined by local density
Zunder et al. *Cell Stem Cell*, 2015.

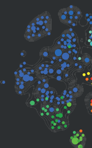


PhenoGraph
Partitioning phenotypic similar cells into clustering graph based on social network algorithms
Levine et al. *Cell*, 2015.
- Human HSC

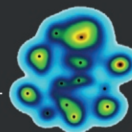
Scaffold map
Force-directed, clustering cell graph using landmark populations
Spitzer et al. *Science*, 2015.
- Human T cells across various tissues



PhenoGraph + *t*-SNE

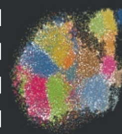


ACCENSE
Probability density-based clustering with peak detection
Shekhar et al. *PNAS*, 2014.
- Mouse CD8^{+} T cells



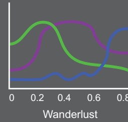
Training clusters

DensVM



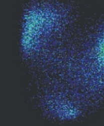
Machine learning SVM with boundary detection
Becher et al. *Nat Immunol*, 2014.
Wong et al. *Cell Reports*, 2015.
- Human CD4^{+} T cells, Peripheral and tonsilar TFH

Wanderlust
Connect waypoint cells with the shortest path using *k*-NN graph
Bendall et al. *Cell*, 2014.
- Progression of human B cell development



2. Dimensionality reduction algorithm

PCA

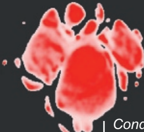


Reduce dimensionality into manageable variables
Newell et al. *Immunity*, 2012.
- Human CD8^{+} T cells continuum, T cell differentiation progression, antigen-specific T cells (CMV, EBV, influenza, etc.)

Linear

Nonlinear

***t*-SNE**

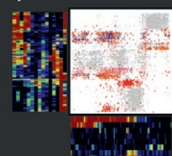


Perform pairwise comparison of cells on embedding map
Amir et al. *Nat Biotechnol*, 2013.
Becher et al. *Nat Immunol*, 2014.
Wong et al. *Cell Reports*, 2015.
- Human CD4^{+} T cells and TFH

Condense dimensions

Find peak maxima

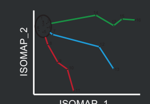
One-SENSE



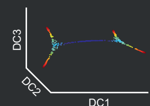
Cellular categorical analysis using 1D *t*-SNE
Cheng et al. *J Immunol*, 2016.
- Human Treg & CD8^{+} T cells

Cell trajectory analysis

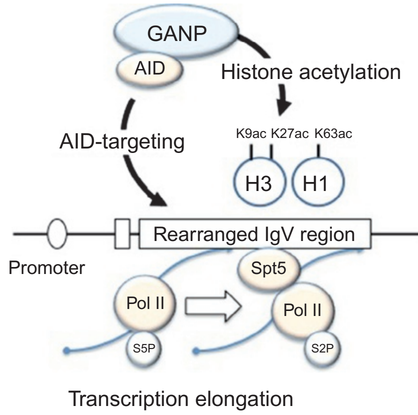
ISOMAP
Reserve local metric and embed cell in geodesic distance
Wong et al. *Cell Reports*, 2015.
- Phenotypic progression of human CD4^{+} T cells and TFH



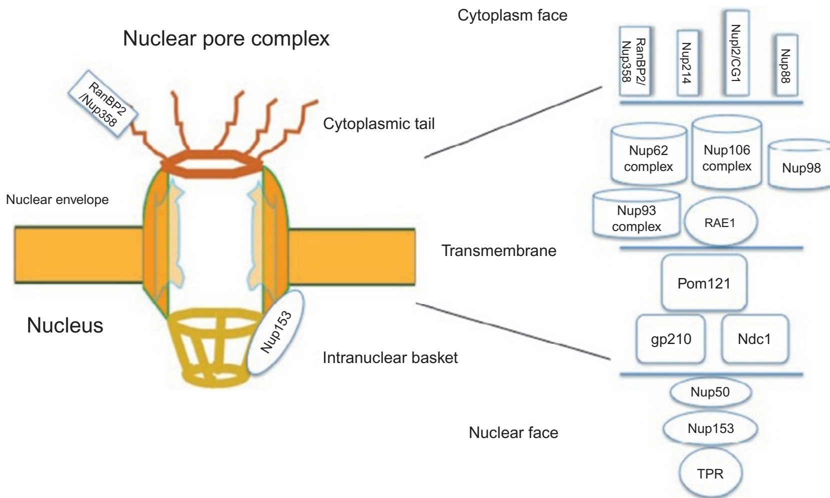
Diffusion map
Calculate all paths using diffusion distance
Haghverdi et al. *Bioinformatics*, 2015.



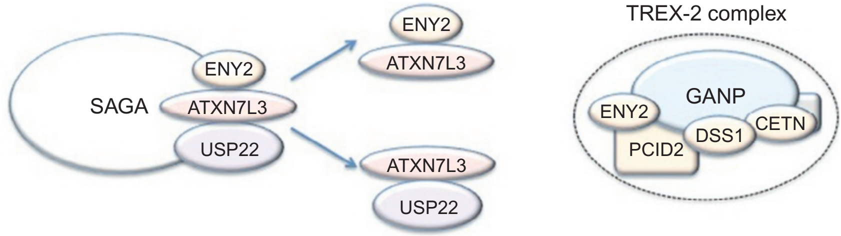
Chapter 3, Fig. 2 (See page 116 of this volume.)



Chapter 4, Fig. 1 (See page 149 of this volume.)



Chapter 4, Fig. 2 (See page 154 of this volume.)

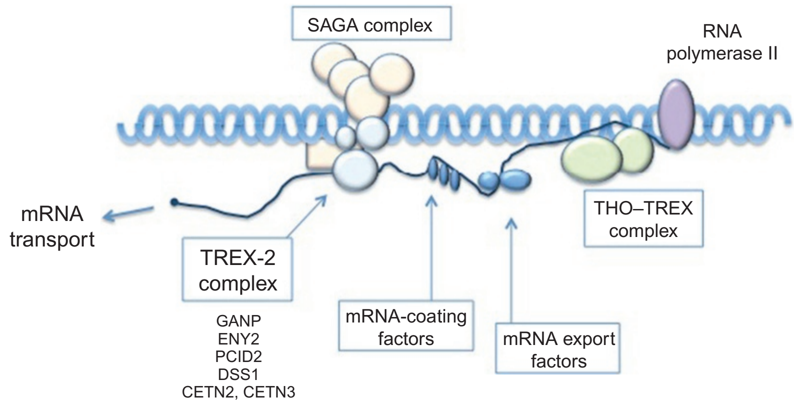


SAGA deubiquitin module

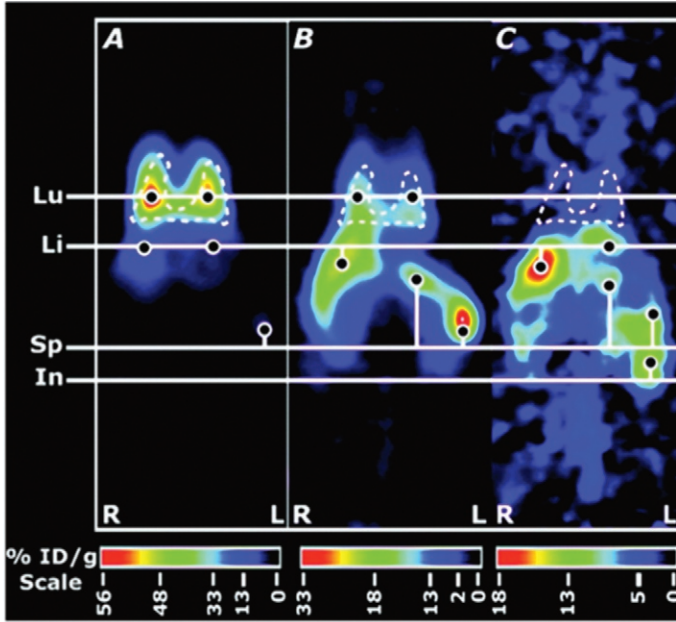
ATXN: ataxin
ENY2: human homologue of enhancer of yellow 2
USP22: ubiquitin-specific peptidase 22

Human	Yeast
GANP	ySac3
ENY2	ySus1
PCID2	yThp1
DSS1	ySem1
CETN2y	Cdc31
CETN3	yCdc31

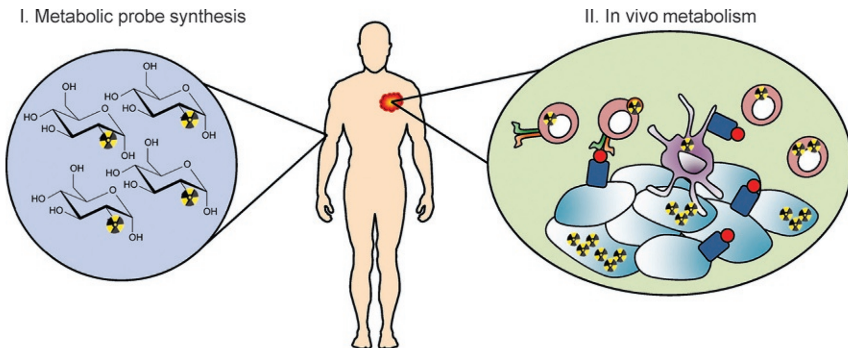
Chapter 4, Fig. 3 **(See page 158 of this volume.)**



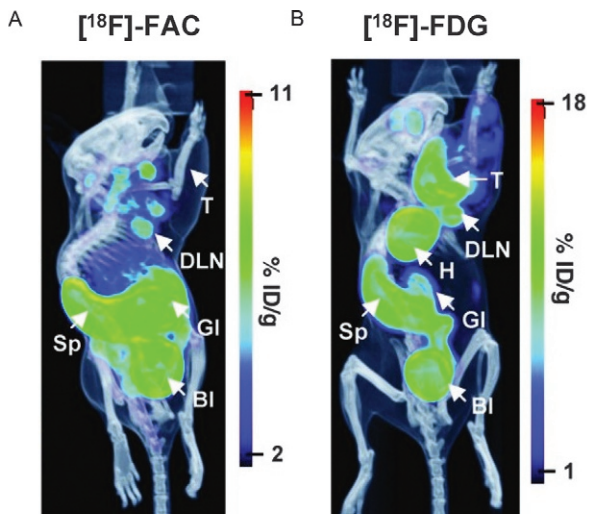
Chapter 4, Fig. 4 **(See page 167 of this volume.)**



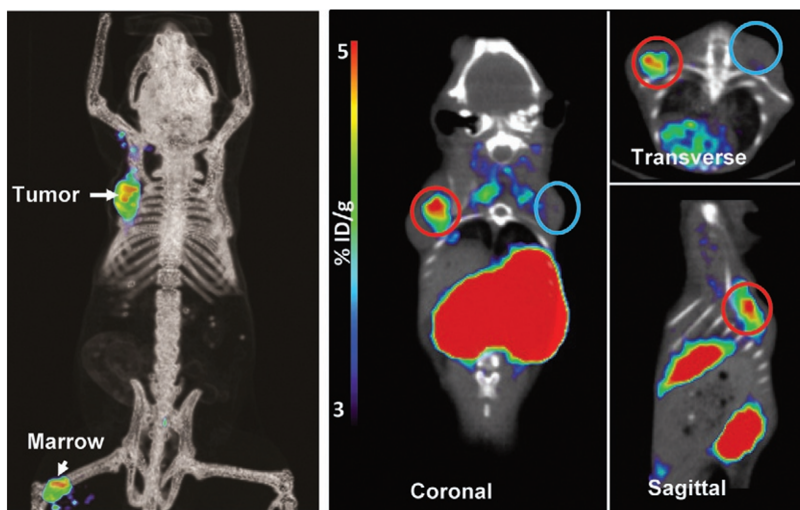
Chapter 5, Fig. 2 (See page 194 of this volume.)



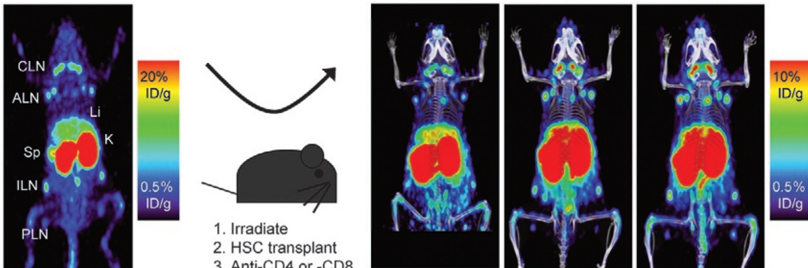
Chapter 5, Fig. 3 (See page 198 of this volume.)

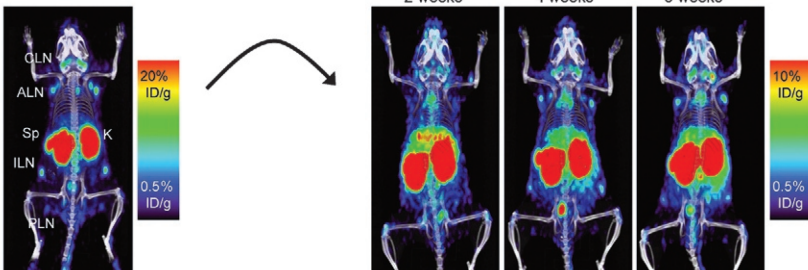


Chapter 5, Fig. 4 (See page 200 of this volume.)

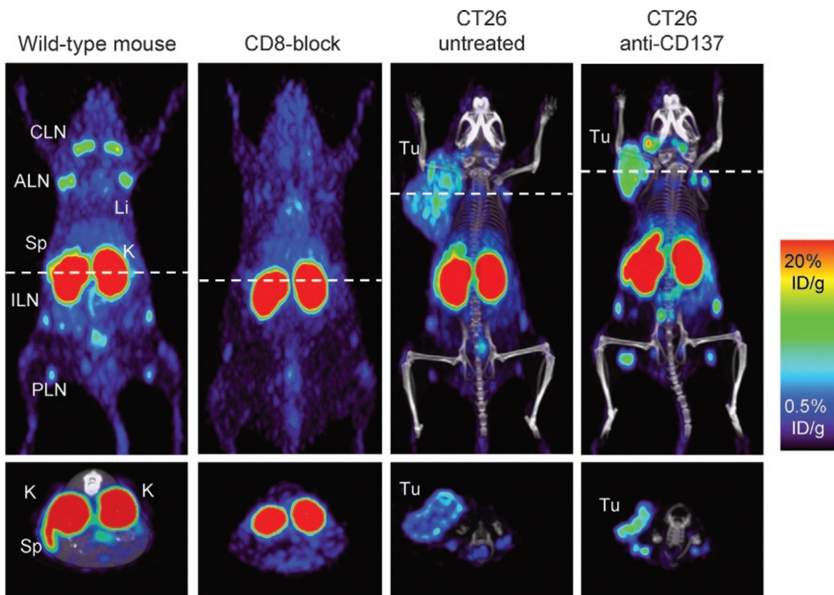


Chapter 5, Fig. 7 (See page 209 of this volume.)





Chapter 5, Fig. 10 (See page 218 of this volume.)



Chapter 5, Fig. 11 (See page 219 of this volume.)

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