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Pathogenic follicular CD8 T cells promote autoimmune disease.

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Quantitative and Systems Biology

by

Kristen Marie Valentine

Committee in charge:

Professor Marcos E. García-Ojeda, Chair

Professor Katrina K. Hoyer,

Professor Kirk D.C. Jensen

Professor Jennifer O. Manilay

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The Dissertation of Kristen Marie Valentine is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

Katrina K. Hoyer

_____

Kirk D. C. Jensen

_____

Jennifer O. Manilay

_____

Marcos E. García-Ojeda, Chair

University of California, Merced

2019

This work, as a culmination of my organized education is dedicated to my entire family, who have always supported by goals and dreams. Special dedication to my parents:

Dean M. Valentine

LeAnn W. Downing, and

Robert A. Downing, II

Thank you for giving me courage, strength and love. I wouldn't have even tried without you.

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LIST OF ABBREVIATIONS

AA, aplastic anemia

AIHA, autoimmune hemolytic anemia

Bcl6, B cell lymphoma 6 protein

BFA, brefeldin A

BMF, bone marrow failure

CBC, complete blood count

CD, cluster of differentiation

CD8 Tfc, CXCR5+PD-1+ CD8 effector T (CD8 T follicular)

CFA, complete Freund's adjuvant

CIA, collagen-induced arthritis

CTL, cytotoxic CD8 T cells

CTLA-4, cytotoxic T lymphocyte antigen 4

CXCR5, C-X-C motif chemokine receptor 5

dKO, double knockout

Fas^{lpr}, MRL/MpJ-Faslpr/J

FBS, fetal bovine serum

Foxp3, forkhead box p3

GC, germinal center

HBV, hepatitis B viral

HET, heterozygous

HIV, human immunodeficiency virus

i.p., intraperitoneal

ICOS, inducible T-cell co-stimulator

IFA, incomplete Freund's adjuvant

IFNγ, interferon gamma

IL, interleukin

IL-21R, B6N.129-Il21rtm1Kopf/J

IL-2-KO, IL-2 deficient

Imm. WT, KLH-immunized WT

KLH, keyhole limpet hemocyanin

KO, knockout

LCMV, lymphocytic choriomeningitis virus

LFC, log fold change

LN, lymph node

MDS, myelodysplastic syndromes

MFI, mean fluorescence intensity

non-Tfc, CD8 CXCR5-PD-1lo/-

non-Tfh, CD4 CXCR5-PD-1lo/-

NP, 4-Hydroxy-3-nitrophenylacetyl

O.C.T, optimal cutting temperature compound

OVA, ovalbumin

PBS, phosphate buffered saline

PCA, principal component analysis

PD-1, programmed cell death 1

PMA, phorbol 12-myristate 13-acetate

RBC, red blood cells

RNAseq, RNA sequencing

RT, room temperature

SA, streptavidin

scurfy aIL-2, scurfy mice treated with anti-IL-2 antibody

scurfy, BALB/c hemizygous male Foxp3sf/Y

scurfy-HET, heterozygous female Foxp3sf/+

SIV, simian immunodeficiency virus

SLE, systemic lupus erythematosus

Spl, spleen

Tc, CD8 T cytotoxic

TCF-1, T cell factor 1

Tem, T effector memory

Tfh, CD4 T follicular helper

Tfreg, T follicular regulatory cells

Th, CD4 T helper

Treg, T regulatory

UC, University of California

WT, wild type

LIST OF SYMBOLS

α, alpha; antibody against

β, beta

°, degree

ε, epsilon

γ, gamma

μ, micro

n, nano

+, positive expression

-, negative expression

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Chapter 1: Introduction

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Chapter 3:

Authors: Kristen M Valentine, Genevieve N. Mullins, Oscar A. Davalos, Travis J Lawrence, Katrina K Hoyer.

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CURRICULUM VITA

EDUCATION AND RESEARCH

2010-2014	Bachelor of Science, Human Biology University of California, Merced School of Natural Sciences
2011-2013	University of California, Merced School of Natural Sciences Laboratory of Dr. Monica Medina
2013-2014	University of California, Merced School of Social Sciences, Humanities and Arts Laboratory of Dr. Jeffery Gilger
2014-2019	Doctor of Philosophy, Quantitative and Systems Biology University of California, Merced School of Natural Sciences Laboratory of Dr. Katrina Hoyer

GRANTS, FELLOWSHIPS AND AWARDS

2014-2018	Teaching Assistant School of Natural Sciences University of California, Merced
2015	QSB Summer Research Fellowship University of California, Merced
2015	Aplastic Anemia (AA) and Myelodysplastic syndromes (MDS) International Foundation Summer Fellowship
2016	Graduate Immunology Research Fellowship University of California, Merced
2017	QSB Summer Research Fellowship University of California, Merced
2018-2019	Fletcher Jones Fellowship University of California, Merced
2018-2019	Presidential Dissertation Year Fellowship University of California

PUBLICATIONS AND PRESENTATIONS

Valentine KM, Díaz-Almeyda E, Medina M. *Symbiodinium* Selection Effects on *Montastraea faveolata.* Abstract presented as a poster at the UC Merced Research Week (2012). Merced, CA

Valentine KM, Díaz-Almeyda E, Medina M. *Montastraea faveolata* Fitness During the Onset of Symbiosis. Abstract presented as a poster at the Society for Developmental Biology West Coast Conference (2013). Cambria, CA

Valentine KM, Gravano DM, Hoyer KK. Defining Antigen in Systemic Autoimmune Disease. Abstract presented as a poster at the UC Merced Research Week (2015). Merced CA

Valentine KM, Gravano DM, Hoyer KK. Defining Antigen in Systemic Autoimmune Disease. Abstract presented as a poster at the UC Merced Annual QSB Spring Retreat (2015). Yosemite CA

Valentine KM, Gravano DM, Isakson SH, Davis JK, Cha H, Sindi SS, Hoyer KK. Dysregulated CD8+ T cells resemble Tfh-like cells to promote B cell activation during autoimmunity. Abstract presented as a poster at the UCSF and UC Berkeley Immunology Retreat (2015). Asilomar, CA

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PATHOGENIC FOLLICULAR CD8 T CELLS PROMOTE AUTOIMMUNE DISEASE.

by

Kristen Marie Valentine

Doctor of Philosophy in Quantitative and Systems Biology

University of California, Merced, 2019

Professor Katrina K. Hoyer

The contribution of CD8 T cells to autoimmune disease remains in debate. We show autoimmune CD8 T cells which induce antibody class switching and plasma cell differentiation act synergistically with CD4 T cells in promoting germinal center reactions. We have identified CXCR5+PD-1+ CD8 effector T (CD8 T follicular; Tfc) cells, within the germinal center, that expand during late autoimmune disease progression. We show that CD8 Tfc cells transcriptionally and phenotypically resemble CD4 T follicular helper (Tfh) cells in multiple models of spontaneous autoantibody-mediated disease including IL-2 deficient (IL-2-KO), scurfy and MRL/MpJ-FAS^{lpr} mice. CD8 Tfc cells maintain the capacity to produce significant amounts of cytotoxic proteins granzyme B, CD107a and TNFα, and helper-associated cytokines IL-21, IFNγ and IL-4. Functionally, CD8 Tfc cells promote cytokine-mediated antibody class switch using mechanisms largely independent of IFNγ or IL-21. When adoptively transferred CD8 Tfc cells in combination with CD4 Tfh cell promote autoantibody production. Our results indicate that CD8 Tfc cells contribute to autoimmune disease synergistically with CD4 Tfh cells to induce B cell class switching and autoantibodies during disease progression. Autoimmune disease is a novel immune setting during which CXCR5+ CD8 T cells develop beyond situations of chronic viral infection and cancer. Thus, pathogenic CD8 T cells influence germinal center reactions and promote autoimmune responses.

CHAPTER 1

Background and historical context

CHAPTER 1: BACKGROUND AND HISTORICAL CONTEXT

AUTOIMMUNITY AND IL-2

Autoimmune diseases, combined, affect more than 5% of the world population (1). Autoimmune diseases (such as diabetes or rheumatoid arthritis) are a group of often disabling illnesses that develop when the immune system attacks normal body tissues. Both CD4 helper and CD8 cytotoxic T cells are involved in the initiation and promotion of autoimmune disease. CD4 T helper (Th) cell subsets including Th1, Th2, Th17, T follicular helper (Tfh), and T regulatory (Treg) cells, arise and contribute to multiple autoimmune diseases (2). Similarly, although less well-described, CD8 T cytotoxic (Tc) cell subsets, including Tc1, Tc2, Tc17, and Treg cells, influence autoimmune disease (3). Despite this emerging assessment of T cell responses, a defined role for self-reactive auto-antigen specific T cells remains elusive. A breakdown of functional tolerance mechanisms allows for self-reactive responses to develop, including dysfunctional thymic tolerance mechanisms during T cell development (4), and the reduction or absence of functional Tregs in the periphery (5).

The discovery of Foxp3+ Tregs that suppress inappropriately activated self-reactive T cells provides another platform to evaluate autoimmune control mechanisms and therapeutics. During development, Tregs require IL-2 cytokine signaling via STAT5 to upregulate the transcription factor Foxp3 (6, 7). Once generated, Treg cells employ multiple mechanisms to maintain self-reactive responses, including cytotoxic T lymphocyte antigen 4 (CTLA-4) co-inhibitory interactions, IL-10 and TGFβ cytokine mediated suppression, and by controlling IL-2 availability to prevent IL-2-induced T cell activation (8). In the absence of IL-2, fewer functional Tregs develop, releasing control of self-reactive T cell allowing autoimmunity (9).

A number of autoimmune diseases are associated with defects in IL-2 and IL-2Rα cytokine signaling (10). In murine models, the absence of IL-2 or Foxp3 allows systemic autoimmune disease to develop (7, 11). This dissertation evaluates multiple models of autoimmune disease with particular emphasis on BALB/c IL-2-deficient (IL-2-KO) mice. In the absence of functional Treg cells, these mice develop systemic lymphoproliferation, autoimmune hemolytic anemia (AIHA), and bone marrow failure (BMF) associated with increased activation and proliferation of B and T cells (12-14).

Autoantibodies binding to red blood cells (RBC) drives RBC destruction via Fc-mediated phagocytosis, and this loss of mature RBCs in combination with dysfunction in the hematopoietic stem cell compartment during BMF facilitates rapid autoimmune disease and death. In the absence of IL-2, IL-7 promotes T cell survival and proliferation in IL-2-KO mice (15). BMF is mediated by CD4 T cell responses but is enhanced when combined with CD8 T cells (14). AIHA is similarly regulated by CD4 and CD8 T cells via IFNγ (16). Co-stimulators CD28 and CD40L regulate disease pathology; CD28 likely

acts during T cell activation promoting lymphoproliferation, and CD40L interactions between T and B cells act to promote autoantibody responses (17).

GERMINAL CENTER REACTIONS

During an immune response, CD4 Tfh cells are required for B cell germinal center reactions by promoting antibody production, B cell maturation and class switching (18). CD4 Tfh cells upregulate PD-1, ICOS, the germinal center chemokine receptor CXCR5, and their principal transcription factor Bcl-6. Together this expression licenses the CD4 Tfh to enter the B cell zone and upregulate the expression of CD40L, FasL, and IL-21 to facilitate B cell responses (19). A specialized germinal center localized Treg subset expressing Foxp3 and Bcl-6, T follicular regulatory cells (Tfreg), control germinal center size, Tfh accumulation, T-B interactions and antibody production (20-22). In the absence of Tfreg, Tfh cells accumulate and B cell responses become less antigen specific (23, 24). Within an autoimmune setting, CD4 Tfh cells promote autoantibody production similar to normal infection responses. CD4 Tfh cells interact with B cells to promote autoantibody production to ubiquitous antigen (25). CXCR5+ Tfh cells are required for autoantibody production and the progression of rheumatoid arthritis in mouse models (26). In humans, circulating CD4 Tfh promote autoantibody production via CD40L interactions in lupus (27).

Only recently have CD8 T cells been recognized as players in germinal center reactions (28). However, the capacity for follicular CD8 T cells to promote pathogenesis versus protection during an immune response remains to be resolved. To address the gap in knowledge, we assessed CD8 T cell capacity to enhance or block antibody-mediated disease. This dissertation demonstrates that CD8 T cells enhance and promote antibody responses associated with AIHA.

CXCR5+ CD8 T CELLS

Introduction

As CD8 T cells have been identified both phenotypically and functionally into distinct subsets beyond that of the classical cytotoxic CD8 T cells (CTL), it follows that novel CD8 T cell subsets may yet still emerge. A recent focus on CD8 T cells has highlighted a diversity of functional responses. Like CD4 T cells, CD8 T cells differentiate into multiple subsets that are customized to a specific infection and immune settings (3, 29) (Figure 1). CD8 Tc1 cells comprise the canonical CTL subset, producing IFNγ, perforin, and granzymes involved in targeted cell killing (30). CD8 Tc1 cells arise predominately in response to viral and intracellular infections but also in some autoimmune diseases to induce pathogenic tissue destruction. CD8 Tc2 cells are implicated in response to specific allergens and typically exhibit reduced CTL function

and produce IL-4 and IL-5. CD8 T regulatory cells (Tregs) identified in the context of self-reactive responses are less well defined and may have multiple phenotypes (31, 32). Some CD8 Tregs localize to the B cell zone but are also found in circulation (33-35). Beyond these effector subsets, at least three memory CD8 T cell types (T effector memory (Tem), tissue resident memory, and central memory) have been extensively described (36).

This thesis characterizes a novel subset of CXCR5+ CD8 T cells capable of infiltrating the B cell follicle in settings of chronic antigen exposure and inflammation. CXCR5+ CD8 T cells maintain an independent phenotype from their CXCR5- CD8 T cell counterparts. Their functional role largely depends on the immune setting, yet they maintain a cytotoxic capacity that aids in the control of viral infection, tumor growth inhibition, or the promotion of inflammation and autoimmune responses. Finally, CXCR5+ CD8 T cells have a unique developmental profile, utilizing genes similar to both CD4 Tfh cell development and CD8 effector memory or memory-stem cell differentiation. We propose that some of the gene variation found in CXCR5+ CD8 T cells is dependent upon the conditions under which these cells arise.

Part I: A novel T cell subset: CXCR5+ CD8 T cells.

CXCR5+ CD8 T cells develop under several conditions of chronic antigen and inflammation. They are transcriptionally and phenotypically distinct from other CD8 T cell subsets. Most studies find that CXCR5+ CD8 T cells gain entry into the B cell follicle (28). Yet, there is no clear consensus defining the function of this CXCR5+ CD8 T cell subset.

A distinct phenotype

Transcriptional and phenotypic profiling, in combination with a variety of functional responses, indicate several possible classifications for CXCR5+ CD8 T cells including: cytolytic, Tem/stem cell, exhausted, and follicular helper CD8 T cells (28, 35-37). The differences leading to these classifications likely depend on the particular immune setting and the subsequent functional responsiveness of CXCR5+ CD8 T cells.

During chronic viral infections, CD8 T cells are frequently associated with an exhausted profile including reductions in IL-2 production, cytolytic function and proliferation. This shift toward exhaustion is associated with increased expression of the co-inhibitory molecules PD-1, 2B4, Tim3, KLRG1, CD160, and Lag3, among others (37). When CXCR5+ CD8 T cells were evaluated for an exhausted phenotype, gene expression profiles reveal that CXCR5+ CD8 T cells have increased *pdcd1* (PD-1) expression but reduced *cd95l* (FasL), *ctla4*, *lag3*, *havcr2* (Tim-3), and *cd244* (2B4) in some studies (38-41). In other studies, CXCR5+ CD8 T cells express elevated PD-1 and FasL with variable CTLA-4, Lag3 and Tim-3, but reduced 2B4 expression (40-44) (Figure 2A). Cytolytic functionality, as measured by granzyme B, perforin, and CD107a expression, provides a mixed picture for CXCR5+ CD8 T cells as a non-exhausted population. CXCR5+ CD8 T cells express decreased *grzma*, *grzmb*, and *prf1* gene

expression when compared to CXCR5- CD8 T cells in viral infection (40). Yet, tumor-infiltrating and virus-specific CXCR5+ CD8 T cells appear to maintain cytolytic capacity upon ex vivo stimulation (41, 43, 45). However, considering the variability in exhaustion marker expression as well and the maintenance of cytolytic capacity (described in Section II of this chapter), CXCR5+ CD8 T cells are likely not functionally exhausted. Specifically, CXCR5+ CD8 T cells express elevated KLRG1, CD44, T-bet, and Blimp-1 compared to CXCR5- and naïve CD8 T cells indicative of an activated, fully differentiated cytolytic subset (40-42) (Figure 2B).

While CXCR5+ CD8 T cells appear to maintain a cytolytic phenotype, this phenotype does not account for the upregulation of *cd127* (IL-7Rα), *tcf7* (TCF-1), *id3*, *eomes*, and *cd44* that are commonly associated with an effector memory phenotype (40, 41) (Figure 2C). Im et al. defined lymphocytic choriomeningitis virus (LCMV)-specific CXCR5+ CD8 T cells as stem-like CD8 Tem that proliferated into both CXCR5+ and CXCR5- CD8 T cell subsets (40). Similarly, CXCR5+ CD8 T cells isolated from PBMCs of cancer patients proliferate more than CXCR5- CD8 T cells after TCR stimulation (44, 45). Leong et al. defined CXCR5+ CD8 T cells in LCMV infection as an effector memory-like (CD62L+ IL-7R+) population by RNA sequencing (41). Perhaps, most convincingly, in SIV infection CXCR5+ CD8 T cells and CD8 T cells under autoimmune conditions express significantly more *bcl6* and less *prmd1* (Blimp-1) (38, 46). The Tem phenotypic description attributed to CXCR5+ CD8 T cells is probably indicative of the chronic antigen exposure under which these cells have thus far shown to arise.

Alternatively, although not completely counter to evidence of an effector memory subset, CXCR5+ CD8 T cells share a transcriptional profile similar to that of CD4 Tfh cells in SIV infection as shown by RNA sequencing of virus specific CXCR5+ CD8 T cells (38). CXCR5 is most commonly associated with B cell zone migration and homing and has been described extensively on B cells and CD4 Tfh cells (47). CXCR5+ CD8 T cells express costimulatory, transcription factors, inhibitory genes and proteins similar to CD4 Tfh, including: increased *cd200*, *icos*, *cd28*, *bcl6*, *id3*, *ctla4*, *pdcd1* and *cd95l* (FasL) and reduced *prdm1*, *id2*, and *havcr2* (Tim-3) (19, 38, 40-42) (Figure 2D). These data are supported by seminal research in the inflammatory environment of human nasal polyps, in which a population of CXCR5+ CD8 T cells expressing FasL, CD28, OX-40 and ICOS post-ex vivo stimulation (48). While in an autoimmune disease setting, CXCR5+ CD8 T cells express cytolytic molecules associated with canonical CD8 T cell function but also express *cxcr5*, *icos*, *bcl6*, *pdcd1*, *cd40l* and *il21* (46). More specifically, in Hodgkin lymphomas, a set of genes identified by RNA sequencing, commonly upregulated by CXCR5+ ICOS+ CD8 T cells and CD4 Tfh cells are sufficient to cluster CXCR5+ICOS+CD8 T cells with CD4 Tfh and not other T cell subsets (49).

A CD8 Treg population that maintains germinal center (GC) reactions and controls autoimmune disease has been described within the B cell follicle (33). CD8 Tregs can express FoxP3 or a transcription factor Helios as described on non-canonical Qa-1 restricted CD8 Treg (32, 35). When identified as CXCR5+, CD8 Tregs express ICOSL, CD44 and CD122 (31, 33) (Figure 2E). He et al. reported that in LCMV infection, CXCR5+ CD8 T cells were ICOSL and Helios deficient, but CD44+ (42).

Similarly, in the context of autoimmune disease, CXCR5+ CD8 T cells largely lack ICOSL, FoxP3, and Helios expression (Valentine and Hoyer, unpublished data). It is possible that CXCR5+ CD8 T cells in some situations are CD8 Treg cells (34) but most reports suggest an effector phenotype for these cells. Together, transcriptional profiling and subsequent validation by flow cytometric analysis of protein expression, identify a CXCR5+ CD8 T cell population with the potential to behave as cytotoxic canonical CD8 T cells, promote CD4 Tfh helper type responses and respond as CD8 Tem (Table 1).

Antigen-specific CXCR5+ CD8 T cell responses and localization

As the principal chemokine receptor that facilitates entry into the B cell zone. CD8 T cells, by CCR7 upregulation and the absence of CXCR5 expression, in some instances are excluded from the B cell follicle (50). However, under particular immune activating conditions this expression pattern changes. CXCR5 expression on CD8 T cells is closely associated with proximity and responsiveness to CXCL13 in the lymph node (43, 48). CXCR5 expression on CD4 Tfh is required to migrate towards CXCL13 and facilitate GC development (51). Some CD4 Tfh developmental signals do not require B cell help initially to induce the CD4 Tfh transcription factor, Bcl6 (52, 53). However, once at the B-T border, CD4 Tfh cells interact with B cells to gain entry into the GC and solidify their transcriptional profile via Bcl6 using ICOS and PD-1 interactions (27, 54, 55). CXCR5+ CD8 T cells may also require B cell interactions to enter the follicle, as CXCR5+ CD8 T cells that maintain *ccr7* gene expression retain their capacity to localize to the T cell zone and are excluded from the GC (40). A requirement for B-T cell interaction has yet to be directly investigated in CXCR5+ CD8 T cell development and function.

CXCR5+ CD8 T cell accumulation in the follicle does not appear to be dictated by antigen concentration, but rather by conditions of chronic inflammation and immune activation (43, 46, 48). In chronic infections, both high and low viral load correlate with expanded CXCR5+ CD8 T cell populations in the GC (50, 56, 57). Chronic human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and LCMV infection studies identified antigen-specific CXCR5+ CD8 T cells (38, 40-42, 58). Peripheral blood and lymphoid extrafollicular CXCR5+ CD8 T cells maintain a robust cytolytic phenotype (40, 42, 58, 59) in comparison to lymphoid populations (60), whereas CXCR5- CD8 T cells are excluded from the B cell zone and are more likely to be exhausted (40-42) (Figure 3A).

Interaction time and specific signals during GC interactions may redirect the transcription of CXCR5+ CD8 T cells and alter effector functions as described for CD4 Tfh cells (61). In HIV infection, some CXCR5+ CD8 T cells demonstrate high lytic potential in GC regardless of antigen specificity (43), while other antigen-specific CXCR5+ CD8 T cells are less lytic (59). The frequency of CXCR5+ CD8 T cells isolated from pancreatic and colorectal cancer tumor masses correlate with improved patient outcomes suggestive of tumor control (39, 62). Viral-specific or tumor infiltrating CXCR5+ CD8 T cells likely utilize cytolytic mechanisms to control viral infection and tumor growth in secondary lymphoid organs, ectopic GC and the tumor microenvironment.

The frequency of antigen-specific CXCR5+ CD8 T cells within the follicle and extrafollicular space are comparable (41). The presence of antigen-specific CD8 T cells in the extrafollicular space and at the B-T border suggests that CXCR5+ CD8 T cells directly interact with virally infected CD4 Tfh cells. The potential for cytolytic responses may explain the negative correlation observed between the frequency of CXCR5+ CD8 T cells and reduced CD4 Tfh cell frequency and viral load (38, 41, 43, 56, 60) (Figure 3B).

In the context of chronic inflammation and autoimmune disease, CXCR5+ CD8 T cells likely employ diverse mechanisms to promote inflammatory responses and advance disease pathogenesis at the site of autoreactive responses within ectopic GCs or lymphoid tissue. Influenza-specific CD8 T cells migrate to lung ectopic GCs and interact with B cells following intranasal infection (44, 63). CD40L+ CD8 T cells within the synovial fluid, that are likely antigen-specific for joint proteins, are required for the formation and maintenance of ectopic GCs in rheumatoid arthritis inflammation (64, 65). Autoimmune CXCR5+PD-1^{hi} CD8 T cells expressing CD40L and GL-7 promote antibody responses (46). CXCR5+ CD8 T cells in human nasal polyps that localize to the B cell zone promote inflammatory damage (66). Together, these data reveal a pattern of CXCR5+ CD8 T cell homing related to antigen accumulation and the site of local immunological responses (Figure 3C).

Part II: CXCR5+ CD8 T cell function.

Viral infection:

CXCR5+ CD8 T cells have been predominately explored in the context of chronic viral infections. In chronic LCMV and SIV infection, CXCR5+ CD8 T cell frequency inversely correlates with viral load and associates with a reduction in virus-producing cells attributing cytolytic function to CXCR5+ CD8 T cells (41, 42, 50, 56, 58). However, in similar studies of chronic SIV and LCMV infection, CXCR5+ CD8 T cells display reduced cytolytic protein expression coupled with a more stem-like effector memory phenotype (40).

There are a number of possible explanations for differences in cytolytic activity across the existing viral CXCR5+ CD8 T cell literature (67). Emerging evidence indicates an effector memory-like CD8 T cell population that develops in situations of chronic antigen and cell exhaustion, with the capacity for cytolytic function. Inhibitory receptors such as PD-1, frequently used to describe exhausted CD8 T cells, may also denote a follicular-helper like subset of CD8 T cells that maintains its cytotoxic effector function and elicits GC entry (43, 59). Adoptively transferred LCMV-specific CXCR5+ CD8 T cells rapidly expanded to reseed the exhausted CXCR5- CD8 T cell niche (40) and significantly reduced viral load following PD-1 blockade (42). Paradoxically, during short PD-1 blockade treatments in HIV infection, a PD-1+ subset of CXCR5+ CD8 T cells, instead produced less TNFα and IFNγ cytokines (59). Within the CXCR5+ population there likely exists multiple effector functions similar to differences observed in CD4 Tfh cell function as it relates to PD-1 expression, follicular localization, and terminal differentiation.

In addition to a cytolytic role in controlling infection, direct interactions with infected B cells and CD4 Tfh cells may also facilitate CD8 T helper-like functions in the follicle. Human CXCR5+ CD8 T cells from chronic hepatitis B viral (HBV) infection produce IFNγ and influence IgG and IgA production when co-cultured with naïve B cells or memory B cells (68). CD8 T cells infiltrate influenza infected lungs and promote IL-21 dependent antibody class switching and prolonged B cell survival (63). This follicular helper type function may also act to promote a tissue specific antiviral response on CD4 Tfh cells differing from the cytolytic response facilitated by chronic viral infection reservoirs in secondary lymphoid organs.

Cancer:

Cancer represents a situation of chronic, low-level self-antigen much like the situation induced by chronic viral infection. In B cell lymphoma-bearing mice and diffuse large B cell lymphoma patients, CXCR5+ CD8 T cells likely arise to directly target cancer cells (41, 69). Whereas, in HBV-related hepatocellular carcinoma, viral responses may initially induce CXCR5+ CD8 T cells that then target cancer cells (44). In colorectal and pancreatic cancer, CXCR5+ CD8 T cells arise and respond to cancer cells (39, 45, 62) suggesting a prevalent role for chronic antigen exposure in the development of tumor-specific CXCR5+ CD8 T cells.

CXCR5+ CD8 T cells isolated during immune responses to cancer maintain cytolytic potential toward tumor cells despite protein expression typically indicative of exhaustion. Circulating CXCR5+ CD8 T cells isolated from patients with HBV-related hepatocellular carcinoma and diffuse large B cell lymphoma expressed granzyme B and CD107a that likely contributed to tumor cell and B cell lysis (44). Circulating, tumor infiltrating and lymphoid CXCR5+ CD8 T cells also express PD-1 and Tim-3 but are functionally less exhausted than CXCR5- CD8 T cells (39, 44). Yet, combined blockade of Tim-3 and PD-1 augment CXCR5+ CD8 T cell specific lysis of tumor cell targets indicating reduced lytic potential (44, 62). Further, CXCR5+ CD8 T cells in colorectal cancer maintain a cytolytic capacity to directly lyse tumor cells but can also influence B cell secretion of IgG, suggesting multiple mechanisms for tumor control by these cells (45).

In spite of the fairly robust cytolytic potential and activity by CXCR5+ CD8 T cells, tumor cells likely employ inhibitory mechanisms to suppress CXCR5+ CD8 T cell function. In vitro neutralization of IL-10 or IL-10R pathway improved granzyme A, granzyme B, and perforin-mediated cytotoxicity by CXCR5+ CD8 T cells (69). IL-10 or PD-1L blockade induced CXCR5+ CD8 T cell targeted specific cell lysis of autologous tumor cells (44). Enhancing specific cell lysis by preventing tumor suppression of CXCR5+ CD8 T cells or by improved CXCR5+ CD8 T cell function provides a new potential target for existing cancer therapeutics. As disease-free survival time in pancreatic and colorectal cancer is positively correlated with CXCR5+ CD8 T cell frequency (62), the maintenance of a CXCR5+ CD8 T cell population may prolong cancer treatment efficacy.

Inflammation and Autoimmune Disease:

The mechanisms by which CD8 T cells mediate autoimmune disease pathology remain largely unresolved, but inflammation and autoimmune disease studies suggest a helper function for CXCR5+ CD8 T cells. In the absence of CD8 T cells, GC formation is prevented in rheumatoid arthritis and disease is delayed in spontaneous auto-antibody mediated disease (46, 65). Differential synovial ectopic GC formation is associated with CD8 T cell recruitment in a CD40L dependent manner (64), and follicular dendritic cells could not be retained in synovial GCs grafted into NOD-SCID mice in the absence of CD8 T cells (65).

While CD40L+ CD8 T cells appear to have a role in mediating ectopic GC formation, they do not produce the cytolytic proteins perforin and granzyme A, but maintain expression of IFNγ and TNFα (65). CXCR5+ CD8 T cells identified in human tonsils express IFNγ, TNFα, granzyme A and IL-2 (48). Similar to CXCR5+ CD4 T cells, human tonsil CD8 T cells co-cultured with B cells promoted B cell survival and induced IgG class switching (48). IL-21-producing CD8 T cells from human nasal polyps co-express IFNγ and IL-21 to induce B cell class switch to IgG when co-cultured with B cells (66). IFNγ is a known mediator of B class switch to IgG2a/c, yet CXCR5+ CD8 T cells that arise in spontaneous autoimmune disease induced B cell class switch to predominately IgG1. When transferred into TCRα deficient mice, IL-2-deficient CD8 T cells alone did not induce B cell differentiation or class switching. Instead, CD8 T cells together with CD4 T cells enhanced plasma cell differentiation and induced IgG1 and IgG2b expression (46).

Whether CD8 T: B cell interactions are driven primarily within the GC or in extrafollicular foci, and whether the mechanisms promoting antibody class switch result from direct contact or secreted cytokines require additional exploration. Although yet untested, CXCR5+ CD8 T cell function in autoimmune disease likely includes canonical cytotoxic mechanisms in addition to acquired Tfh mechanisms. In contrast to most chronic viral infections and cancer, autoimmune and inflammatory CXCR5+ CD8 T cells likely promote the disease state, although the mechanisms that alter or advance GC reactions, in addition to direct cell lysis, may be similar.

Part III: Distinct developmental pathways for CXCR5+ CD8 T cells.

The similarity of CXCR5+ CD8 T cell phenotype and function to other CD8 T cell subsets described in Figure 2 likely provide overlapping, if not identical models, for differentiation and function of these cells described in other excellent reviews (28, 36, 37). Here, we propose a transcriptional network that explains the gene expression and function observed in CXCR5+ CD8 T cells (Figure 4).

To explore CXCR5+ CD8 T cell regulation, the cxcr5 promoter has been evaluated by chromatin immunoprecipitation deep sequencing. The *cxcr5* promoter contains two Blimp-1 binding sites and one E2A binding site, in addition to binding sites at the bcl6 and tcf7 promoter. CXCR5+ CD8 T cells under conditions of chronic viral infection are permissive for E2A binding and more differentially expressed genes bound by E2A and TCF-1 similar to that of CD8 effector memory T cells and CD4 Tfh cells

(41, 42, 70). Retroviral Bcl6 induction of LCMV-specific donor cells increased CXCR5+ CD8 T cell frequency with a corresponding TCF-1 upregulation and Blimp-1 repression (41), suggesting a regulatory connection between TCF-1 and Bcl6 antagonism of Blimp-1 expression (53, 71). Further, CXCR5+ CD8 T cells do not arise in the absence of *tcf7* (TCF-1) during LCMV infection (40, 41). Experiments to test the significance of Blimp-1 regulation in CXCR5+ CD8 T cells using mixed bone marrow chimeras of Blimp-1 deficient : WT showed that CXCR5+ CD8 T cells preferentially expand indicating that, like CD4 Tfh cells, CXCR5 expression is in part regulated by the absence of Blimp-1 mediated transcription (41).

Id2 and Id3 regulate E2A and other e-family proteins responsible for regulating gene transcription in CD4 T cells (70, 72). E2A overexpression enhances CXCR5 expression increasing cytotoxic responses via CD107a expression and PD-1 downregulation, producing a less exhausted phenotype (42). In CXCR5+ CD8 T cells, Id2 is downregulated and Id3 is upregulated relative to CXCR5- CD8 T cells (40-42). T cell specific deletion of Id2 results in a dramatic expansion of CXCR5+ CD8 T cells (41, 42, 73). Because Id2 is significantly downregulated in CXCR5+ CD8 T cells, the expression of Id2 may block the development of CXCR5+ CD8 T cells during early CD8 T cell activation. Id3 upregulation in CXCR5+ CD8 T cells may restrain CXCR5+ CD8 T cell development, perhaps after initial subset differentiation. Thus, Id2 restricts, and Id3 maintains, E2A induction of Bcl6, TCF-1 and CXCR5 to stabilize the CXCR5+ CD8 T cell phenotype (Figure 4).

Early immunological signals that prompt development of a CXCR5+ CD8 T cell population remain in question. Some evidence for specific cytokine and cellular interactions exists but is largely circumstantial via in vitro culture assays. In vitro cultures of SIV+ CD8 T cells with IL-12, IL-23 and TGFβ promote CXCR5+ CD8 T cell expansion relative to IL-12 or IL-23 alone (38). When cultured with IL-6, CD8 T cells produce IL-21 similar to CD4 T cells cultured with IL-6 (63). However, in CD8 T cells IL-6 induction of IL-21 via STAT3, inhibits IFNγ and IL-2 production (63), unlike the robust IFNγ responses in LCMV-specific CXCR5+ CD8 T cells. Although, thus far, CXCR5+ CD8 T cells respond to similar stimuli as CD4 Tfh cells (74-76), these stimuli may induce a context specific response in CXCR5+ CD8 T cells that has yet to be carefully resolved.

In the context of chronic antigen, CD4 Tregs control inappropriate self-reactive responses (20). Within the GC, follicular Tregs maintain T-B interactions to promote B cell differentiation (77). Foxp3+ Tregs localize in close proximity to follicular and extrafollicular CXCR5+ CD8 T cells, but with higher frequency to extrafollicular CXCR5+ CD8 T cells (58). During low SIV viremia, CXCR5+ CD8 T cell frequency is negatively correlated with viral load and positively correlated with follicular Tregs. Whereas in high SIV viremia, the frequency of CXCR5+ CD8 T cells is negatively correlated with follicular Tregs. Together, this suggests that Treg control of CXCR5+ CD8 T cells inhibits function rather than development within the GC, and the efficacy of that inhibition likely relates to viral control (56).

CD8 T cells can be found within the GC of several murine models of spontaneous autoimmune disease including in IL-2-deficient and scurfy mutant autoimmune disease. In these mice, both CD4 Tfh and CXCR5+ PD-1+ CD8 T cells are significantly expanded (46). One common feature of these autoimmune models is a defect in functional Tregs. In the absence of functional Tregs or in conditions of high chronic antigen and inflammation, CXCR5+ CD8 T cells have the capacity to expand and maintain robust effector function by cytokine secretion or direct B cell interactions.

Conclusions.

CXCR5+ CD8 T cells have been found under a number of pathogenic conditions with varied functional capacity. CXCR5+ CD8 T cells promote cell lysis in viral infection as well as in some cancers, while in inflammation and autoimmunity CXCR5+ CD8 T cells function as helper cells, thus promoting disease pathogenesis. The presence of CD8 T cells within the B cell zone, in combination with their cytolytic and helper functionality, provides the potential for unique interactions with CD4 Tfh cells, B cells and follicular dendritic cells and access to infected CD4 T cells and cancerous B cells that have yet to be fully explored.

Treatments to influence effector responses require a clear analysis of CXCR5+ CD8 T cell function in multiple immune settings that facilitate specific cell interactions. Engineering CD8 T cells to express CXCR5 promotes migration to the B cell follicle (81). While the use of bispecific antibodies optimizes CXCR5+ CD8 T cell targeting of HIV-infected cells via cell specific lysis (43). A combination therapy to optimize CXCR5+ CD8 T cell responses in a patient specific manner will address challenges currently identified in immune non-responding patients to existing HIV treatments. CXCR5+ CD8 T cell activities within and near the follicle provide clues about the immune response that may explain class switch choices, the development of broadly neutralizing antibodies, and promote a paradigm shift in the nuances of GC reactions.

SUMMARY

In this dissertation, we seek to understand the contribution of CD8 T cells to autoimmune disease in the absence of functional Tregs. We identified a novel CXCR5+PD-1+ CD8 T cell population (termed CD8 Tfc cells) that influences germinal center reactions and autoimmune disease progression. Together, these studies contribute to a growing body of evidence defining CD8 T cells responses to chronic antigen and inflammation.

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TABLES

Table 1-1: CXCR5+ CD8 T cells maintain antigen-specific transcription and localization. CXCR5+ CD8 T cells have been identified under at least four antigen conditions including SIV/HIV infection, LCMV infection, Cancer and autoimmunity/inflammatory. * Gene transcription compared WT CD8 T cells and IL-2 deficient CD8 T cells in autoimmunity.

	Transcription		Location	Reported Functional Capacity	References
	High	Low			
SIV/HIV	*bcl6*, *cd28*, *cd40*, *cd83*, *cd200*, *ctla4, il2*, *irf4*	*cd244*, *grzma*, *grzmb*, *id2*, *runx3*, *Prdm1* (Blimp-1)	GC, follicle and extrafollicular space of lymph node and spleen	Less cytotoxic effector CD8 T cell that controls infection	(Connick et al., 2014; Leong et al., 2016; Li et al., 2014; Mylvaganam et al., 2017; Rahman et al., 2018)
LCMV	*bcl6, cd200*, *icos*, *id3*, *il2*, *il7rα* (CD127), *sell* (CD62L), *tcf7* (TCF-1)	*cd244*, *fasl*, *grzma*, *grzmb*, *havcr2* (Tim-3), *id2*, *prdm1*, *prf1*	B cell follicle and T cell zone of splenic white pulp	Less cytotoxic effector CD8 T cell that controls infection and maintains proliferative capacity.	(He et al., 2016; Im et al., 2016; Leong et al., 2016)
Cancer	*bcl6*, *grzma*, *grzmb, ifnγ*, *il2*, *pdcd1*, *prdm1*, *prf1*, *tbx21 (T-bet)*, *tnf*	*ctla4*, *havcr2* (Tim-3), *lag3*	Peripheral blood, tumor infiltrating, and tumor draining lymph node	Non-exhausted cytotoxic effector CD8 T cell that promotes tumor suppression	(Bai et al., 2017; E et al., 2018; Jin et al., 2017; Tang et al., 2017; Xing et al., 2017)
Autoimmunity and inflammation	*bcl6*, *ccr7*, *cd200*, *ctla4*, *cxcr5*, *eomes*, *fasl*, *grzma*, *grzmb*, *havcr2*, *icos*, *ifng*, *il21*, *irf4*, *maf*, *pdcd1*, *prdm1*, *sh2d1a* (sap)*	Not tested	B cell follicle lymph node and spleen, ectopic GC in tissue-specific disease	Promotes autoimmune antibody responses	(Kang et al., 2002; Quigley et al., 2007; Valentine et al., 2018; Wagner et al., 1998; Xiao et al., 2016)

FIGURES AND FIGURE LEGENDS

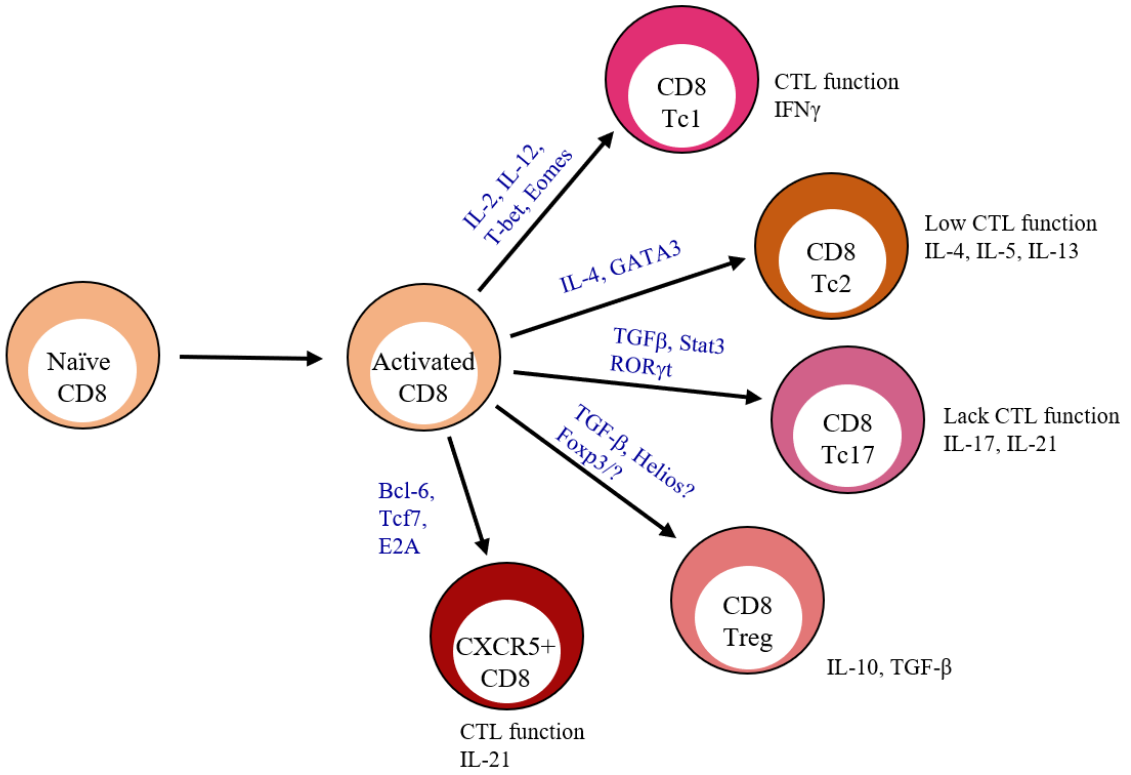

Figure 1-1: CD8 T cell differentiation subsets. After antigen recognition, activated CD8 T cells that receive specific TCR interactions, cytokine signaling, and dendritic cell signals upregulate transcription factors that program terminal differentiation outcomes including CD8 T cytotoxic (Tc) 1, Tc2. Tc17, Treg, and CXCR5+ subsets.

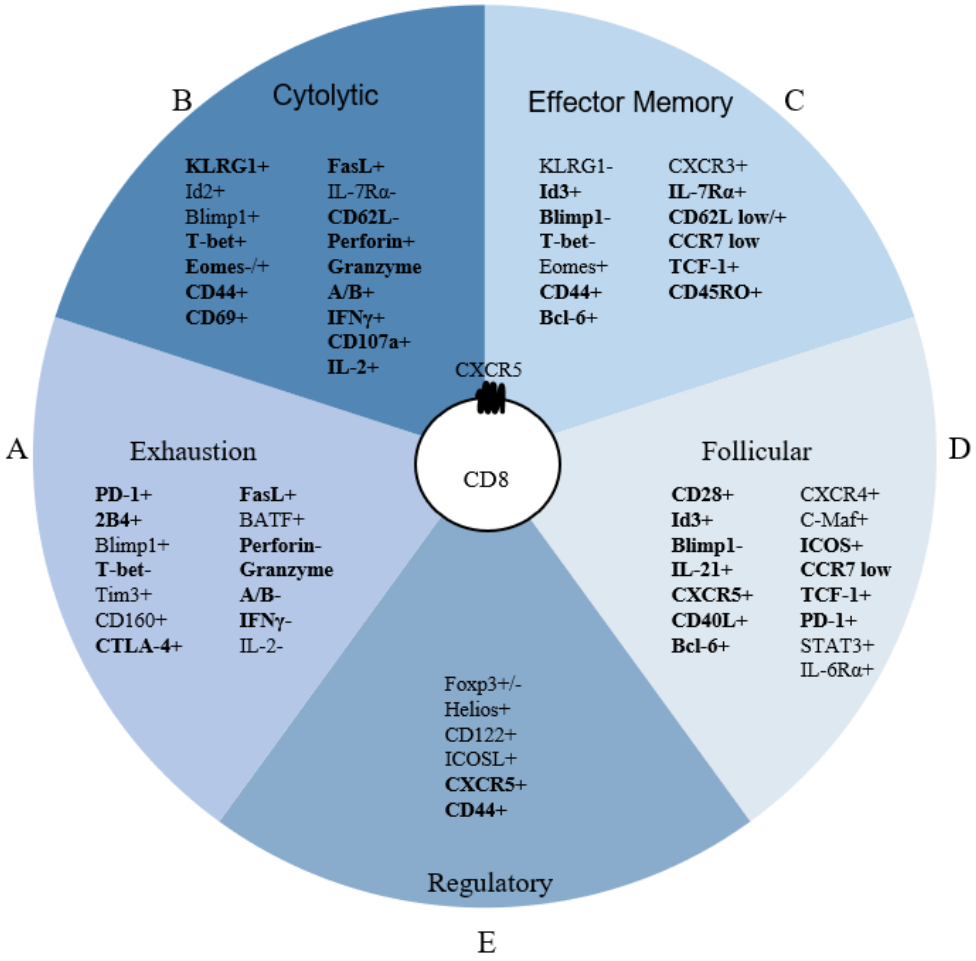

Figure 1-2: CXCR5+ CD8 T cell maintain a distinct expression pattern. CXCR5+ CD8 T cell protein expression relative to T cell subsets; A) CD8 T cell exhaustion (37), B) CD8 cytotoxic T cell, C) CD8 T effector memory (Tem) (36), D) CD4 T follicular helper (Tfh) (11), and E) CD8 T regulatory cell (Treg) (35). Bold indicated literature confirmed protein expression in CXCR5+ CD8 T cells.

Cytolytic: KLRG1+ (19), T-bet+ (20, 22), Eomes- (22), CD44+ (19-21), CD69+ (4), FasL+ (4), CD62L- (4, 20), Perforin+ (22, 25, 28, 39, 40), Granzyme A+ (4, 40), Granzyme B+ (5, 22, 25, 28, 40-42), IFNγ+ (3-5, 19, 20, 23, 27, 39, 43), CD107a+ (19, 27, 39, 40, 42), IL-2+ (4, 20, 27). Exhaustion: PD-1+ (1, 20, 21, 23, 28, 29, 39, 42), 2B4+ (29), T-bet- (5), CTLA-4+ (27), FasL+ (4), Perforin- (3, 4, 21, 27, 41, 44), Granzyme A- (3, 21), Granzyme B- (20, 21, 27, 39), IFNγ- (21, 29). Effector Memory: Id3+ (21), Blimp1- (5, 21), T-bet- (5), CD44+ (19-21), Bcl6+ (1, 5, 20, 21, 27), IL-7Rα+ (4, 20, 21), CD62L- (4, 20), CD62L+ (21), CCR7- (4, 21, 25, 28, 29), TCF-1+ (20, 21), CD45RO+ (4, 22). Follicular: CD28+ (4, 20, 27), Id3+ (21), Blimp1- (5, 21), IL-21+ (1, 43), CD40L+ (1), Bcl6+ (1, 5, 20, 21, 27), ICOS+ (1, 4, 20, 21), CCR7- (4, 21, 25, 28, 29), TCF-1+ (20, 21), PD-1+ (1, 20, 21, 23, 28, 29, 39, 42). Regulatory: CD44+ (19-21)

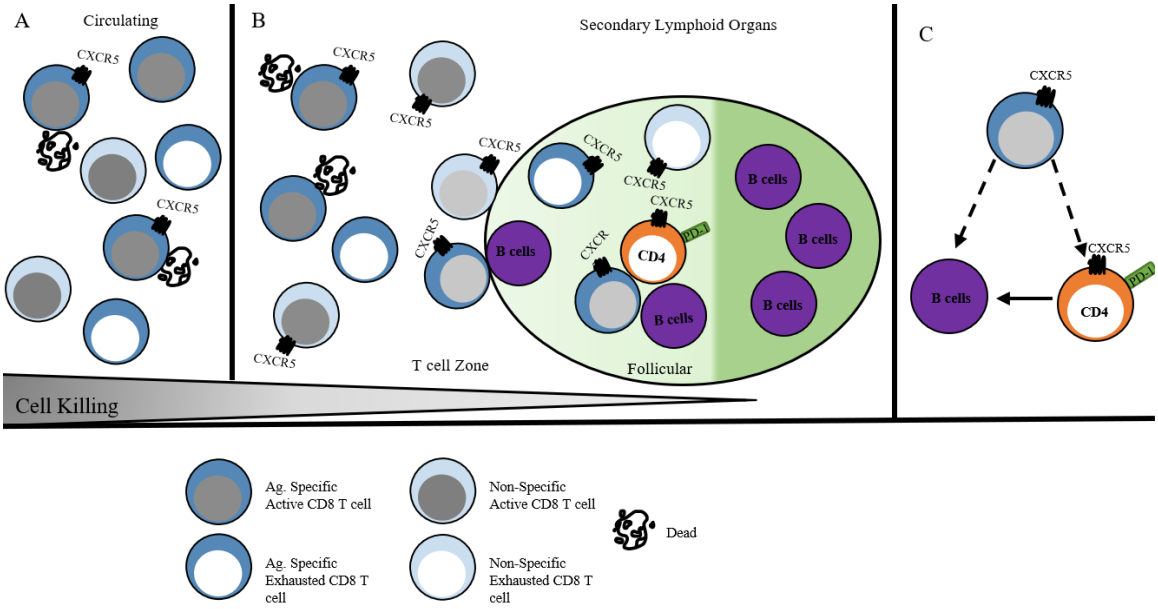

Figure 1-3: CXCR5+ CD8 T cell function is anatomically instructed. A) Antigen-specific CXCR5+ CD8 T cells in peripheral blood circulation induce direct cell killing, whereas other CD8 T cells may be functionally exhausted. B) CXCR5+ CD8 T cells within secondary lymphoid organs that localize to the extrafollicular space maintain higher direct cell killing capacity than CXCR5+ CD8 T cells within the follicle. Antigen-specific CXCR5+ CD8 T cells are maintained at similar frequency in the extrafollicular space and follicle. C) Follicular CXCR5+ CD8 T cells maintain the capacity for cytotoxicity; different antigen scenarios likely elicit various functional outcomes that promote direct B cell or CD4 Tfh cell interactions. Dark blue cells indicate antigen specific T cells. Dashed lines indicate potential interactions.

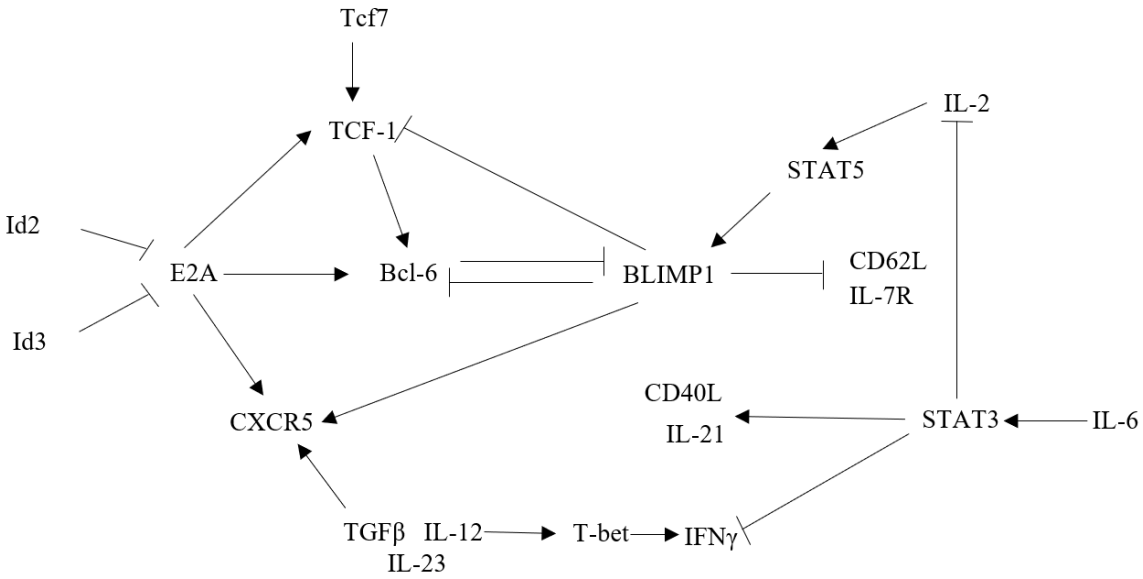

Figure 1-4: CXCR5+ CD8 T cells acquire a unique transcriptional profile from other CD8 T cells. CXCR5+ CD8 T cells express the transcription factor Bcl6 that is maintained by TCF-1 and E2A. This transcriptional interaction allows for CXCR5 upregulation and expression of functional proteins. Arrows indicate promoting interaction. Blunted lines indicate inhibitory interactions.

CHAPTER 2

CD8 follicular T cells promote B cell antibody class switch in autoimmune disease

CD8 Follicular T Cells Promote B Cell Antibody Class Switch in Autoimmune Disease

Kristen M. Valentine,* Dan Davini,† Travis J. Lawrence,* Genevieve N. Mullins,* Miguel Manansala,‡ Mufadhal Al-Kuhlani,† James M. Pinney,† Jason K. Davis,§ Anna E. Beaudin,† Suzanne S. Sindi,§ David M. Gravano,‡ and Katrina K. Hoyer†

CD8 T cells can play both a protective and pathogenic role in inflammation and autoimmune development. Recent studies have highlighted the ability of CD8 T cells to function as T follicular helper (Tfh) cells in the germinal center in the context of infection. However, whether this phenomenon occurs in autoimmunity and contributes to autoimmune pathogenesis is largely unexplored. In this study, we show that CD8 T cells acquire a CD4 Tfh profile in the absence of functional regulatory T cells in both the IL-2–deficient and scurfy mouse models. Depletion of CD8 T cells mitigates autoimmune pathogenesis in IL-2–deficient mice. CD8 T cells express the B cell follicle–localizing chemokine receptor CXCR5, a principal Tfh transcription factor Bcl6, and the Tfh effector cytokine IL-21. CD8 T cells localize to the B cell follicle, express B cell costimulatory proteins, and promote B cell differentiation and Ab isotype class switching. These data reveal a novel contribution of autoreactive CD8 T cells to autoimmune disease, in part, through CD4 follicular-like differentiation and functionality. ***The Journal of Immunology*****, 2018, 201: 000–000.**

The development of autoimmunity involves both a breakdown in tolerance control mechanisms and complex interactions between immune cells. Some of these cells promote disease, whereas others act to block this dysregulation. As disease progresses, immune activation is amplified, self-perpetuating the lymphoproliferation, inflammation, and self-destruction associated with autoimmunity. The process initiated by inflammatory and Ag signaling promotes T cell proliferation, differentiation, and acquisition of T cell effector functions. Autoimmunity in the IL-2–deficient (knockout [KO]) mouse model results from reduced T regulatory cell (Treg) frequency and functionality (1–3), promoting dysregulation of the T effector response. CD4 Th1 cells subsequently promote the production of anti-RBC IgG Abs and bone marrow failure dependent on IFN-γ secretion (4–6). Although there is a clearly established role for CD4 T cells in promoting Ab-mediated disease, the importance of CD8 T cells in these diseases has been less explored.

B cell responses to self-antigens during autoimmune disease are induced and enhanced by germinal center (GC) reactions in the peripheral lymphoid organs (7, 8). Within the GC, activated, Ag-specific B cells undergo clonal expansion, BCR somatic mutation, affinity maturation and Ab class switching, and differentiate into memory and long-lived plasma cells. GC reactions begin at the border of the B cell follicle and the T cell zone, where CD4 T follicular helper (Tfh) cells interact with B cells (9). CXCR5 upregulation and CCR7 downregulation facilitates migration of CD4 Tfh and activated B cells into the follicle. Engagement of several interactions between the activated CD4 Tfh and B cells (including ICOS-ICOSL and CD40-CD40L) ensures optimal GC reactions and CD4 Tfh cell development by promoting the transcription factor, Bcl6 (10). CD4 Tfh cells are required for the production of high avidity, class-switched Abs (11).

Abnormal activation of CD4 Tfh cells, or loss of regulation, can promote Ab-mediated autoimmune disease (12–14). Follicular CD4 Tregs are essential inhibitors of GC interactions by mediating T cell help to B cells (7). In the absence of CD4 follicular Tregs, CD4 Tfh cell expansion results in autoantibody generation and autoimmune disease (13, 15). Similarly, CD8 Tregs also control self-reactive cells, and their elimination exacerbates autoimmune disease (16, 17). Like CD4 T cells, CD8 T cells differentiate into effector subsets based upon their transcription factor expression and cytokine production, and these cytokines may amplify CD4 T cell responses or act through mechanisms unique to CD8 T effectors (17). However, regulation and function of distinct CD8 T cell subsets are less clearly delineated as compared with CD4 helper cells.

CD8 T cell effectors located within the GC have recently been described (18–22). In rheumatoid arthritis synovial ectopic follicles, CD8 T cells make up the majority of the infiltrating T cells and express CD40L, which is important in B cell GC reactions (19). Furthermore, the CD8 T cells are required for the formation and maintenance of ectopic GCs (18). CD8 T cells expressing CXCR5 also develop in chronic viral infection and under inflammatory conditions (20, 21, 23–25). $CXCR5^+$ CD8 T cells are localized in the B cell follicle in human tonsil, and these cells support

*Quantitative and Systems Biology Graduate Program, University of California, Merced, Merced, CA 95343; †Department of Molecular and Cell Biology, School of Natural Sciences, University of California, Merced, Merced, CA 95343; ‡Stem Cell Instrumentation Foundry, University of California, Merced, Merced, CA 95343; and §Department of Applied Mathematics, University of California, Merced, Merced, CA 95343

ORCIDs: 0000-0002-7380-489X (T.J.L.); 0000-0003-1646-5710 (G.N.M.); 0000-0002-4514-0774 (J.K.D.).

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The sequences presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE112540.

Address correspondence and reprint requests to Dr. Katrina K. Hoyer, Department of Molecular and Cell Biology, University of California, Merced, 5200 N. Lake Road, Merced, CA 95343. E-mail address: khoyer2@ucmerced.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: GC, germinal center; HET, heterozygous; KLH, keyhole limpet hemocyanin; KO, knockout; LN, lymph node; MFI, mean fluorescence intensity; NP, nitrophenylacetyl; Tfc, T follicular; Tfh, T follicular helper; Treg, T regulatory cell; UC, University of California; WT, wild-type.

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B cell survival in ex vivo culture (26). CXCR5$^+$ CD8 memory T cells within the GC control viral load in lymphocytic choriomeningitis virus and SIV infections and express many of the genes associated with CD4 Tfh cell differentiation and function (20, 23). Together, these data suggest that under specific disease conditions, CD8 T cells may acquire unique functionality within the GC. Whether CD8 T cells function in GC reactions in autoimmune disease is largely unexplored.

Materials and Methods

Mice, immunizations, and Ab depletions

BALB/c IL-2–KO mice, wild-type (WT), and IL-2–heterozygous (HET) littermate controls (IL-2–HET and WT; referred to as WT) were used. IL-2–KO autoimmune disease is not gender specific, only age is used to determine data inclusion, as specified in each figure legend. BALB/c hemizygous male Foxp3^{sfDY} (scurfy) mice and HET female FoxP^{sfDy} (scurfy-HET) mice were purchased from The Jackson Laboratory (27). Both gender and age are used to determine data inclusion in scurfy disease. Our breeding setup is restricted to scurfy male mice, and age-matched female scurfy-HET littermates were used as controls where indicated. For immunization, mice were treated by i.p. injection with 100–200 μg keyhole limpet hemocyanin (KLH) in CFA at −15 to −26 d, followed by a second i.p. injection at −5 d, as previously described (7, 8). CD4 or CD8 depletions were performed by i.p. injection of 20 μg anti-CD4 (GK1.5) or anti-CD8 (2.43) Ab per gram weight three times per week, from day 8 to 16. IL-2 depletions were performed by i.p. injection of 20 μg anti–IL-2 (JES6-1A12) Ab per gram weight three times per week between days 7 and 15. Abs were purchased from the University of California (UC), San Francisco mAb Core or Bio X Cell. All mice were bred and maintained in our specific pathogen-free facility in accordance with the guidelines of the Department of Animal Research Services at UC Merced. The UC Merced Institutional Animal Care and Use Committee approved all animal procedures.

Complete blood counts

Cardiac punctures or eye bleeds were performed immediately following cervical dislocation, and blood was collected in heparinized tubes (28). Complete blood counts were evaluated within 24 h on a Hemavet 950 Veterinary Hematology System.

Microscopy and immunofluorescence

Spleens were embedded in optimal cutting temperature compound (O.C.T. Compound; Fisher Scientific) and snap frozen in the vapor phase of liquid nitrogen. Fifteen micrometer sections were generated, then fixed with 100% ice cold acetone, followed by blocking with PBS/5% BSA. Sections were stained overnight with anti–CD8α-FITC (53-6.7; eBioscience), anti–IgD-PE-Dazzle594 (11-26c.2a; BioLegend), and anti–GL7-eFluor450 (GL-7; eBioscience), and imaged using single-plane confocal microscopy on a Zeiss LSM 880 confocal system with a 10× objective. Confocal images were processed in ImageJ to adjust for contrast and pseudocolored in red, green, and blue. GL-7$^+$ GCs were traced in ImageJ using the freehand selection tool, and the area was determined. CD8$^+$ cells within the GC were marked using the multipoint selection tool when a dark center (nucleus) surrounded by CD8 surface staining could be identified. GC CD8 T cells within the GC area were quantified manually.

Flow cytometry and cell sorting

Splenocytes and peripheral lymph nodes (LNs) were stained with fluorochrome-conjugated Abs (eBioscience, unless otherwise noted) following incubation with Fc-block (anti-CD16/CD32; 2.4G2). Cell viability was determined by DAPI, Fixable Viability Dye eFluor780, or Fixable Viability Dye eFluor506 (both from eBioscience). For CD8 T follicular (Tfc) cell identification, cells were stained with anti–CXCR5-biotin (SPRCL5), then stained with Streptavidin-BUV395 (BD Biosciences), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD278 (ICOS; C398.4A), anti–GL-7 (GL-7), anti-CD279 (PD-1; J43; BioLegend), anti-CD11c (N418), anti-CD11b (M1/70), anti–Ly-6G (Gr-1; RB6-8C5), and anti-CD45R (B220; RA3-6B2), as previously defined (29). For intracellular proteins, cells were stained as above, fixed using the Foxp3/Transcription Factor Fixation/Permeabilization Kit (eBioscience), and stained with anti-Bcl6 (BCL-DWN) and SA-BUV395 (BD Biosciences) or anti–IL-2-PE (JES6-5H4; BioLegend). Flow cytometry was performed on a Becton Dickinson LSR-II, and data were analyzed using FCS Express with Diva Version 4.07.0005 (De Novo Software) or FlowJo v10.1 (FlowJo).

Prior to cell sorting, pooled splenocytes and lymphocytes were depleted of non–T cells using EasySep Mouse PE Selection Kit according to the manufacturer's instructions (STEMCELL Technologies) to remove B220-PE$^+$, CD11c-PE$^+$, CD11b-PE$^+$, and Gr-1-PE$^+$ cells. CD4 Tfh (CXCR5$^+$PD-1^{hi}) and CD8 Tfc cells (CXCR5$^+$PD-1^{hi}) were sorted from IL-2–KO mice, CD4 Tfh cells (CXCR5$^+$PD-1^{hi}) were sorted from KLH-immunized mice, and bulk CD4 and CD8 T cells were sorted from WT or IL-2–KO mice, as indicated. B cells (CD19$^+$TCRβ$^-$CD11c$^-$CD11b$^-$Gr-1$^-$) were sorted from pooled WT spleens. All sorts were performed with >90% purity on the Aria II cell sorter (BD Biosciences).

RBC Ab detection

Serum RBC Ab levels were detected, as previously described (30). Freshly isolated RBCs were washed three times in PBS and resuspended to 1% RBCs. Ten microliters of 1% RBCs were incubated with anti-mouse IgM-FITC (1:150; on ice) or anti-mouse IgG-FITC (1:50; at 37°C; Jackson ImmunoResearch Laboratories). The percentage of RBCs bound by Ab was determined by flow cytometry.

T cell stimulations

Harvested cells were stimulated at 37°C with 50 ng/ml PMA and 500 ng/ml ionomycin for 5 h with Brefeldin A or monensin added during the final 4 h. IL-2 cytokine production was determined by intracellular flow cytometry. Anti-CD40L (MR1) was added directly to cells during the stimulation, as previously described (31). Cells were stained poststimulation for CD4 Tfh and CD8 Tfc cell markers (CXCR5$^+$PD-1^{hi}). IL-21 cytokine production was determined in CD4 Tfh and CD8 Tfc cells, as previously described, with recombinant mouse IL-21R subunit Fc chimera (R&D Systems) and PE-conjugated F(ab')$_2$ goat anti-human IgG (Jackson ImmunoResearch Laboratories) at 4°C (32).

In vitro T cell and B cell culture assays

T and B cell stimulation was performed, as previously described (22). Indicated T cell populations were plated at 5×10^4 cells per well and activated with 5 μg/ml soluble anti-CD3ε (145-2C11; BioLegend) and 1 μg/ml anti-CD28 (37.51; BioLegend) for 72 h. Supernatant from activated T cells were plated with 5×10^4 sorted WT B cells per well and with 1 μg/ml anti-CD40 (IC10) and 5 μg/ml F(ab')$_2$ goat anti-mouse IgMμ (Jackson ImmunoResearch Laboratories) for 6 d. B cell supernatant was analyzed for Ab production by ELISA.

T cell adoptive transfer assays

IL-2–KO T cells were adoptively transferred, as previously described (4). 1×10^6 CD4 T cells, 2×10^6 CD8 T cells, or a combination of each were transferred into TCRα-KO mice via eye injection. Two days after cell transfer, mice were immunized i.p with 200 μg of KLH in CFA. Seven days after cell transfer, mice were reimmunized i.p. with 100 μg of 4-Hydroxy-3-nitrophenylacetyl (NP)–conjugated KLH in IFA.

Ab ELISA

Total IgG was determined by ELISA, as described (28). Serum and culture supernatant samples were prepared in PBS/1% BSA. Serum from depletion experiments was prepared at 1:50,000 dilution, a standard curve of purified mouse IgG (SouthernBiotech), stimulated B cell supernatants were prepared at 1:50 dilution, and serum from adoptive transfer experiments was diluted to 1:10,000 dilution for IgG2a and IgG2b or 1:100,000 dilution for IgG1. Abs were detected with HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, or IgG3 (SouthernBiotech), then developed with TMB Peroxidase Substrate (Vector Laboratories) following manufacturers' instructions. Plates were stopped with 1 N sulfuric acid and read on a Victor 3 1420 Multilabel Counter plate reader (PerkinElmer) at 450 nm. When applicable, a standard curve of purified mouse IgG (SouthernBiotech) was used to interpolate IgG concentrations from a sigmoidal standard protein curve.

RNA isolation and analysis of RNA next-generation sequencing

CD8 cells were sorted from 12-d-old IL-2–KO and WT mice to ≥85% purity. Samples were quick frozen and shipped to Expression Analysis for total RNA isolation using Illumina TrueSeq Stranded Total RNA Sample Preparation Kit. Eight samples were sequenced, four biological replicates each for IL-2–KO and WT mice, producing 2 × 50 paired-end reads using the Illumina HiSeq 2500 platform. Raw reads were provided by Expression Analysis and were used for further analyses. Adapter removal and quality trimming at the Q20 level were performed using Atropos v1.1.17 (33) with Python v3.6.2. Read pairs were removed if either read was <20 bp trimming. Rsubread v1.28.1 (34) was used to perform read alignment, reporting up to 10 equally likely mapping locations. Read pairs that could not be aligned together were aligned individually. The genome used for alignment was C57BL/6J of GRCm38/mm10 (GCF_000001635.20). Read summarization was performed on the gene level

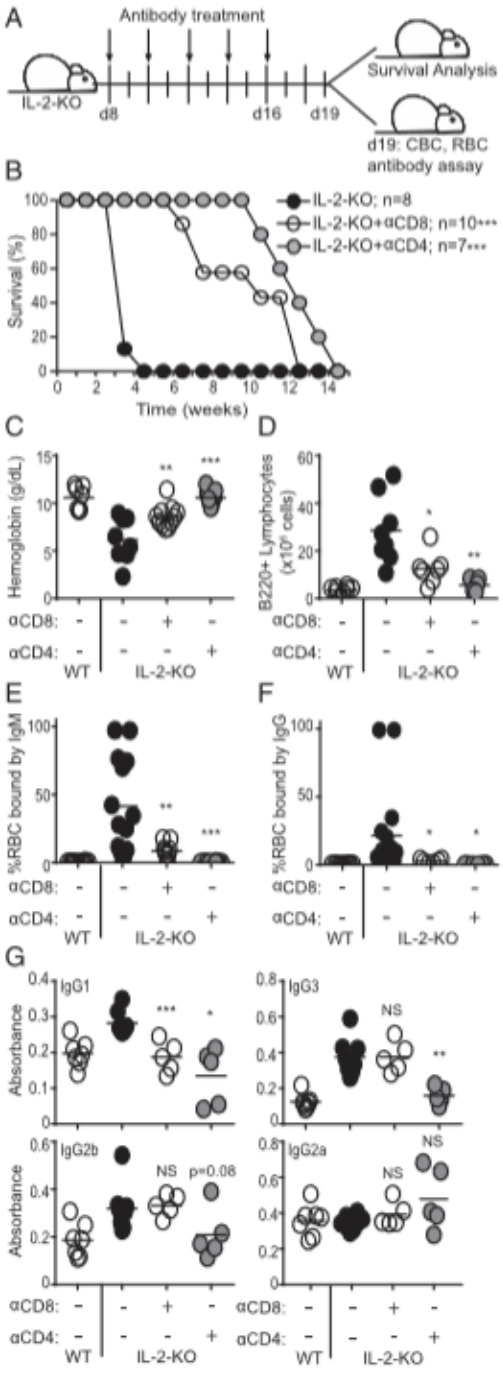

FIGURE 1. Depletion of IL-2–KO CD4 or CD8 T cells prolongs survival and delays Ab production. (**A**) Schematic describing the Ab depletion. Peripheral blood and LN cells were isolated at 18–21 d of age. (**B**) Kaplan–Meier survival plots. Statistics were performed to test differences relative to untreated IL-2–KO mice. (**C**) Hemoglobin levels were measured from peripheral blood by complete blood count (CBC) at day 19 of age. (**D**) Total LN B220^{+} B cell numbers were determined by flow cytometry and cell counting. (**E** and **F**) RBCs were stained with anti-mouse IgM-FITC (E) or IgG-FITC (F) and analyzed by flow cytometry to detect the percentage of RBC bound by Abs. (**G**) Serum IgG1, IgG3, IgG2b, and IgG2a levels were determined by ELISA. (C–G) Each symbol indicates an individual animal. Statistics tests were performed relative to IL-2–KO and unpaired Student *t* test with a Welch correction. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *(Figure legend continues)*

using featureCounts (35) using annotations from a modified version of the annotations for GRCm38/mm10 containing only protein coding genes. Multimapping reads were treated as fractional counts when mapping to several genes, mapping across more than one gene, and read pairs in which ends mapped to different chromosomes were discarded. Genes that had <1 count per million in three to four samples were discarded. Remaining gene counts were normalized using trimmed mean of M-values (36). Differential expression analysis was performed using limma v3.34.9 (37) with voom-transformed read counts (38). Genes were considered differentially expressed if their p value was <0.05 after the false discovery rate was controlled (39). Read mapping, summarization, and differential expression analysis were performed using R v3.4.3. Differentially expressed genes were annotated with their biological process Gene Ontology group using Panther 13.1 (40) and the Gene Ontology database released on February 2, 2018. Sequence data were uploaded to National Center for Biotechnology Information GEO under accession number GSE112540 at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112540.

Real-time PCR

Total RNA was isolated from cells using an RNeasy kit (Qiagen), and cDNA syntheses were conducted according to the manufacturer's instructions with Superscript III First-Strand Synthesis SuperMix for RT-PCR (Invitrogen). Real-time PCR analysis was conducted in duplicates using Mx3000P (Stratagene California) with Fast SYBR Green RT-PCR Master Mix (Bio-Rad). Averages of the collected data were normalized to β-actin or HPRT. Relative expressions (ΔΔCt) were calculated to the indicated cell population.

Statistics

GraphPad (Prism) was used for statistics. Differences between two experimental groups were determined by unpaired Student *t* test unless otherwise noted. A Mantel–Cox log-rank test was used to analyze Kaplan–Meier survival curves. Protein concentrations from a standard protein curve were interpolated using a sigmoidal four parameter logistic standard curve with x as log(concentration). Statistically significant differences in adoptive transfer and supernatant culture experiments were determined by one-way ANOVA with Bonferroni correction.

Results

Depletion of either CD4 or CD8 T cells reduces anemia and anti-RBC Abs and prolongs survival

We have previously demonstrated that early death in IL-2–KO mice is driven in part by autoimmune hemolytic anemia that requires IFN-γ and is CD4 Th1–mediated (5, 6). We found that CD8 T cell numbers were increased on average by 3-fold in the LNs and spleen of IL-2–KO mice (Supplemental Fig. 1A). Both splenic and LN CD8 T cells expressed decreased CD62L and elevated CD44 and CD69, indicating an activated state (Supplemental Fig. 1B, 1C). CD8 T cells have been shown to promote and inhibit autoimmunity in multiple models due to both regulatory and effector responses (4, 8, 19, 41). As the population of IL-2–KO CD8 T cells was activated and expanded, we next determined how and to what extent CD8 T cells contributed to autoimmunity in IL-2–KO mice.

To evaluate the contribution of CD8 T cells to autoimmune disease, we eliminated CD8 T cells or CD4 T cells as a control, prior to disease onset, by treating with anti-CD4– or anti-CD8–depleting Abs (Fig. 1A). As expected, depletion of CD4 T cells significantly delayed disease onset with a median survival of 14 wk, as compared with 19–25 d survival for PBS-treated IL-2–KO mice (Fig. 1B). Surprisingly, depletion of CD8 T cells also significantly prolonged survival, with a median survival of 12 wk. In concordance with augmented survival, IL-2–KO mice with depleted CD4 or CD8 T cells had increased hemoglobin levels

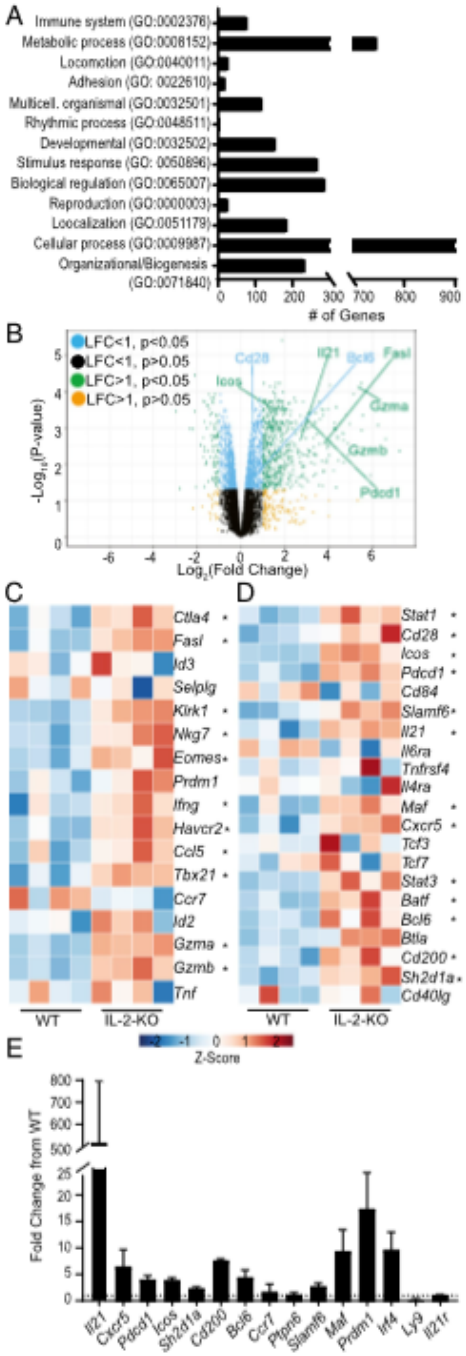

FIGURE 2. Cytolytic and follicular helper profile of IL-2–KO CD8 T cells. RNA sequencing of four independent WT and IL-2–KO CD8 T cell samples pooled from LN of 12-d-old mice. **(A)** Gene Ontology analysis of all 2226 differentially expressed genes in IL-2–KO CD8 T cells relative to WT. **(B)** Volcano plots displaying log2 fold change gene expression of IL-2–KO relative to WT CD8 T cells versus log10 p value. Select differentially expressed genes are labeled in the plot. Data are organized by color to indicate both log fold change (LFC) and p value. (**C** and **D**) Heat maps showing CD8 T cell expression data. Color *(Figure legend continues)*

relative to PBS-treated IL-2–KO mice (Fig. 1C). Both T cell depletions also significantly reduced B cell numbers (Fig. 1D) and frequency of IgM and IgG autoantibodies bound to RBCs (Fig. 1E, 1F) relative to PBS-treated IL-2–KO mice. In the absence of CD8 T cells, serum IgG1 (but not IgG3, IgG2b, or IgG2a) was significantly reduced in comparison with PBS-treated IL-2–KO mice (Fig. 1G). Although these data confirm that CD4 T cells play a critical role in autoimmune progression, they also reveal the contribution of CD8 T cells to the rapid autoimmunity that occurs in IL-2–KO mice. Furthermore, these data demonstrate that CD8 T cells facilitate enhanced B cell expansion and Ab production in IL-2–KO mice, as these outcomes are significantly reduced when CD8 T cells are eliminated.

Transcriptional profiling of CD8 T cells during early autoimmunity

To determine mechanistically how CD8 T cells contribute to Ab-mediated autoimmune development in the absence of functional Tregs, we examined early gene dysregulation in IL-2–KO CD8 T cells. We performed RNA sequencing of CD8 T cells sorted from 12-d-old mice, as this is the earliest time point at which IL-2–KO CD8 T cells have been shown to be activated (42). Bulk CD8 T cells were sorted from the peripheral LNs of four sets of pooled IL-2–KO and littermate control WT mice. Differential expression analysis identified 2226 genes (1290 upregulated; 936 downregulated) that showed significant ($p < 0.05$) differences in expression in IL-2–KO CD8 T cells relative to WT CD8 T cells (Supplemental Table I). Differentially expressed genes were grouped into several biological process Gene Ontology categories, including metabolic processes, cellular processes, and biological regulation (Fig. 2A). Several genes upregulated in IL-2–KO CD8 T cells were genes involved in cytolytic function (*granzymes*, *tbx21*, *fasl*), but we also identified differential expression of costimulatory molecules and follicular helper-associated genes such as *icos*, *cd28*, *il21*, and *bcl6* (Fig. 2B). Further evaluation of RNA sequencing data confirmed the profiles of cytolytic gene expression in individual IL-2–KO mice (Fig. 2C). Based on the observed reduction in B cell numbers and Ab production in the absence of CD8 T cells (Fig. 1), we focused our analysis on the expression of costimulatory molecules and genes involved in B cells. This analysis revealed a profile of gene expression in CD8 T cells comparable to that described in CD4 Tfh cells (Fig. 2D). IL-2–KO CD8 T cells expressed elevated *cxcr5*, *sh2d1a* (SAP), *icos*, *bcl6*, *il21*, and several other genes that define CD4 Tfh cells, as further confirmed by real-time PCR (Fig. 2E). Thus, during early autoimmune initiation, CD8 T cells acquire a gene expression profile associated with varying functional roles, including a CD4 Tfh cell–like role.

CD8 Tfc cells develop during systemic autoimmune disease

We next evaluated protein expression of CD4 Tfh cell–associated genes in IL-2–KO CD8 T cells. In naive mice, a small population (0.1–0.5%) of CXCR5^{+}PD-1^{hi} CD4 Tfh cells has been described (43, 44). We used this population and percentage range, in addition to fluorescence-minus-one controls, to confirm our gating strategy for CD8 T cells. Using these stringent conditions, a very small population of ≤0.2% CD8 T cells expressed CXCR5 and PD-1 in naive WT mice. In contrast, the same markers were

indicates gene expression by Z-score, and * indicates IL-2–KO gene expression with statistical significance relative to WT. Differential expression of select cytolytic-associated genes (C) and CD4 Tfh-associated genes (D). (**E**) mRNA expression of select genes in two independent experiments from 12-d-old IL-2–KO CD8 T cells relative to WT CD8 T cells. Dashed line indicates a fold change of 1, and error bars indicate SD.

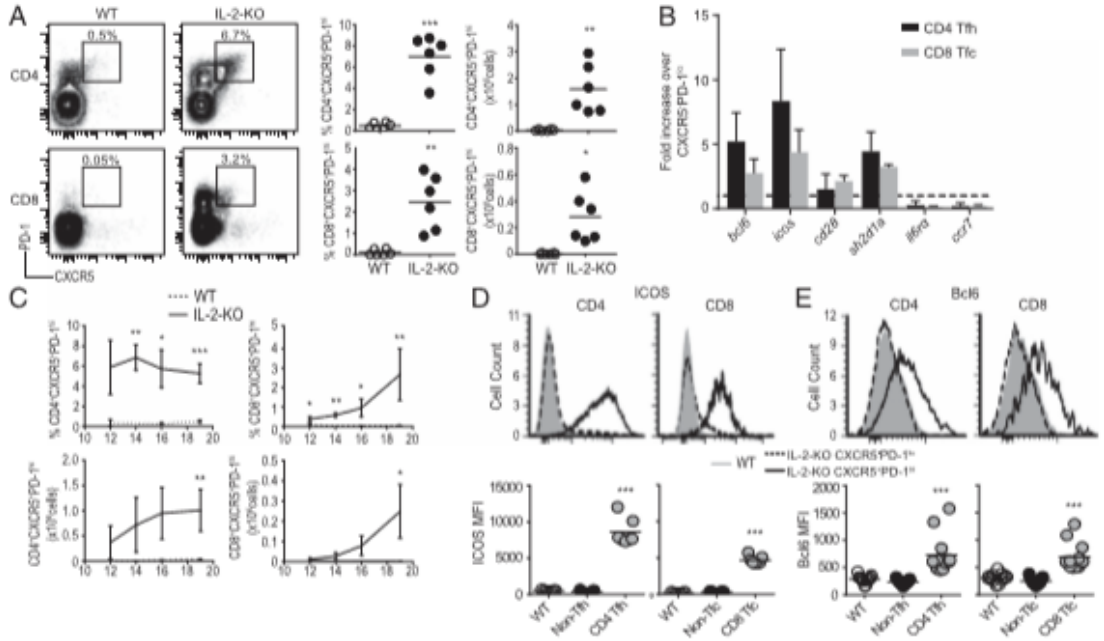

FIGURE 3. CD8 T cells express markers of follicular helpers. **(A)** Flow cytometric analysis of CXCR5 and PD-1 expression on CD4 or CD8 T cells from 18- to 21-d-old IL-2–KO or WT LN gated on B220$^-$ CD11c$^-$ CD11b$^-$ GR-1$^-$. Representative flow plots, frequency, and total number of CXCR5$^+$PD-1^{hi} CD4 and CD8 T cells are shown. **(B)** Real-time PCR comparing relative gene expression in IL-2–KO CD4 Tfh or CD8 Tfc cells (CXCR5$^+$PD-1^{hi}) with IL-2–KO CD4 CXCR5$^-$ PD-1^{lo} (non-Tfh) or CD8 CXCR5$^-$ PD-1^{lo} (non-Tfc) cells, respectively. Dashed line indicates fold change of 1, and error bars indicate SD. **(C)** Percent and total number of CD4 Tfh and CD8 Tfc cells in IL-2–KO and WT mice from 12 to 20 d of age. **(D and E)** Representative flow plots and MFI quantification of surface expression of ICOS (D) and Bcl6 (E) in WT naive bulk CD4 and CD8 T cells, IL-2–KO CD4 non-Tfh or CD8 non-Tfc cells, and IL-2–KO CD4 Tfh and CD8 Tfc cells. (A, D, and E) Each symbol indicates an individual animal. (A and C–E) Data are representative of three to six independent experiments. (B) Data are representative of two independent experiments. Statistics: unpaired Student *t* test relative to WT with a Welch correction. $*p < 0.05$, $**p < 0.01$, $***p \leq 0.001$.

significantly expanded among both CD4 and CD8 T cells in IL-2–KO LN (7.0 ± 1.8% of CD4 T cells and 2.5 ± 1.2% of CD8 T cells; Fig. 3A). Similarly, CXCR5$^+$PD-1^{hi} CD4 and CXCR5$^+$ PD-1^{hi} CD8 T cells significantly increased in frequency and total number in the spleens of IL-2–KO mice relative to naive WT (Supplemental Fig. 2A). To confirm select gene expression patterns identified in the bulk CD8 T cell RNA sequencing, we performed real-time PCR analysis of IL-2–KO CD4 Tfh and CD8 Tfc cells relative to IL-2–KO CD4 non-Tfh cells and CD8 non-Tfc cells, respectively. CD8 Tfc cell mRNA levels of *bcl6*, *icos*, *cd28*, *sh2d1a*, *il6ra*, and *ccr7* were comparable to CD4 Tfh cells (Fig. 3B). Thus, based on their surface phenotype and gene expression profile, we defined the CXCR5$^+$PD-1^{hi} CD8 T cells as CD8 Tfc cells.

A detectable population of CD8 Tfc cells was identified in IL-2–KO mice at day 12 by flow cytometry and continued to expand in both frequency and total number over time (Fig. 3C). Although, CXCR5$^+$PD-1^{hi} T cells comprise only a small fraction of the expanded CD4 and CD8 T cell population observed in IL-2–KO mice (Supplemental Fig. 2B). We next evaluated CD8 Tfc cells for the expression of other proteins known to be involved in B cell interactions within the follicle. ICOS is highly expressed during CD4 Tfh cell differentiation and promotes the expression of Bcl6, a master regulator of CD4 Tfh cell fate that promotes CD4 Tfh/B cell interactions (45, 46). IL-2–KO CD8 Tfc cells expressed increased ICOS and Bcl6, similar to IL-2–KO CD4 Tfh cells, in comparison with both naive WT CD8 T cells and IL-2–KO CD8 non-Tfc cells (Fig. 3D, 3E, Supplemental Fig. 2C, 2D). Together, mRNA and surface expression of effector proteins that typically describe CD4 Tfh cells confirm the identity of CD8 Tfc cells in systemic autoimmunity.

Next, we validated that CD8 Tfc cells develop under IL-2–sufficient autoimmune conditions (47, 48). Scurfy mice lack functional Treg development, resulting in early systemic autoimmune disease (48). We first confirmed that, despite a reduction in the total number of IL-2–producing CD4 and CD8 T cells in the scurfy mice, the frequency of IL-2–producing cells was comparable to WT mice (Fig. 4A) (47). We identified a significantly expanded population of CXCR5$^+$PD-1^{hi} CD4 Tfh cells (3.0 ± 0.6%) and CXCR5$^+$PD-1^{hi} CD8 Tfc cells (1.0 ± 0.3%) in scurfy LNs compared with scurfy-HET LNs (Fig. 4B). Depleting IL-2 in scurfy mice using a neutralizing Ab or by genetic cross to the IL-2–KO background resulted in a higher percentage of CD4 Tfh and CD8 Tfc cells as compared with scurfy mutants but not IL-2–KO mice (data not shown). Similar to IL-2–KO mice, CD8 Tfc cells in scurfy mice expressed ICOS and Bcl6 (Fig. 4C, 4D). Together, these data demonstrate even under IL-2–sufficient conditions that CD8 Tfc cells can be identified during autoimmunity induced by the absence of functional Tregs. However, differences in CD8 Tfc cell frequency between the IL-2–KO and scurfy mice suggest that IL-2 may play a role in regulating the expansion of this distinct CD8 T cell population.

GC localization of CD8 T cells

CXCR5 expression by B cells and CD4 Tfh cells allows colocalization of these cells into the follicle, providing a site for productive T cell/B cell interactions. During chronic viral infection,

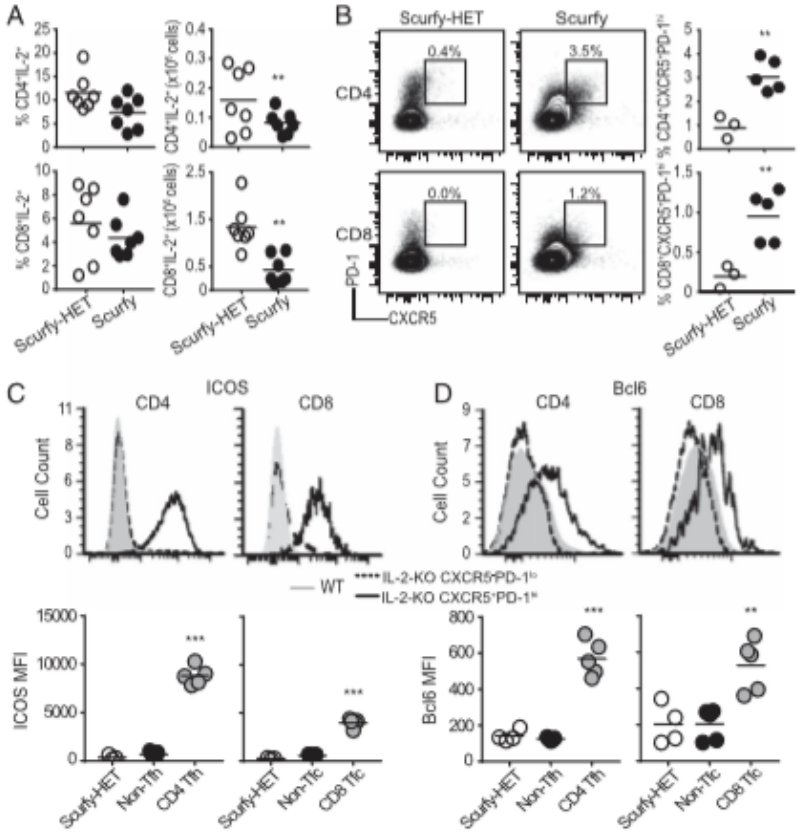

FIGURE 4. Autoimmune scurfy mice develop CD8 Tfc cells. Flow cytometric analysis of scurfy and scurfy-HET littermate control LNs. **(A)** Quantification of IL-2–producing CD4 and CD8 T cell frequency and total cell number after PMA and ionomycin stimulation. **(B)** CXCR5 and PD-1 expression on CD4 or CD8 T cells from 18- to 19-d-old scurfy or scurfy-HET gated on live B220$^-$ CD11c$^-$ CD11b$^-$ GR-1$^-$. Representative flow plots, frequency, and total number of CXCR5$^+$ PD-1^{hi} CD4 and CD8 T cells are shown. (**C** and **D**) Representative flow plots and quantification of MFI of expression of ICOS (C) and Bcl6 (D) in scurfy-HET naive bulk CD4 and CD8 T cells, scurfy CD4 non-Tfh or CD8 non-Tfc cells, and scurfy CD4 Tfh and CD8 Tfc cells (CXCR5$^+$PD-1^{hi}), following stimulation with PMA and ionomycin. Each symbol indicates an individual animal. Data are representative of three to six independent experiments. Statistics: unpaired Student *t* test relative to scurfy-HET (A, B, and D) with a Welch correction in (C). $^{**}p < 0.01$, $^{***}p \leq 0.001$.

CXCR5$^+$CD8 T cells have been reported both within the B cell follicle and, primarily, excluded from the follicle (20, 21, 23). CXCR5$^+$CD8 T cells localize to the follicle in ectopic GCs but not in the spleen and LN during arthritis and influenza infection (18, 22). We investigated whether CD8 T cells enter the B cell follicle during systemic autoimmunity. As IL-2–KO mice develop abnormal GC structure during late-stage disease (42, 49), we selected early disease–stage IL-2–KO mice with normal, albeit large, splenic gross morphology. To determine if CD8 Tfc cells were capable of GC localization, we evaluated GL-7 expression, a known GC-specific marker (50). Both IL-2–KO CD4 Tfh and CD8 Tfc cells express elevated levels of GL-7 (Fig. 5A). To examine CD8 T cell localization within IL-2–KO GCs, we compared immunized WT spleens with IL-2–KO spleens. GCs were defined as GL-7$^+$ GC B cells within IgD$^+$ B cell follicles (Fig. 5B). IL-2–KO spleens had significantly larger GC areas compared with immunized WT spleens. Nonetheless, 3.9-fold more CD8 T cells were present within a comparable area of IL-2–KO GC relative to those observed in immunized WT GCs (Fig. 5C). To determine if CD8 Tfc cells are capable of providing B cell help, we tested for the expression of CD154 (CD40L), as CD40/CD40L interactions are known to be crucial for B cell activation and Ab class switching in normal and autoimmune settings (51, 52). IL-2–KO CD8 Tfc cells were found to express CD40L upon stimulation (Fig. 5D). These results indicate that IL-2–KO CD8 T cells are capable of coexpressing B cell zone-specific markers (CXCR5 and GL-7), localizing to the GC, and expressing helper proteins, which may promote CD8 Tfc and B cell interactions that influence autoimmune disease.

CD8 Tfc cells promote B cell Ab class switch

To determine the influence of IL-2–KO CD8 Tfc cells on B cell activities, we examined CD8 Tfc cell production of the cytokine IL-21, an effector cytokine produced by CD4 Tfh cells (10). Stimulation of CD8 Tfc cells yielded similar levels of IL-21 (average mean fluorescence intensity [MFI] of 120), as compared with IL-2–KO CD4 Tfh cells (average MFI of 136), and significantly more than naive WT cells or IL-2–KO non-Tfc cells (Fig. 6A). Consistent with higher IL-21 production, both CD4 Tfh cells and CD8 Tfc cells expressed higher levels of IL-21 mRNA as compared with their CXCR5$^-$PD-1^{lo} counterparts (Fig. 6B). We next evaluated whether CD8 Tfc cells secreted proteins capable of influencing B cell Ab class switching using in vitro culture assays. WT B cells were cultured with and without αIgM and αCD40 plus supernatant from activated T cells (Fig. 6C). Supernatant from IL-2–KO CD8 Tfc cells induced significant amounts of total IgG and IgG1 production by B cells, comparable to levels produced by B cells with immunized WT CD4 Tfh cell and IL-2–KO CD4 Tfh cell supernatants (Fig. 6D). These data confirm that IL-2–KO CD8 Tfc cells are capable of inducing Ab class-switch recombination independent of CD4 Tfh cells.

To next evaluate the impact of CD8 T cells on B cell responses in vivo, we adoptively transferred IL-2–KO CD4 T cells, CD8 T cells, or a combination of both into TCRα-KO mice that were then immunized with KLH and reimmunized with NP-KLH (Fig. 6E). Fourteen days posttransfer, Fas$^+$GL-7$^+$ GC B cell expansion was measured relative to PBS-treated TCRα-KO mice. Transferred IL-2–KO CD4 T cells alone produced significant GC

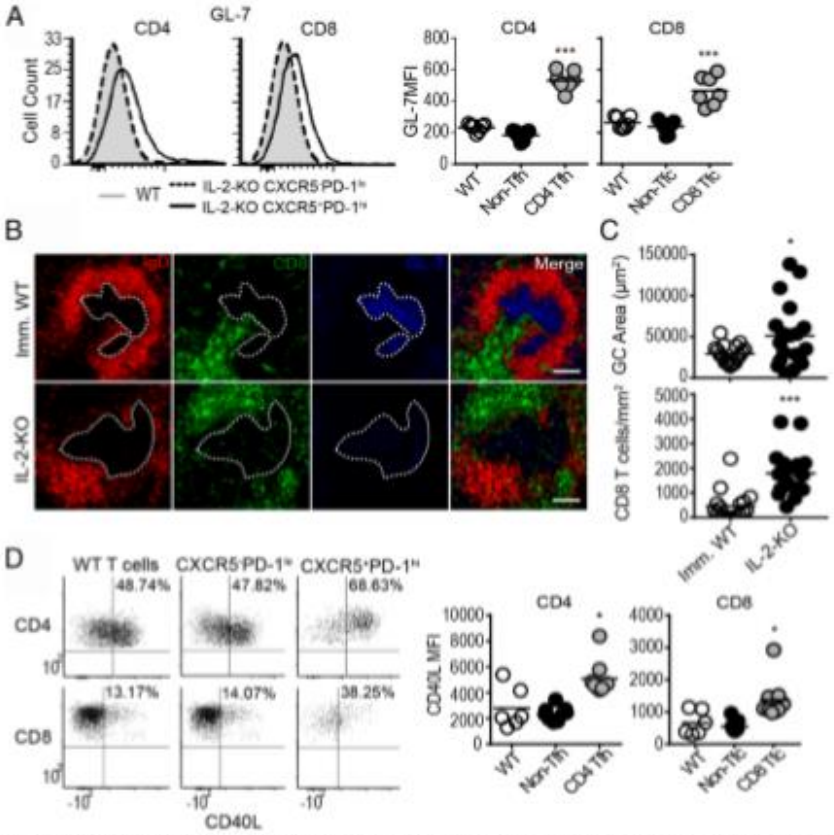

FIGURE 5. CD8 T cells localize to the GC during autoimmune disease. **(A)** Representative flow plots and MFI quantification of GL-7 expression in IL-2–KO and WT splenocytes in the indicated populations. **(B)** Immunofluorescence staining of B cells (red, IgD), GCs (blue, GL-7), and CD8 (green, CD8α) of spleens from 18- to 21-d-old IL-2–KO and KLH-immunized WT (Imm. WT) mice. White dotted lines indicate the GC outline. White scale bar, 100 μm. **(C)** Quantification of GC area (square micrometer) and CD8 T cells (per square millimeter). **(D)** Representative flow plots and MFI quantification of CD40L expression in IL-2–KO and WT CD4 and CD8 T cells stimulated with PMA and ionomycin. (A, C, and D) Each symbol indicates an individual animal. (A) Data from four independent experiments. (B and C) Data are representative of 5 spleens and 19 GCs from IL-2–KO mice or 4 spleens and 17 GCs in Imm. WT mice. (D) Data are representative of three independent experiments. Statistics: unpaired Student *t* test relative to WT (A) with a Welch correction in (C) and (D). $*p < 0.05$, $***p < 0.001$.

B cell expansion, whereas transfer of IL-2–KO CD8 T cells alone did not. A combination of IL-2–KO CD4 and CD8 T cells yielded similar GC B cell frequency as compared with IL-2–KO CD4 T cells alone (Fig. 6F). IL-2–KO CD4 and CD8 T cells, when transferred independently, induced a similar plasma cell expansion. Transfer of IL-2–KO CD4 and CD8 T cells together induced significantly more plasma cells than either population alone (Fig. 6G). Interestingly, when transferred alone, CD8 T cells did not induce Ab class switching. However, when transferred with CD4 T cells, CD8 T cells promoted an increase in IgG1 and significantly increased IgG2b as compared with the transfer of CD4 T cells alone (Fig. 6H). Together, these data demonstrate that CD8 T cells act synergistically with CD4 T cells to enhance B cells differentiation and specific class switching. CD8 Tfc cells, therefore, have the potential to provide a helper-like interaction within the GC to facilitate B cell Ab production during systemic autoimmune disease.

Discussion

In this study, to our knowledge, we provide the first evidence that a new class of CD8 Tfc cells develop during autoimmune disease in the absence of functional Tregs. In the setting of inflammation and autoimmunity, CD8 Tfc cells acquire CD4 Tfh cell–like functionality by producing Tfh cell effector cytokines and coreceptors and promoting B cell Ab class switching. CD8 Tfc cells promote Ab class-switch recombination at a level comparable to CD4 Tfh cells in vitro and synergize with CD4 Tfh cell responses in vivo. Our findings concur with recent reports describing the generation of similar CD8 Tfc cells during chronic infection (20, 21, 23, 53).

CD8 Tfc cells affected class-switch recombination in B cells through a cell-secreted factor in the supernatant. One likely factor is the cytokine IL-21, as it is produced by IL-2–KO CD8 Tfc cells. IL-21 is known to induce plasma cell differentiation, Ig production, and class switching. In vitro–derived CXCR5$^+$CD8 cells also produce IL-21 that promotes influenza-specific IgG production that is reduced in IL-21R–deficient B cells (22). In addition to the role of IL-21 in developing CD4 Tfh cell populations, other CD4 Tfh cell–secreted cytokines promote specialized Ab class switch, for example, IFN-γ supports IgG2a, and IL-4 supports IgG1 switching (54, 55). Although IL-2–KO disease has been described as a Th1-mediated disease (5), IFN-γ–mediated class switching to IgG2a was only detected when IL-2–KO CD4 T cells were transferred alone and was only moderately affected by cotransfer with CD8 T cells (Fig. 6H). In contrast, both CD8 depletion and cotransfer of CD4 and CD8 T cells most

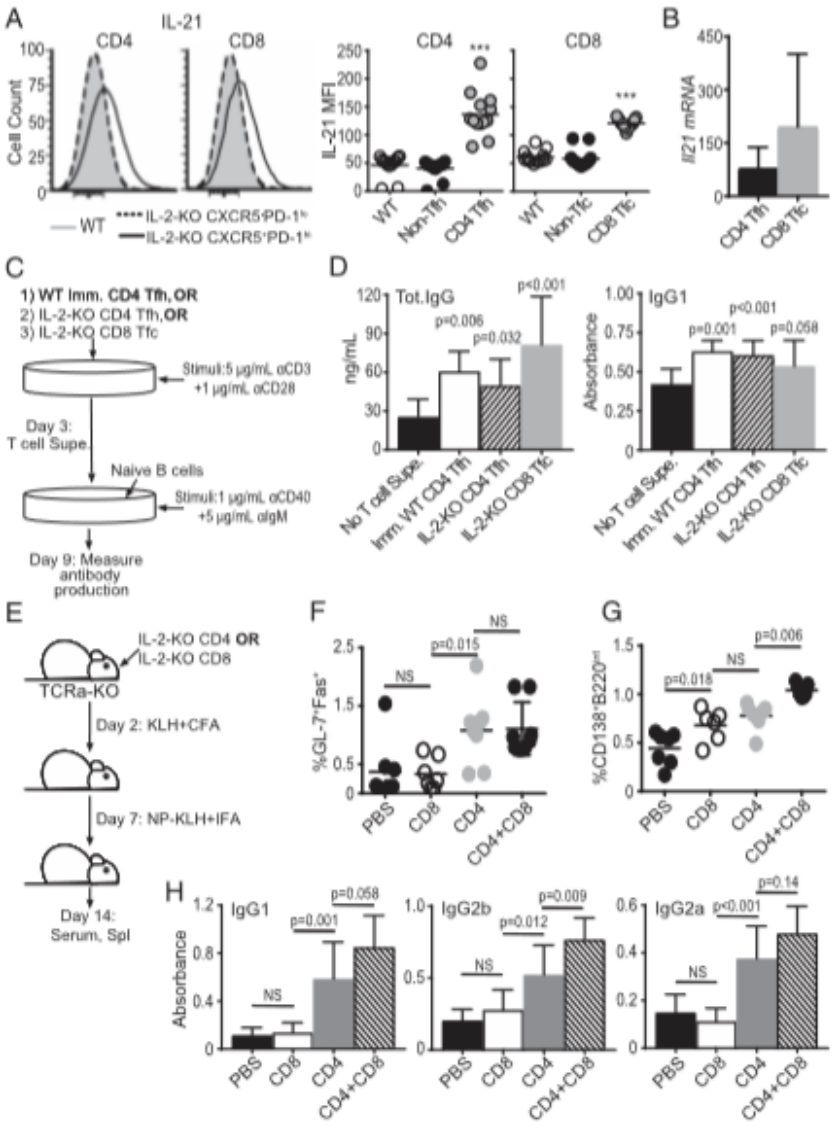

FIGURE 6. IL-2–KO CD8 Tfc cells promote Ab class switch by B cells. **(A)** IL-2–KO and WT lymphocytes were stimulated with PMA and ionomycin. CD8 and CD4 T cells were gated on the indicated populations and analyzed for IL-21 expression by flow cytometry. **(B)** IL-21 mRNA expression in sorted IL-2–KO CD4 Tfh or CD8 Tfc cells relative to IL-2–KO CD4 non-Tfh or CD8 non-Tfc cells, respectively. **(C)** Schematic describing the assay for T cell stimulation and B cell Ab induction. B cell supernatant was analyzed for total IgG and IgG1 by ELISA. **(D)** Total IgG concentration or IgG1 levels of stimulated B cells with and without stimulated IL-2–KO CD4 Tfh cells, IL-2–KO CD8 Tfc cells, or KLH-immunized WT (Imm. WT) CD4 Tfh cell supernatants were determined by ELISA. **(E)** Schematic describing IL-2–KO T cell transfer and B cell induction. Sorted IL-2–KO CD4 or CD8 T cells were adoptively transferred to TCRα-KO mice, immunized with KLH in CFA, and reimmunized with NP-KLH in IFA. **(F)** $B220^{+}GL\text{-}7^{+}Fas^{+}$ GC B cells frequency and **(G)** $B220^{int}CD138^{+}$ plasma cell frequency from TCRα-KO recipient spleens. **(H)** IgG1, IgG2b, and IgG2a levels determined by ELISA from TCRα-KO recipient serum. (A, F, and G) Each symbol indicates an individual animal. Data are representative of four to six independent experiments. Statistics: unpaired Student *t* test relative to WT with a Welsh correction (A) and ordinary one-way ANOVA with select comparisons and a Bonferroni correction (D and F–H). $^{***}p \leq 0.001$.

significantly impacted class switching to IgG1. Autoimmune interactions in the IL-2–KO mouse, and specifically in the GC, may be governed by a combination of cytokines, including IL-21 and IL-4, in contrast to the IFN-γ production found systemically. Additionally, elevated CD40L and ICOS expression by IL-2–KO CD8 Tfc cells was observed and may promote B cell function via direct cell/cell interactions. In IL-2–KO mice, CD8 T cells localize to the B cell follicle and can be identified within the GC. CD8 T cells migrate into proximity with B cells, providing the localization necessary for influencing GC B cell reactions.

We report that autoimmune disease is delayed in IL-2–KO mice in the absence of CD8 T cells. Expansion of CD8 Tfc cells is also delayed in comparison with the observed expansion of CD4 Tfh cells during disease progression. The kinetics suggest that CD4 T cell dysfunction proceeding CD8 Tfc cell expansion may promote a transition to more rapid, lethal autoimmunity. Our reported differences in the frequency of CD4 Tfh and CD8 Tfc cells in the IL-2–KO and scurfy mouse models also suggest a role for IL-2 in CD8 Tfc cell development. The absence of IL-2 contributes to a lymphoproliferative disorder in IL-2–KO mice that is not seen in scurfy mice, which may partially account for observed differences (48). However, IL-2 is known to suppress CD4 Tfh cell differentiation via Bcl6 expression in vivo (56). Thus, the difference in CD8 Tfc cell frequency between IL-2–KO mice and scurfy mice may be explained in part by the reduced numbers of IL-2–expressing cells in addition to differences in lymphoproliferation. As scurfy mice produce fairly normal levels of IL-2 (Fig. 4A, 47), other factors in addition to IL-2 loss likely contribute to CD8 Tfc cell development.

As a reduction or impairment of Tregs is the driving defect underlying autoimmunity in both IL-2–KO (2) and scurfy mice (47), the expansion of CD8 Tfc cells in both models is likely due, in part, to the breakdown in immune tolerance mechanisms that precedes autoimmune disease. During chronic inflammation and situations of high localized Ag, especially when immune regulation is compromised, CD4 Tfh cells expand (13, 16). Cellular expansion and

inflammation occurs in the IL-2–KO mouse because of reduced function and frequency of Foxp3$^+$ Tregs (2). Both CD4 and CD8 Tregs have been identified as essential regulators of GC tolerance (7, 8). The absence of CD4 Tfc regulatory cells has been shown to promote autoimmune disease and aging via increased CD4 Tfh cells, GC B cells, and Ab class switching (7, 57). Impaired Treg function may similarly allow for the development of CD8 Tfc cells during chronic inflammatory conditions.

Together, this study adds to a growing body of research supporting a helper role for CD8 T cells during chronic Ag exposure and inflammation, including chronic viral infections, cancer, and autoimmune disease. Our data provide a unique perspective on the role of CD8 T cells in GC interactions that may promote, amplify, or shift the autoimmune disease process. Future studies are needed to reveal the overlapping and distinct immune stages, roles, and influences of CD4 Tfh and CD8 Tfc cells during autoimmune disease progression. The identification of CD8 Tfc cell interactions within the GC provides many avenues for continuing research, including defining the contribution of CD8 Tfc cells to immunity and disease. An understanding of the types of Abs generated and the contribution to affinity maturation throughout the kinetics of CD8 Tfc cell development may unveil a new paradigm for GC reactions and a deeper insight into strategies for manipulating these processes.

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Disclosures

The authors have no financial conflicts of interest.

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CHAPTER 3

CD8 follicular T cells maintain diverse function during autoimmune promotion.

CHAPTER 3:CD8 follicular T cells maintain diverse function during autoimmune disease progression.

Introduction

A population of CXCR5+ CD8 T cells has recently been detailed during situations of chronic antigen and inflammation, including models of chronic viral infection, multiple cancer types, and antibody-mediated autoimmune disease (Chapter 1). In situations of chronic viral infection CXCR5+ CD8 T cells maintain an effector memory phenotype, capable of reseeding the CD8 T cell niche and controlling viral infection (1, 2). CXCR5+ CD8 T cells isolated from patients with hepatocellular carcinoma have cytolytic capacity towards tumor cells yet, cells isolated from colon cancer promote antibody-mediated immune responses (3, 4). Similarly, inflammatory CXCR5+ CD8 T cells maintain cytolytic function but produce helper-like cytokines included IL-21 and IFNγ (5, 6). Despite the emergence of comprehensive and robust research, a consistent description of CXCR5+ CD8 T cell functional responsiveness and developmental requirements remains elusive.

Like effector memory populations observed in chronic viral infections, CXCR5+ CD8 T cells express the transcriptional repressor bcl6 (1, 7). This is likely regulated by TCF-1, E2A, and e-related proteins Id2 and Id3 (2, 8). As in CD4 T follicular helper (Tfh) cells, CXCR5 provides a homing signal that facilitates CXCR5+ CD8 T cell migration to the CXCL13 expressing B cell follicles (5, 9, 10). Once in the germinal center, CD4 Tfh and CXCR5+ CD8 T cells via cell: cell contact and by cytokine mediated responses influence B cell differentiation, antibody class switch and memory (5, 6, 11).

In the context of autoimmunity, we have shown that PD-1+ CXCR5+ CD8 T cells (herein termed CD8 T follicular (Tfc) cells), localize to the germinal center, and are capable of mediating antibody class switch (12). CD8 Tfc cells, in the IL-2-deficient mouse model, arise in the absence of functional CD4 T regulatory cells (Treg) (13, 14). IL-2 signaling limits germinal center formation via impaired CD4 Tfh cell function and development (15). As CD4 Tfh cells display a reduced IL-2 dependency (16, 17), germinal center reactions may be altered, in part, by reduced frequency or function of CD4 Treg including CD4 T follicular regulatory cells (Tfreg). CD4 Treg and Tfreg absence enhances B cell mediated autoimmunity (18, 19). The role of IL-2 and Tregs on CXCR5+ CD8 T cell development, especially in the context of autoimmune disease, is not fully defined.

Here we show that autoimmune CD8 Tfc cells develop regardless of IL-2 production, but rather depend on inflammation and the absence of Tregs. Similar to data described for CXCR5+ CD8 T cells in viral infection and cancer, CD8 Tfc cells maintain cytolytic capacity but lack direct cell lysis. However, within the germinal center CD8 Tfc cells employ multiple cytokines and direct contact-mediated mechanisms to facilitate B cell responses. These data provide further evidence that CD8 Tfc cell development

requires exposure to chronic antigen and inflammation but maintain context specific function.

Materials and Methods

Mice, antibody depletions, and immunizations.

IL-2 knockout (KO), CD25 (IL-2Rα)-KO, IL-2xIFNγ-KO, and IL-2xCD28-KO were used with littermate IL-2 wildtype or heterozygous (WT) controls. IL-2 mediated autoimmune disease is not gender specific such that only age is used to determine disease and is indicated for each experiment. BALB/c hemizygous male Foxp3sf/Y (scurfy) mice and HET female FoxPsf/+ (scurfy-HET) littermate controls were used. Scurfy and scurfy-HET mice were depleted of IL-2 by 5 intraperitoneal (i.p.) injections from day 7-16 of age using 20µg/g mouse of anti-IL-2 (JES6-1A12; BioXcell) or PBS (Omega). MRL/MpJ-*Fas*^{lpr}/J (Fas^{lpr}) mice (JAX) aged 17 weeks or more. MRL/MpJ mice, as controls for Mrl.lpr mice were generously donated by Dr. Gabriela Loots (Lawrence Livermore National Laboratory). Diseased NOD mice generously donated by Dr. Hans Dooms (Boston University). Collagen-induced arthritis (CIA) mice were generated as described (20) using 6-8 week old C57BL/6 mice (JAX) injected intradermally with 100µg type II chicken collagen in complete Freund's adjuvant (CFA) containing 200µg tuberculosis mycobacterium supplemented at 2 weeks with a collagen boost of 50µg type II chicken collagen in incomplete Freund's adjuvant (IFA). Mice were monitored for 7 weeks post disease onset for use in experiments. B6N.129-*Il21r*^{tm1Kopf}/J (IL-21R-KO mice; JAX and donated by Mercedes Rincon (University of Vermont)) were used to isolate B cells. All mice were housed and bred in specific-pathogen free conditions in accordance with UC Merced's Department of Animal Research Services and approved by the UC Merced Institutional Animal Care and Use Committee.

Flow cytometry and cell sorting.

All antibodies were purchased from eBioscience at Fisher Scientific unless otherwise specified. Lymphocytes and splenocytes were processed and stained in 1% fetal bovine serum (FBS; Omega) phosphate buffered saline (PBS; Fisher Scientific). Splenocyte red blood cell (RBC) lysis was performed in 3mL of 1x ammonium chloride lysis buffer for 1 minutes at room temperature (RT). CXCR5+ PD-1+ CD4 Tfh or CD8 Tfc cells were identified as previously described (12). In brief, lymphocytes and splenocytes were stained first for CXCR5-biotin for 1 hour at RT, then stained for CD4, CD8, PD-1 (Biolegend), ICOS, fixable viability 506, streptavidin (SA; BD Biosciences), CD11c, CD11b, Ly6G (Gr-1), and B220 for 30 min at 4°C. CD4 Tfh and CD8 Tfc cells were gated by fluorescence^{-1} for CXCR5+PD-1+ cells. CD4 non-Tfh and CD8 non-Tfc cells were determined as CXCR5-PD-1-. Chemokine receptors CCR7 and CXCR4 (BD Bioscience) were identified on CD8 Tfc cells in comparison to CD8 non-Tfc cells by staining with CXCR5-biotin, CXCR4 and CCR7 for 45 min at 37°C as previously described (21) and gated by fluorescence^{-1} for CXCR4 and CCR7. Then cells were stained for surface proteins for 20 min at 4°C.

To isolate CD4 Tfh and CD8 Tfc cells, splenocytes and lymphocytes were processed and negatively selected for T cells using a murine PE-selection kit (Stem Cell Technologies) using CD11c, CD11b, Ly6G (Gr-1) and B220 in PE. T cells were then stained for CXCR5 and PD-1 and sorted at >85% purity. WT or IL-21R-KO naive B cells were isolated from splenocytes by staining for CD19, TCRβ, CD11c, CD11b, Gr-1 and fixable viability dye 506 for 30 min at 4°C. B cells were sorted at >95% purity. All flow cytometry and cell sorting were done on Becton Dickinson LSR-II and BD Biosciences Aria II cell sorter, respectively. Flow cytometric analysis was performed using FCS Express with Diva Version 4.07.005 (DeNovo Software) or FlowJo Version 10.1 (FlowJo).

T cell stimulations.

Processed cells as described were stimulated with 50ng/ml Phorbol 12-myristate 13-acetate (PMA) and ionomycin with brefeldin A (BFA) for 5 hours at 37°C, 5% CO2. Post-stimulatory staining was categorized into three panels; 1) cytolytic, 2) regulatory, 3) follicular. For all panels, cells were surface stained as follicular cells according to panels above except SA was used after fixations. Then cells were fixed using the Foxp3 fixation and permeabilization kit (eBioscience) then stained for intracellular cytokines as follows: 1) cytolytic, cells were stained with SA, perforin, granzyme B, and IFNγ or SA, granzyme B and IFNγ for 45 minutes at RT; 2) regulatory, cells were stained with IL-21R-chimeria for 1 hour at RT then with SA, IL-10, IFNγ, and IL-21R for 45 minutes at RT; 3) follicular, cells were stained tertiarily with IL-21R-chimeria for 1 hour at RT then with SA, IL-21R, IL-4, and IFNγ or with SA, IL-21R, IL-17, and IFNγ for 45 minutes at RT. To stain for CD107a, 5 µl in 200 µl stimuli was added to the culture during the 5 hour stimulation without BFA. BFA was added after the first hour.

RNA isolation and analysis of RNA next generation sequencing

RNA sequencing (RNAseq) of 4 independent WT and IL-2-KO Bulk CD8 T cell samples available at NCBI GEO (accession number GSE112540) and previously published (12) was downloaded for analysis. Two independent WT Bulk CD4 T cells available at NCBI GEO (accession number GSE34550 and GSE110598) and previously published (22, 23) were similarly processed, collected, and downloaded for analysis.IL-2-KO CD4 and CD8 Tfc cells were sorted from at least 6 pooled 15-16 day old lymph node (LN) and spleens. Samples were quick frozen and shipped to Expression Analysis for total RNA isolation and TruSeq stranded mRNA sequencing. Four samples were sequenced with 2 biological replicates for IL-2-KO CD4 Tfh and IL-2-KO CD8 Tfc cells. Raw reads were provided by Expression Analysis (Morrisville, NC) and were used for further analysis.

Adapter contamination removal and quality trimming were performed using Atropos v. 1.1.21 (24) with Python v3.7.2. Adapter sequences were removed using the heuristic algorithm and quality trimming was performed at the Q20 level. Read pairs were removed if either read was less than 20 bp after adapter removal and quality trimming. Read pairs were mapped to the primary assembly (C57BL/6J) of GRCm38/mm10 NCBI build 38.1 (GCF_000001635.20) using Rsubread v1.32.2 (25) reporting up to ten mapping locations with equal MAPQ scores. Reads pairs that failed to

map together had each read in the pair mapped independently. Read summarization was performed on the gene level using featureCounts (26) using annotations from a modified version of the RefSeq top level feature annotations for GRCm38/mm10 NCBI build 38.1 containing only protein coding genes. Multi-mapping reads were treated as fractional counts when mapping to several genes, reads mapping across more than one gene and read pairs where ends mapped to different chromosomes were discarded. Genes with less than one count per million in at least three samples were discarded, and read counts were TMM normalized (27). Differential expression analysis was performed using limma v3.38.3 (28) using voom transformed read counts (29). Genes were considered differential expressed if their p-value was less than 0.05 after multiple testing correction using Benjamini-Hochberg (30). Read mapping, summarization, and differential expression analysis were performed using R v3.5.2 and R v3.5.1. Differential expressed were annotated with their biological process with the enrichGO function in the clusterProfiler package using the org.Mm.eg.db packaged released on 2019-04-016.

In vitro T cell and B cell culture assays.

B cell responses to T cell supernatant were measured as described previously (12). Sorted IL-2-KO or IL-2.IFNγ dKO CD8 Tfc cells or CD4 Tfh cells were stimulated for 3 days with 5μg/ml anti-CD3ε and 1μg/ml anti-CD28 at 37°C. Naïve B cells sorted from the spleens of WT or IL-21R-KO mice were then incubated with T cell supernatant, 5μg/ml anti-IgM and 1μg/ml anti-CD40 for 6 days at 37°C. Supernatant from T and B supernatant cultures were analyzed for IgG and IgM antibody titers. B cell supernatant were prepared at a dilution of 1:50. Antibodies were detected with HRP-conjugated antibodies for total IgG and IgG1. Standard curves generated from purified mouse total IgG or IgG1 were used to calculated concentrations.

T cell adoptive transfer assays.

$0.5x10^6$ CD8 Tfc cells, $0.25x10^6$ CD4 Tfh cells or a combination of both were transferred on day 0 into TCRα-KO mice by retroorbital injection. Two days post-transfer mice were immunized with 200μg keyhole limpet hemocyanin (KLH; Sigma) in CFA by i.p. injection. Seven days post-transfer mice were boosted with 4-Hydroxy-3-nitrophenylacetyl (NP)-KLH (Sigma). Splenocytes and peripheral blood were isolated at day 14 post-transfer.

RBC antibody detections.

Peripheral blood isolated from WT, IL-2-KO or adoptive transfer mice was evaluated for RBC-reactive antibody titers as previously described. WT whole blood was isolated and washed 3 times in PBS and resuspended at 1% RBCs. 10μl of 1% RBC were incubated with anti-mouse IgG-FITC (Jackson ImmunoResearch Laboratories) at 1:50 dilution at 37°C or anti-mouse IgM-FITC (Jackson ImmunoResearch Laboratories) at 1:100 dilution at 4°C and assessed by flow cytometry.

Statistics.

All statistics, except for RNAseq, were performed on GraphPad Prism (Version 8.0.0). Differences between two means was assessed by unpaired Student *t* test with a

Welsh correction if indicated. Differences between multiple groups was performed by ordinary 1-way ANOVA with a Bonferroni correction. ELISA protein concentrations from a standard protein curve were interpolated using a sigmoidal four parameter logistic standard curve analysis. Statistical measurements were indicated in each figure legend.

Results

CD8 Tfc cells are a distinct CD8 T cell population in systemic autoimmunity.

Multiple studies, especially in cancer and chronic viral infection, utilize CXCR5 expression as the primary marker to delineate a population of follicular CD8 T cells (Chapter 1). We have defined a subset of the total CXCR5+ CD8 T cell population as PD-1+CXCR5+ CD8 Tfc cells in IL-2-KO autoimmune mice (12). In an effort to resolve differences in the current nomenclature; we now define the functional capacity of CD8 Tfc cells in comparison to PD-1-CXCR5+ cells. CD4 and CD8 T cells maintain similar total CXCR5+ T cell frequencies, yet CD8 T cells have reduced CXCR5+ CD8 T cell total numbers compared to that of the CXCR5+ CD4 T cells. CD4 CXCR5+PD-1+ CD4 Tfh cells comprise 50% of the total CXCR5+ CD4 T cell population while CXCR5+PD-1+ CD8 Tfc cell population comprise only 37% of the total CXCR5+ CD8 T cell frequency (Fig. 1A). Yet, CD8 Tfc cells express higher ICOS costimulatory molecules (Fig. 1B) and Bcl-6 transcription factor (Fig. 1C) than the CXCR5+ PD-1- CD8 T cells. The capacity of CXCR5+PD-1+ CD8 Tfc cells to maintain higher Tfh cell proteins, suggests potential functional differences. These may explain the reported functional differences reported between disease models (cancer, viral infection, and autoimmune disease) and cell types (CXCR5+PD-1+ versus CXCR5+PD-1-).

We next assessed CD8 Tfc cells differentiation in systemic and tissue specific autoimmune models. First, as expected, IL-2-KO CD8 Tfc cells frequency and total numbers were elevated when compared to wild-type (WT), naïve mice (Fig. 2). Similarly, IL-2Rα-KO mice, a counterpart for IL-2 signaling deficiencies, display increased CD4 Tfh and CD8 Tfc frequency and total number (data not shown). Previously, we demonstrated that CD8 Tfc cells arise in IL-2 sufficient scurfy systemic autoimmune mice (12). To further assess systemic autoimmunity unrelated to IL-2 deficiency, we evaluated Fas^{lpr} lupus mice. Fas^{lpr} mice develop severe lymphoproliferation and antibody-mediated systemic lupus erythematosus (SLE) associated with expanded Tfh cell frequency (31). CD4 Tfh cells promote B cell responses and antibody production in Fas^{lpr} mice primarily through extrafollicular interactions (32) although the exact mechanisms of interaction remain unclear. Splenic CD8 Tfc cell frequency (10.84+/-5.4%) and total number ($0.3x10^6$+/- $0.2x10^6$ cells) are expanded in aged Fas^{lpr} mice, similar to but less than, CD4 Tfh cells when compared to aged MRL.MpJ control mice (Fig. 2). However, when we assessed tissue-specific models autoimmunity including NOD and CIA CD8 Tfc cells did not arise despite a detectable CD4 Tfh cell population (Fig 2). Together these data suggest that CD8 Tfc cells arise in response to systemic antigen and lymphoproliferation. However, it is possible that CD8 Tfc cells may develop within ectopic germinal centers or draining lymph nodes during tissue-specific responses that have not yet been fully assessed.

Cytokines control CD8 Tfc cell differentiation.

CD4 Tfh cell development is mediated by antigen and costimulatory interactions from B:T interactions (11). To evaluate the influence of co-stimulatory and inflammatory cytokines, we first used IL-2.CD28 and IL-2.IFNγ double KO (dKO) mice. IL-2.CD28 dKO mice, in addition to reduced functional Tregs, are lymphopenic and have delayed autoantibody production until 3 months of age compared to IL-2-KO mice (33). IL-2.IFNγ dKO mice are similarly delayed in autoantibody production until 3 months of age yet develop early severe lymphoproliferative disorder (34). CD8 Tfc cell frequency is significantly reduced in IL-2.CD28 dKO (LN: 0.51+/-0.36%; Spl 0.59+/-0.25%) and IL-2.IFNγ dKO (LN:1.0+/- 0.34%; Spl 1.4+/-0.86%) late stage disease (12 weeks) relative to IL-2-KO (3.6+/-1.2%) late stage disease (3 weeks). Because autoimmunity develops late in IL-2.IFNγ dKO mice, CD8 Tfc cell expansion occurs late in disease, thus explaining autoantibody development at 3 months. CD8 Tfc cell presence alone does not explain the delayed autoantibody production as a comparable population of CD8 Tfc cell was detected during early disease (3 weeks) and late disease (12 week) timepoints (Fig. 3B). However, late disease CD8 Tfc cell total numbers are significantly expanded in comparison to early disease, as the LN and spleen continue to enlarge. These data suggest a role for CD28 and IFNγ signaling in CD8 Tfc cell development.

CD8 Tfc cells also develop in the IL-2-sufficient, Treg-deficient scurfy mice (12). However, the CD8 Tfc cell expansion in IL-2-KO mice is larger than in scurfy mice likely due to differences in lymphoproliferation (35), the presence of some Tregs in IL-2-KO mice (13) or the absence of IL-2. To begin defining the role of Treg deficiency and IL-2 on CD8 Tfc cell development, we depleted IL-2 in scurfy mice using anti-IL-2 antibodies. Splenocytes from IL-2-depleted scurfy mice maintain a similar CD8 Tfc cell frequency (1.3+/-0.71%) to untreated scurfy controls (0.6+/-0.15%), both of which are significantly reduced in comparison to IL-2-KO mice (Fig 2C). To determine if germline IL-2 deficiency may play a greater role in CD8 Tfc cell development, we backcrossed scurfy heterozygous females to WT males for F1 progeny that contained both IL-2-KO and scurfy IL-2-KO (scurfy.IL-2). Scurfy.IL-2 CD8 Tfc splenocytes are mildly elevated relative to scurfy CD8 Tfc cells yet, scurfy.IL-2 CD8 Tfc cells are significantly reduced in comparison to IL-2-KO (Fig. 3D). Further, the total splenocyte number in scurfy, IL-2-depleted scurfy and scurfy.IL-2 mice were reduced compared to IL-2-KO mice (Fig 3E). Although total splenocyte numbers were slightly increased when comparing IL-2-depleted scurfy and scurfy.IL-2 mice against scurfy mice. These data suggest that the development of CD8 Tfc cells is likely due to the absence of functional Tregs rather than IL-2 deficiency. The absence of IL-2 and subsequent lymphoproliferation then induces CD8 Tfc cells expansion.

CD8 Tfc cell transcriptional profile mirrors CD4 Tfh cells.

CD8 Tfc cell maintain a transcriptional phenotype that is distinct from both the naïve CD8 T cell and CXCR5- CD8 T cell transcriptional profiles (1). In autoimmune disease CD8 Tfc cells upregulate genes associated with a CD4 Tfh phenotype (12). To determine how closely CD8 Tfc cells isolated from an autoimmune setting resemble canonical CD8 T cells or CD4 Tfh cells we performed RNAseq of IL-2-KO CD4 Tfh and

IL-2-KO CD8 Tfc cells sorted from day 15-16 of age, the earliest timepoint at which CD8 Tfc cells could be readily isolated. Cells were sorted from at least 6 IL-2-KO pooled spleens and LNs. CD8 Tfc cell transcripts were first compared to day 12 IL-2-KO bulk CD8 T cell RNAseq data from which 5167 genes were significantly differentially expressed (Fig 4A). CD4 Tfh associated genes *cxcr5*, *pdcd1* (PD-1), *icos*, and *bcl6* are significantly upregulated in CD8 Tfc cells compared to IL-2-KO bulk CD8 T cells, validating the CD8 Tfc cell sorting criteria and similarity to CD4 Tfh cells. An additional 208 genes are associated with CD8 T cell differentiation and 355 genes are associated with CD8 T cell activation, including *cd28* and *cd44* upregulation and *s1pr1* and *ccr7* downregulation. Despite the CD4 Tfh-like transcriptional profile of CD8 Tfc cells, a large number CD8 effector transcripts were expressed, such as significantly increased *lamp1* (CD107a), *ctla4*, *fasl*, and *tbx21* (T-bet), and decreased *gzma*. Because CD8 Tfc cells upregulate genes closely associated with canonical cytolytic CD8 T cell function, we sought to compare IL-2-KO CD8 Tfc cell to IL-2-KO CD4 Tfh cells, naïve WT CD8 T cells and WT CD4 T cells. Principal component analysis (PCA) using transcript expression data segregated samples into distinct groups along the first two principal components with IL-2-KO CD8 Tfc cells more closely associated to IL-2-KO CD4 Tfh cells than bulk CD8 T cells (Fig. 4B).

To identify gene transcripts that are specific to differences between CD8 Tfc cells and CD4 Tfh cells rather than genes that define CD8 and CD4 T cell development, we first compared the transcriptional profile of CD8 WT cells to CD8 Tfc cell (CD8WT/CD8Tfc), yielding 3793 significantly expressed genes and CD4 WT cells to CD4 Tfh cell (CD4WT/CD4Tfh), yielding 5618 significantly expressed genes. Then we evaluated the differences between these comparisons (CD8WT/CD8Tfc)/(CD4WT/CD4Tfh), revealing 2146 genes that respond differentially between the two groups. We identified 579 genes that are associated with follicular cells (CXCR5+PD-1+) and 1567 genes that are differentially regulated between CD4 Tfh and CD8 Tfc, but do not define them as CD4 or CD8 T cells (Fig 4C). Of the differential gene expression related to CD8 Tfc cells (3793 genes), 40% are associated with maintaining a CD8 phenotype and 60% are associated with a CD4 Tfh cells. Together these data demonstrate that CD8 Tfc cells maintain a unique transcriptional profile that contains gene expression from both a canonical CD8 T cell and CD4 Tfh cell profile.

CD8 Tfc cells maintain diverse functional capacity

In chronic viral infection and some cancer settings CXCR5+CD8 T cells maintain the capacity for cytolytic function by perforin and granzyme B protein expression (9, 36, 37). As RNAseq analysis revealed that IL-2-KO CD8 Tfc cells transcriptionally express cytolytic genes, we investigated whether CD8 Tfc cells maintain cytolytic capacity during autoimmune disease. We examined the production of granzyme B, perforin, and CD107a. Stimulated IL-2-KO CD8 Tfc cells express significantly more granzyme B, CD107a and TNFα but not perforin by mean fluorescent intensity (MFI) than IL-2-KO CD8 non-Tfc and naïve WT CD8 T cells (Fig. 5A). Thus, CD8 Tfc cells maintain lytic capacity in an autoimmune setting.

CD8 T cells expressing IL-21 in human nasal polyps co-express IL-17 and IFNγ, and promote IgG class switch in vitro (6) suggesting a role for CD8 T cell cytokine mediated antibody class switch. To define a mechanism by which CD8 Tfc cells promote antibody class switch we evaluated germinal center reaction associated cytokines. IL-2-KO CD8 Tfc cells produced significantly more IFNγ and IL-4 but not IL-17 by MFI than IL-2-KO CD8 non-Tfc cells and naïve WT CD8 T cells (Figure 5B). By frequency, CD8 Tfc cells also co-produce IL-21 and IFNγ (14.15%), with a greater frequency expressing IFNγ (69.70%) alone (Figure 5C). CD8 Tfc cells co-produce IL-21 and IL-4 (4.13%), with a greater frequency expressing IL-4 (12.27%) alone (Figure 5D). CD8 Tfc cells maintain a diverse repertoire of cytokine expression with the capacity to be both lytic and promote helper-like responses.

CD8 Tfc cells direct class switching in autoreactive B cells.

IL-2-KO CD8 T cells promote antibody class switch to IgG1 and IgG2b upon in vivo transfer (12). To investigate the mechanisms by which CD8 Tfc cells promote antibody class switch we used a supernatant co-culture system to combine activated CD8 Tfc cell supernatant with B cells. First, given that IgG1 class switch is mediated by CD4 Tfh produced IL-21 and IL-4 (38), we cultured activated CD4 Tfh or CD8 Tfc supernatant with WT B cells or IL-21R-KO B cells to evaluate class switch in the absence of IL-21 signaling. CD4 Tfh cell mediated total IgG and IgG1 antibody class switch in IL-21R-KO B cells is reduced whereas CD8 Tfc cell mediated antibody class switch is largely unaffected (Fig 6A). Given that IL-2-KO CD8 Tfc cells produce significant IFNγ, we evaluated B cell responses mediated by IFNγ from CD8 Tfc cells. Activated IL-2-KO or IL-2.IFNγ dKO CD4 Tfh or CD8 Tfc cell supernatant was incubated with WT B cells to assess antibody class switch in the absence of IFNγ. In BALB/c mice IFNγ mediates IgG2a class switch (39) but we identified no differences in IgG2a between IL-2-KO and IL.2.IFNγ dKO supernatant, and a slight increase in total IgG in the absence of T cell produced IFNγ (Fig 6B). Thus, although CD8 Tfc cells promote cytokine-mediated B cell responses, the specific combination of cytokines mediating these differs from CD4 Tfh cells.

Here we show that CD8 Tfc cells maintain a significant capacity for cytokine production. However, B cell responses to cytokine are often initiated in the extrafollicular region of the B cell follicle for plasma cell development (40) followed by somatic hypermutation and B cell cycling within the germinal center (41). We assessed CD8 Tfc cell potential for localization to and around the germinal center by evaluating chemokine expression. Increased CXCR4 expression coupled with decreased CCR7 expression identifies a population of extrafollicular CD4 T cells in autoimmunity and viral infections (21, 32). CXCR4 and CCR7 staining identify a subset population of CCR7+ CD8 Tfc cells that are likely in the T cell zone or transition to the B cell zone. Of the CD8 Tfc cells that are CCR7- cells only a small proportion are CXCR4+ indicating the potential for extrafollicular interactions. CXCR4+ CCR7- CD8 Tfc cells are reduced in comparison to the CXCR4- IL-2-KO CD8 Tfc cells suggesting that the majority of CD8 Tfc cells are only CXCR5+ indicating a germinal center population (Fig. 6C).

Finally, to assess CD8 Tfc cell influence on autoreactive B cell responses, we adoptively transferred IL-2-KO CD8 Tfc, CD4 Tfh or both into TCRα-KO mice. Fourteen days after KLH and NP-KLH immunizations (to facilitate B-T interactions), we evaluated autoantibodies against RBCs. IL-2-KO CD8 Tfc and CD4 Tfh cells alone did not induce a significant increase in the frequency of RBC bound by either IgM or IgG. Yet, in combination, IL-2-KO CD8 Tfc and CD4 Tfh transfer induced a significant increase in frequency of IgM and IgG antibodies bound to RBC (Fig 6D). Together these data demonstrate that CD8 Tfc cells mediate B cell responses at least in part via cytokine secretion within the germinal center. To facilitate autoantibody responses CD8 Tfc cells likely work in synergy with CD4 Tfh cells.

Discussion

Together, these data demonstrated that CD8 Tfc cells comprise a functionally, non-redundant subset of CXCR5+ CD8 Tfc cells in autoimmune disease. CD8 Tfc cells develop in the absence of functional Tregs and expand during lymphoproliferative responses. CD8 Tfc cells maintain significant cytokine function to promote B cell class switch and antibody production in autoimmune disease.

By transcriptional profile and protein expression, CD8 Tfc cells resemble CD4 Tfh cells. IL-2-KO CD8 Tfc cells maintain the capacity to produce helper cytokines including the co-expression of IL-21 and IL-4. However, CD8 Tfc cells maintain the capacity for cytolytic function and express granzyme B and TNFα, suggesting the potential for functional mechanisms that diverge from CD4 Tfh cells. While both IL-2-KO CD4 Tfh and CD8 Tfc cells promote cytokine-mediated antibody responses, CD8 Tfc cells do not utilize IL-21 to mediate B cell responses. This is in contrast to CD4 Tfh cells that utilize IL-21 for antibody class switching (40). Instead CD8 Tfc cells likely utilize IL-4 interactions to mediate IgG1 class switch and perhaps direct cell contact interactions such as CD40L known to elevated on CD8 Tfc cells (12).

Requirements for CD8 Tfc cell development are similarly conserved between CD4 Tfh and CD8 Tfc cells. CD28 and ICOS, a member of the CD28 costimulatory family, are required for Tfh cell development (42, 43) CD8 Tfc expansion is impaired in IL-2.CD28 dKO mice suggesting that CD28 signals are required for CD8 Tfc development. Alternatively, the lymphopenic environment in IL-2.CD28 dKO mice may block CD8 Tfc differentiation. Further, reduced IFNγ signaling in autoimmune disease prevents germinal center B and Tfh cell development and accumulation (44), and the absence of IFNγ signaling similarly reduces CD8 Tfc cell expansion in IL.2.IFNγ dKO mice. However, despite the reduced frequency of IL.2.IFNγ dKO CD8 Tfc cells, lymphoproliferation leads to expanded CD8 Tfc cell total numbers.

CD8 Tfc cell development is associated with Treg dysfunction in both the IL-2-KO and scurfy mouse models. Yet, some questions remain regarding the contribution of Treg deficiency or IL-2 signaling to CD8 Tfc cell development. IL-2 inhibits CD4 Tfh cell differentiation via STAT5 (15, 16). When IL-2 is depleted in scurfy mice CD8 Tfc cells fail to expand but IL-2-KO.scurfy mice have an increased frequency of CD8 Tfc cells. This contradictory data can be explained, in part, by the lymphoproliferation

exhibited by IL-2.scurfy (35) that is absent in IL-2-depleted scurfy. Together, this supports the hypothesis that Treg deficiency releases CD8 Tfc cell function while IL-2 deficiency promotes lymphoproliferation associated with CD8 Tfc cell expansion. To fully evaluate the role of Treg control on CD8 Tfc cell development additional studies are needed.

CD8 Tfc cells have not been separated from the total CXCR5+ CD8 T cell population in chronic viral infection and cancer studies (Chapter 1). However, CXCR5+PD-1+ CD8 Tfc cells maintain a higher capacity for co-stimulatory and transcription factor expression than CXCR5+PD-1- CD8 T cells in autoimmune disease. The differences in the helper function attributed to autoimmune CD8 Tfc cells as compared to the cytolytic function of CD8 Tfc cells in chronic viral infection may be in part related to different CXCR5+ CD8 T cell subset definitions. A consistent description of CXCR5+ CD8 T cells may resolve remaining conflicts in function and classification across different immune reactions. Although it is still probable that CD8 Tfc cell function is dictated by antigen type and prevalence.

IL-2-KO CD8 Tfc cells alone promote B cell responses in vitro. In vivo, IL-2-KO CD8 Tfc cells only promote autoantibody specific responses in synergy with CD4 Tfh cells. This may result from a CD4 Tfh cell requirement for CD8 Tfc development. CD4 Tfh cell may be similarly required for CD8 Tfc cell survival. CD8 Tfc cells promote autoreactive responses and arise in significant frequency during late stage disease in IL-2-KO mice. CD8 Tfc cell kinetics provide a mechanism by which CD8 Tfc cells promote enhanced antigen-specific B cell responses during the disease progression. CD8 Tfc cells are an effector T cell population closely associated with late-stage autoimmune disease and as such represent a promising avenue for autoimmune treatment.

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FIGURES AND FIGURE LEGENDS

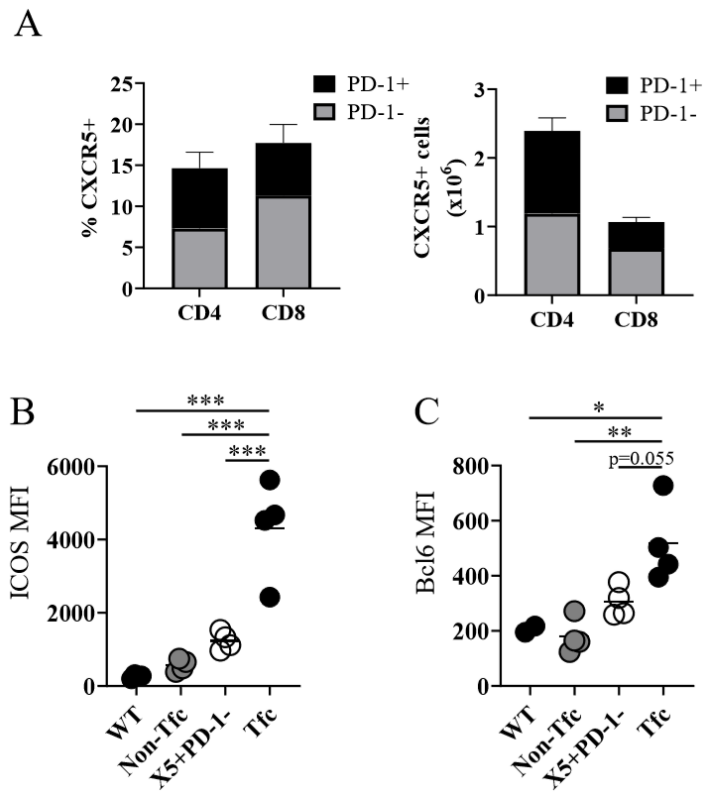

Figure 3-1. CXCR5+ CD8 T cells are comprised of multiple subsets. Flow cytometric analysis of CXCR5 and PD-1 expression on CD4 or CD8 T cells from 17-21-day old IL-2-KO or WT LN gated on live $B220^{-}CD11c^{-}CD11b^{-}GR\text{-}1^{-}$. (**A**) The frequency, and total number of IL-2-KO CXCR5+ CD4 and CD8 T cells are shown as $CXCR5^{+}PD\text{-}1^{+}$ and $CXCR5^{+}PD\text{-}1^{-}$ subsets. (**B, C**) Mean fluorescence intensity (MFI) quantification of surface expression of ICOS (**B**) and intracellular Bcl-6 (**C**) in WT naïve CD8 T cells, IL-2-KO CD8 non-Tfh, IL-2-KO $CXCR5^{+}PD\text{-}1^{-}$ (X5+PD-1-), and IL-2-KO CD8 Tfc cells. (B, C) Each symbol indicates an individual animal. Data is representative of 4 independent experiments in A and B and 2-4 independent experiments in C. Statistics: (A) 2-way ANOVA with multiple comparisons and Bonferroni correction, (B) 1-way ANOVA with multiple comparisons and Bonferroni correction* $p<0.05$; ** $p<0.01$, *** $p\leq0.001$.

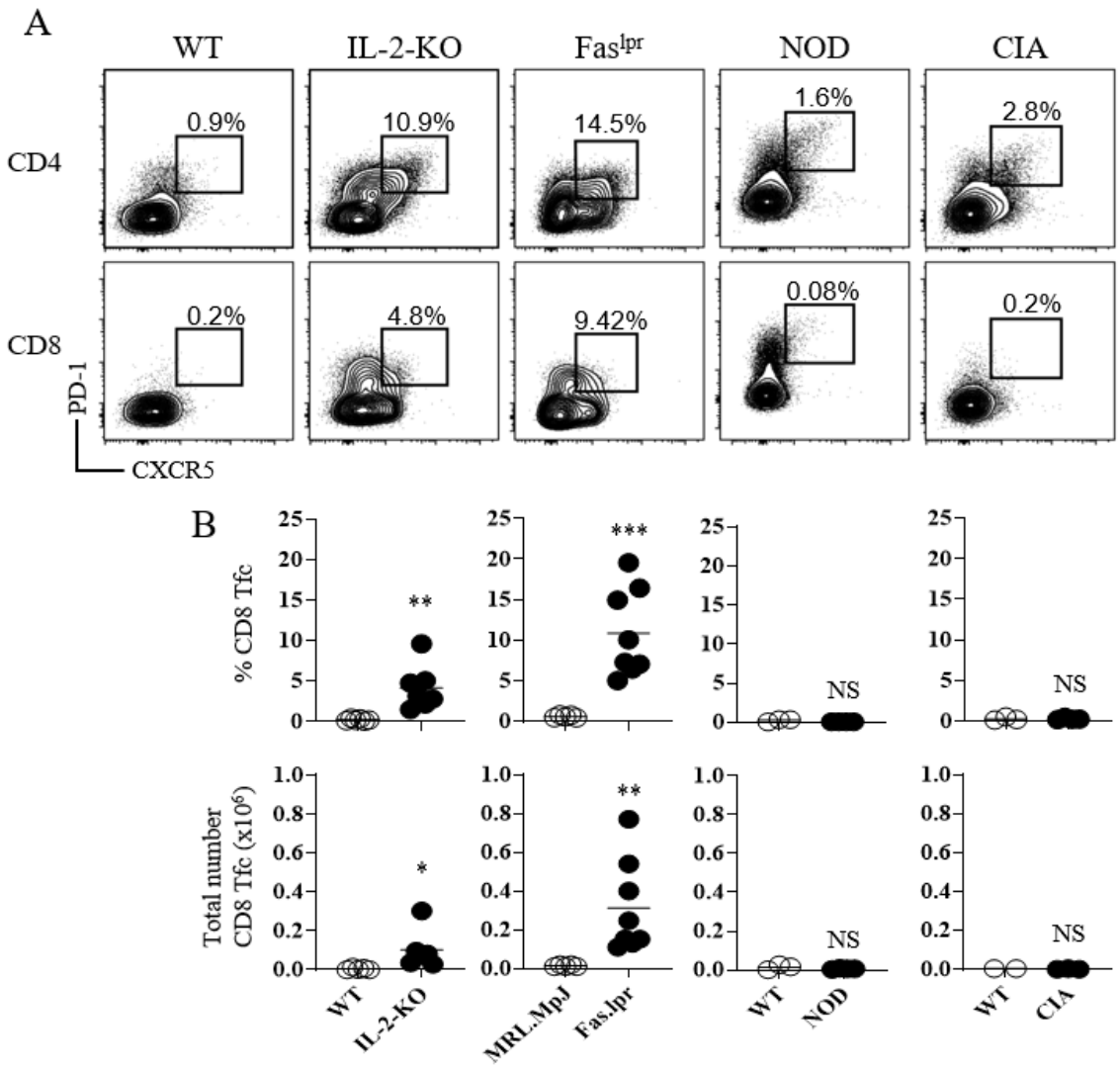

Figure 3-2. CD8 Tfc cells arise in multiple models of autoimmune disease. Flow cytometric analysis of CXCR5 and PD-1 expression on CD4 or CD8 T cells splenocytes from 17-21 day old IL-2-KO and WT littermate controls, 17-23 week old Fas^{lpr} and MRL.MpJ controls, diseased NOD and 6-14 week old WT controls, and 7 week disease CIA and WT PBS control mice gated on live B220^{-}CD11c^{-}CD11b^{-}GR-1^{-}. (A) Representative flow plots of the indicated model are shown for CD4 Tfh and CD8 Tfc cells. (B) Frequency and total number of CD8 Tfc cells in the indicated model are shown. Each symbol indicates an individual animal. Data representative of 1-3 independent experiments per comparison. Statistics: unpaired one-tailed Student's t-test relative to indicated controls with a Welch correction if necessary: NS = not significant, * $p<0.05$; ** $p<0.01$, *** $p\leq0.001$.

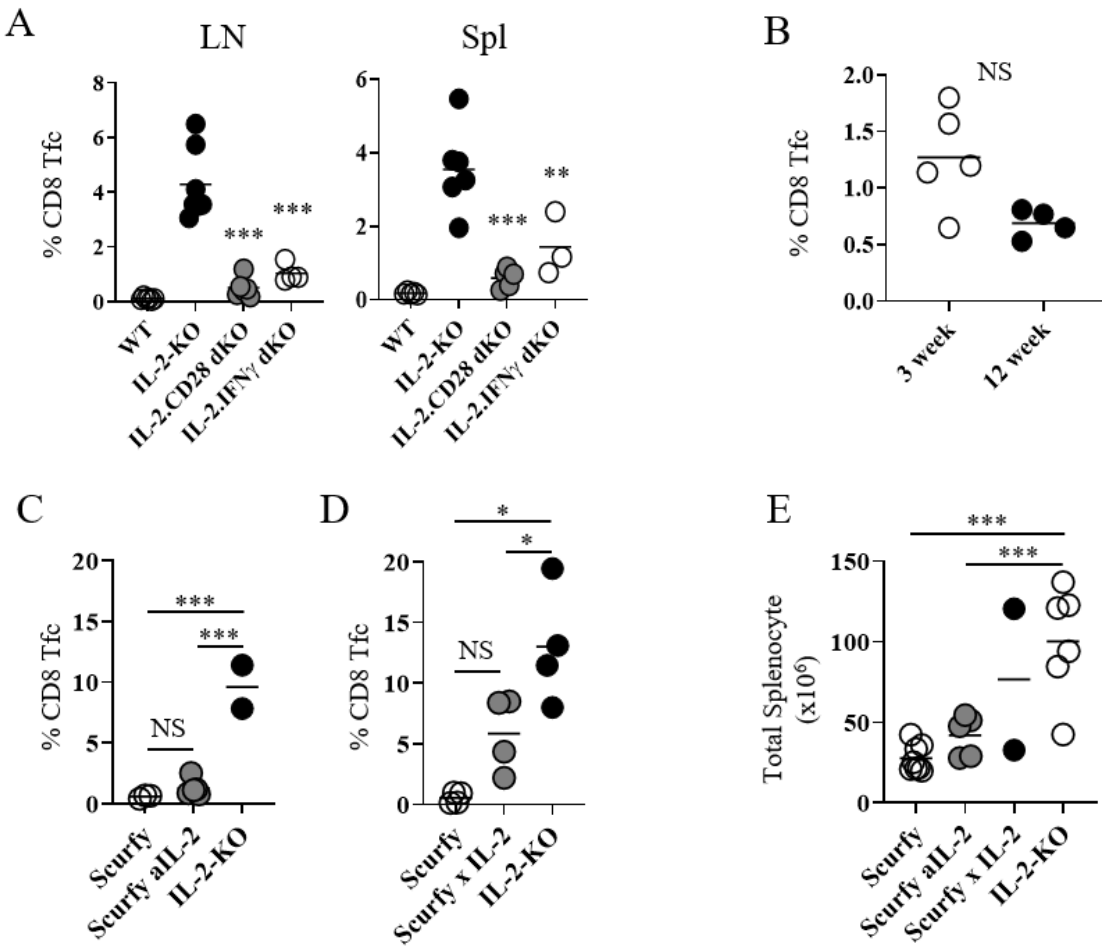

Figure 3-3. Cytokines control CD8 Tfc differentiation. Flow cytometric analysis of CD8 Tfc cells gated on live CXCR5^{+}PD-1^{+}B220^{-}CD11c^{-}CD11b^{-}GR-1^{-}. (**A**) The frequency of CD8 Tfc cells LN and spleen (Spl) of 17-21-day old IL-2-KO and WT, and 12-week old IL-2.CD28 dKO and IL-2.IFNγ dKO mice. (**B**) Splenic CD8 Tfc cell frequency from 3 week and 12-week-old and IL-2.IFNγ dKO mice. (**C**) Splenic CD8 Tfc cells frequency from 17-19-day old IL-2-KO mice and scurfy mice treated 5 times with PBS (scurfy) or anti-IL-2 (scurfy aIL-2). (D) Splenic CD8 Tfc frequency in 18-20-day old IL-2-KO, scurfy, and scurfy.IL-2-KO (Scurfy.IL-2). Each symbol indicates an individual animal. Data representative of 2-4 independent experiments in A, B and 3 independent experiments in C-E. (**D**) Total splenocyte number in scurfy, scurfy aIL-2, Scurfy.IL-2, and IL-2-KO mice described in C and D. Statistics (A, C-E) 1-way ANOVA with multiple comparisons and Bonferroni correction. (B) unpaired Student's t-test with a Welch correction. NS = not significant, * $p<0.05$; ** $p<0.01$, *** $p\leq0.001$.

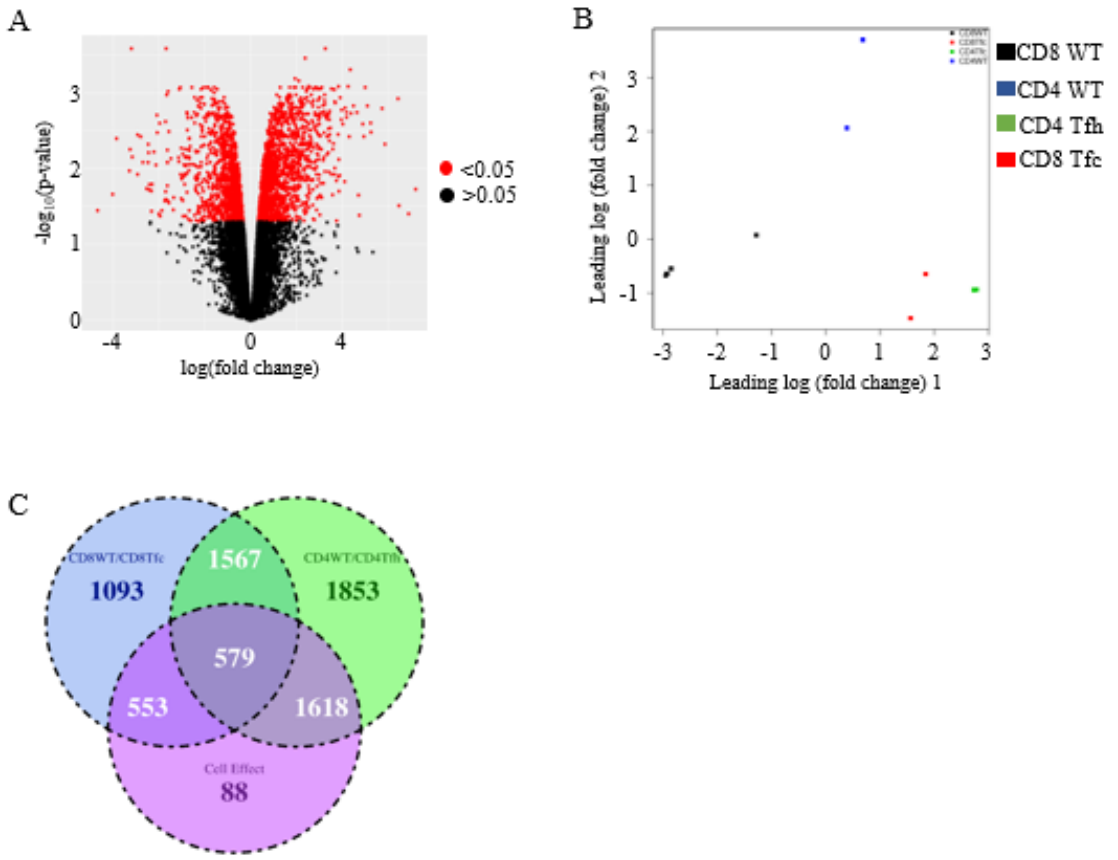

Figure 3-4. CD8 Tfc cell transcriptional profile mirrors CD4 Tfh cells. RNAseq of 4 independent WT and IL-2-KO Bulk CD8 T cell samples available at NCBI GEO (accession number GSE112540), 2 independent WT Bulk CD4 T cells available at NCBI GEO (accession number GSE34550 and GSE110598), and 2 independent IL-2-KO CD4 Tfh and CD8 Tfc from 6-7 pooled LN and Spl. (**A**) Volcano plots displaying log2 fold change gene expression of IL-2-KO Bulk CD8 T cells relative to IL-2-KO CD8 Tfc cells versus log10 p-value. Data is organized by color red to indicate p-value <0.05 and black to indicate p-value >0.05. (**B**) principal component analysis (PCA) comparing WT CD8, WT CD4, IL-2-KO CD4 Tfh and IL-2-KO CD8 Tfc. (**C**) Venn diagram of significantly expressed genes overlapping between WT CD8, WT CD4, IL-2-KO CD4 Tfh and IL-2-KO CD8 Tfc.

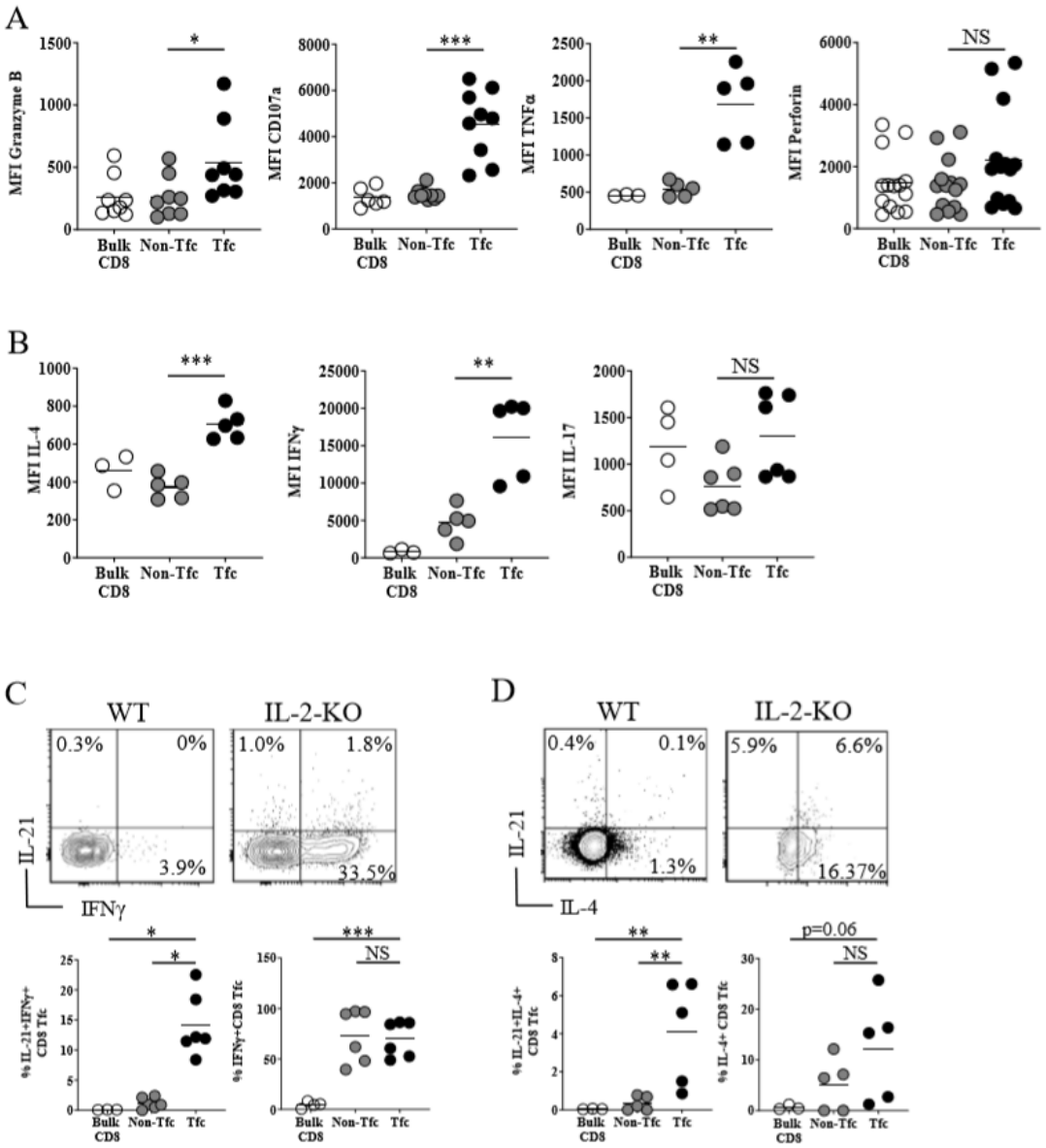

Figure 3-5. CD8 Tfc cells maintain diverse functional capacity. IL-2-KO and WT lymphocytes were stimulated with PMA and ionomycin with BFA stained with indicated cytokines and analyzed for CD8 Tfc cells gated on live B220$^-$CD11c$^-$CD11b$^-$GR-1$^-$. (**A**) MFI of Granzyme B, CD107a, TNFα, and Perforin expression on naïve WT CD8, IL-2-KO CD8 Tfc and IL-2-KO CD8 non-Tfc. (**B**) MF1 of IL-4, IL-17, IL-21 and IFNγ expression on naïve WT CD8, IL-2-KO CD8 Tfc and IL-2-KO CD8 non-Tfc. (**C**) Representative plots and frequency of IL-21 and IFNγ co-producing and IFNγ producing alone in WT CD8 Bulk T cells, IL-2-KO CD8 non-Tfc IL-2-KO CD8 Tfc cells. **D**) Representative plots and frequency of IL-21 and IL-4 co-producing and IL-4 producing alone in WT CD8 Bulk T cells, IL-2-KO CD8 non-Tfc IL-2-KO CD8 Tfc cells. Each symbol indicates an individual animal. Data representative of 3-6 independent experiments. Statistics: unpaired one-tailed Student's t-test relative to WT with a Welch correction if necessary: NS = not significant, * $p<0.05$; ** $p<0.01$, *** $p\leq0.001$.

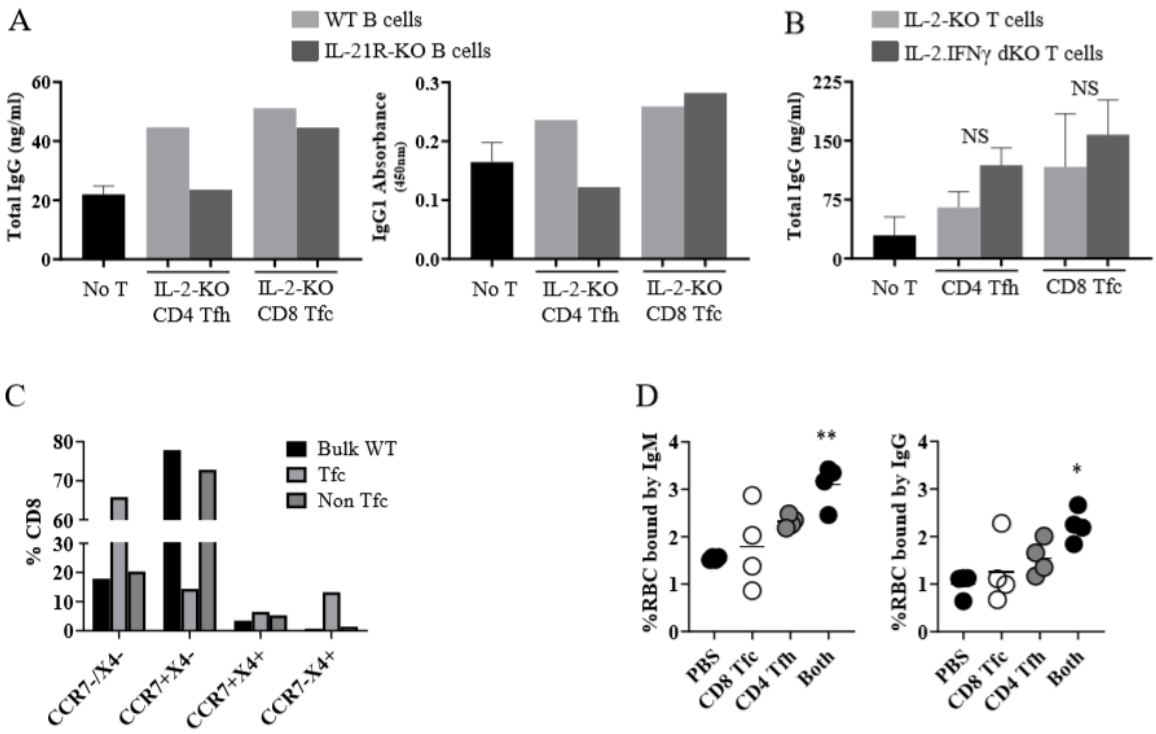

Figure 3-6. CD8 Tfc cells direct class switch in autoreactive B cells. (A, B) Sorted IL-2-KO or IL-2.IFNγ dKO CD4 Tfh and CD8 Tfc cells were stimulated for 3 days with anti-CD3 and anti-CD28. T cell supernatant was then plated with B cells and anti-IgM and anti-CD40 for 6 days. (**A**) Total IgG concentration or IgG1 levels of stimulated WT and IL-21R-KO B cells with and without stimulated IL-2-KO CD4 Tfh or IL-2-KO CD8 Tfc were determined by ELISA. Data is representative of 1 independent experiment (**B**) Total IgG concentration of stimulated WT B cells with and without stimulated IL-2-KO or IL-2.IFNγ dKO CD4 Tfh or CD8 Tfc cells were determined by ELISA. Data representative of 4 independent experiments. (**C**) Frequency of CXCR4 and CCR7 expression WT bulk CD8 T, IL-2-KO CD8 Tfc and CD8 non-Tfc. Data is representative of 1 experiment with 1 WT and 5 pooled IL-2-KO. (**D**) Sorted IL-2-KO CD4 Tfh or CD8 Tfc cells were adoptively transferred to TCRα-KO mice, immunized with KLH in CFA and reimmunized with NP-KLH in IFA. RBCs were stained at 14 days post-transfer with anti-mouse IgM-FITC or IgG-FITC and analyzed by flow cytometry to detect the percentage of RBC bound by antibodies. Each symbol indicates an individual animal. Data representative of 4 independent experiments. Statistics: 1-way ANOVA with multiple comparisons and Bonferroni correction. NS = not significant, * $p<0.05$; ** $p<0.01$.

CHAPTER 4

Conclusion

CHAPTER 4: CONCLUSION AND FUTURE DIRECTIONS

CONTRIBUTIONS TO THE FIELD

CXCR5+ CD8 T cell biology

The work described in this dissertation defines a novel population of follicular CD8 T cells that develops in situations of chronic antigen and autoimmunity. CXCR5+ CD8 T cells have been well described in chronic viral infections and to a lesser extent cancer. This dissertation work is among the first to describe CXCR5+ CD8 T follicular (Tfc) cells in autoimmune disease. Chapter 2 describes how CD8 T cells were identified within systemic autoimmune germinal centers in the absence of functional T regulatory (Treg) cells (1). Prior to this work, CD8 T cells had only been identified within the germinal center in autoimmune and inflammatory conditions in a small number of studies: rheumatoid arthritis (2, 3) and human nasal polyp tissue (4). Chapter 3 describes our study of CD8 Tfc cells in additional models of autoimmune disease and the identification of CD8 Tfc cells in spleens in the context of IL-2Rα-KO systemic autoimmunity and murine lupus but not in non-obese diabetic, or collagen-induced arthritis. This supports the evidence also described in chapter 3 for CD8 Tfc cell development in situations of altered Treg control or function. Chapter 2 further defines autoimmune CD8 Tfc cell function in plasma cell differentiation and antibody class-switch in vivo. CD8 Tfc cell cytokines mediate B cell responses, likely via IL-4 secretion, similar to CXCR5+ CD8 T cells in colon cancer and other inflammatory conditions (4-6).

One key difference between the studies herein, and other studies of CXCR5+ CD8 T cells is an inconsistent phenotypic definition of the CD8 Tfc subset. This dissertation identifies a subset of CXCR5+ CD8 T cells in autoimmune disease that is also PD-1+, whereas many other studies have defined only a single CXCR5+ CD8 T cell subset (Chapter 1). CD8 Tfc cells are associated with a higher production of co-stimulatory molecules and functional proteins, including IL-4 and IL-21 than the encompassing the CXCR5+ CD8 T cells subset or the CXCR5- CD8 T cell subset (Chapter 3). These observations may account for differences described in various immune responses and settings. However, it is likely that CXCR5+ CD8 T cells have diverse functional capacity dictated by immune cues and microenvironmental response.

Germinal center reactions

Nearly two decades ago, the canonical features of B-helper CXCR5+ CD4 T cells were identified (7, 8). Since this seminal work, the field of germinal center biology has rapidly expanded to define the influence of CD4 T follicular helper (Tfh) cells within the B cell follicle. Just eight years ago, bcl6+ CD4 T follicular regulatory cells (Tfreg) were defined within the germinal center and associated with CD4 Tfh and B cell control (9,

10). Our work, in combination other CXCR5+ CD8 T cell studies, identifies another novel follicular population capable of influencing secondary and ectopic germinal center formation. We show that CD8 Tfc cells localize within the germinal center structure during autoimmune disease. However, it is not yet clear where CD8 Tfc cells promote B cell responses. CD8 Tfc cells promote plasma cell differentiation and antibody class-switch, but not germinal center B cell development. T cell-dependent plasma cell responses and antibody class-switch initially occur in the extrafollicular space, while B cell somatic hypermutation and proliferation occur within the germinal center (11-13). Together, these data suggest that CD8 Tfc cells predominately support extrafollicular B cell responses even though they localize within the germinal center. The question of position versus functional capacity provides unique avenues for continued exploration only briefly explored within the field thus far (Chapter 1).

Before the identification of CXCR5+ CD8 T cells, only Qa-1 restricted CD8 Tregs were known to localize to the germinal center (14, 15). Improved identification markers and assay sensitivity continues to facilitate discovery of germinal center subsets with specialized features such as CD8 Tfc cells. While CD8 Tfc cells may not arise in germinal center responses under normal conditions, CD8 Tfc cells development in the absence of functional Tregs and expand in response to lymphoproliferation (Chapter 3). The association between CD8 Tfc cell development and Treg defects reveal an additional role for Treg or Tfreg control in germinal center formation checkpoints. Further evaluating CD8 Tfc cell involvement in germinal center formation, maintenance and function may lead to a shift in the germinal center paradigm, especially during situations of chronic antigen.

Autoimmune Disease

This work evaluates novel mechanisms defining non-canonical CD8 T cell responses in autoimmune disease (16). IL-2-KO CD8 T cells augment CD4 T cell mediated BMF when co-transferred (17). Similarly, CD8 Tfc cell co-transferred with CD4 Tfh cells act in synergy to promote plasma cell differentiation and class switch in vivo (Chapter 3). While, CD8 Tfc cells maintain the capacity to be highly cytolytic, even more so than other lymphoid CD8 T cell populations, CD8 Tfc cells induce B cell class-switch via IL-21 and likely IL-4. These data suggest that CD8 Tfc cells utilize multiple mechanisms to promote autoimmune disease progression.

Several questions still remain regarding the origin of CD8 Tfc cells. Do they develop as a completely novel subset of CD8 T cells similar to that of CD4 Tfh cells, or from an effector memory precursor? One study and chapter 3 of this dissertation directly compare sequencing information from CD8 Tfc cells with that of CD4 Tfh helper populations (18). In contrast, CD8 Tfc cell gene expression profile in cancer and chronic viral infection is more indicative of an effector memory population (4, 19-22). CD8 Tfc cells likely arise independent of other CD8 T cell subsets. However, CD8 Tfc cell development varies and is likely disease specific, as CXCR5+ CD8 T cells correlates

with improved patient outcome in cancer (23, 24) and reduced viral load in chronic viral infections (19, 21, 25-28). CD8 Tfc cells may then represent possible avenues for diagnostic testing and treatment of antibody-mediated autoimmune disease.

Some of these studies have already been conducted in setting of chronic viral infection in which CXCR5+ CD8 T cells controlled viral load and were capable of reseeding the T effector niche when transferred (20). Moreover, CXCR5+ CD8 T cells have been manipulated by virus-specific antibodies to facilitate target cell lysis (29) or engineered in vitro to target functional CD8 T cells to the germinal center (30).

FUTURE DIRECTIONS

As the vast majority of CXCR5+ CD8 T cell research was published only in the past three years, a number of research questions remain regarding CXCR5+ CD8 Tfc cell function. Future studies should continue to evaluate CD8 Tfc cell development, functional mechanisms, and compare autoimmune CD8 Tfc cells to viral- or cancer-specific CXCR5+ CD8 T cells.

This dissertation suggests a relationship between Treg function and the development of CD8 Tfc cells. To dissect the requirement for Treg control of CD8 Tfc cell development, conditional Treg depletion studies using Foxp3-diptheria toxin mice to could be used to evaluate spontaneous CD8 Tfc cell development and autoimmune disease initiation. Further, CD8 Tfc cell development at the loss of Treg cells at different timepoints would illustrate the conditions required for CD8 Tfc cell development beyond that of Treg control mechanisms such as inflammation, or the activation of antigen presentation. To interrogate the requirement for peripheral Treg versus follicular Treg populations on CD8 Tfc cell development, *Bcl6*$^{fl/fl}$*Foxp3-Cre/Cre* mice with specific deletion of Foxp3+bcl-6+ Tfreg, should be evaluated for CD8 Tfc cells (31). These studies could be further expanded to evaluate the suppressive role of peripheral Tregs and Tfregs on CD8 Tfc cell function.

The capacity for CD8 Tfc cells to directly promote or initiate autoimmune disease is unknown. Another challenge in understanding autoimmunity and CD8 Tfc cells in disease has been our inability to identify antigens targeted both early in disease initiation and during disease progression. Chapter 3 of this dissertation illustrates that CD8 Tfc cells respond to red blood cell (RBC) antigen loaded B cells but does not directly identify CD8 Tfc cells as direct mediators of disease progression. IL-2Rα-KO CD8 T cells transferred into a host with T cells reactive to a non-cognate antigen (OVA specific responses in OT-I mice) would illustrate CD8 Tfc cell ability to promote antigen-specific responses. To further evaluate antigen presentation to CD8 Tfc cells, culture experiments in which antigen presenting cells loaded with RBC antigen could be used to stimulate CD8 Tfc cells from late disease or bulk CD8 T cells from early disease and evaluated for cytokine responses. Either DC cells, shown to be required to initiate disease (32), or B

cells, which act as a superior antigen presenting cell in later autoimmune disease progression (33, 34) can be used as the antigen presenting cell to evaluate the role of CD8 Tfc cells at each disease phase. It is likely that CD8 Tfc cells do not survive well in culture and during transfers thus CD4 Tfh cells co-cultures or transfers may be needed to fully address these questions. Finally, and perhaps of most relevance would be to identify CD8 Tfc cells in additional autoimmune disease patients, as CD8 Tfc cells have only been identified in human nasal polyps (4, 6) and rheumatoid arthritis synovium (2, 3).

SUMMARY

Many questions about CD8 Tfc cell biology and CD8 Tfc antigen-specific interactions remain. Moreover, completely defining the function and development of CXCR5+ CD8 T cell population in multiple antigen settings will define the CD8 T cell repertoire and open diverse therapeutic possibilities. In the context of chronic viral infection, targeted cell lysis, as achieved in the context of LCMV (29), may be used to eliminate viral reservoirs. In autoimmune disease, CD8 Tfc cell repression by co-inhibitory treatments, such as PD-1 blockades may represent an avenue for existing therapeutics to reduce autoimmune disease severity or progression. This dissertation defines CD8 Tfc cells in multiple autoimmune disease models, describes possible functions on B cells as well as cytotoxic cells, and suggests Treg control of CD8 Tfc cell development. This research promotes an understanding of CD8 T cells in the germinal center responses. Specifically, defining a novel role for CD8 T cells in multiple models of antibody-mediated autoimmune disease.

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