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Elucidation of the Roles of TNFRSF Receptors in Cutaneous T Cell Responses

by

Kelly Anne Remedios

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

Dedication

This dissertation is dedicated to

Jim and Marjorie Carroll,
who inspired the journey;

Jim Remedios,
who provided the bicycle and let me choose the destination;

and

Josiah Haut,
for riding by my side throughout this crazy adventure.

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Contributions of Co-Authors to Presented Work

All work in this dissertation was performed under the guidance and mentorship of Dr. Michael Rosenblum.

Chapter 2 is in revisions as of July 25, 2018 as: Kelly A. Remedios, Bahar Zirak, Priscila Munoz Sandoval, Margaret M. Lowe, Devi Boda, Evan Henley, Shrishti Bhattarai, Tiffany C. Scharschmidt, Wilson Liao, Haley B. Naik, and Michael D. Rosenblum. The TNFRSF Members, CD27 and OX40, Coordinately Limit Th17 Differentiation in Regulatory T cells. *Science Immunology*. It is presented here in accordance with the policies of AAAS. M. Rosenblum and myself conceived of this project and designed the study together. I wrote and prepared figures for the manuscript with edits by M. Rosenblum. I performed experiments and data analysis, except as specified below. B. Zirak, D. Boda, P. Munoz Sandoval, and S. Bhattarai assisted with mouse experiments. P. Munoz Sandoval, S. Bhattarai, M. Lowe performed the human experiments in Figure 1.6. M. Lowe performed the human RNA-seq experiments. E. Henley assisted with experiments and analyzed data in Supplementary Figure 1.10. W. Liao and H. Naik oversaw and designed the HS and PSO studies. Tiffany C. Scharschmidt contributed the murine RNA-sequencing data in Figure 1.1.

Chapter 3 has been prepared as a manuscript as: Kelly A. Remedios, Lauren Meyer, Bahar Zirak, Mariela L. Pauli, Hong-An Truong, Michael D. Rosenblum. CD27 Promotes Effector T Cell Survival by Suppressing the Cell-extrinsic Apoptosis Pathway. *In Preparation*. M. Rosenblum and myself conceived of and designed this study. I wrote and prepared figures for

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Elucidation of the Roles of TNFRSF Receptors in Cutaneous T Cell Responses

Kelly Anne Remedios

Abstract

Regulatory T cells (Tregs) are closely related to Th17 cells and utilize aspects of the Th17-differentiation program for optimal immune regulation. In several chronic inflammatory human diseases, Tregs express IL-17A, suggesting that dysregulation of Th17-associated pathways in Tregs may result in either loss of suppressive function and/or conversion into pathogenic cells. The pathways that regulate the Th17 program in Tregs are poorly understood. We have identified two TNFRSF members, CD27 and OX40, to be preferentially expressed by skin-resident Tregs. Both CD27 and OX40 signaling suppressed the expression of Th17-associated genes from Tregs in a cell intrinsic manner *in vitro* and *in vivo*. However, only OX40 played a non-redundant role in promoting Treg survival. Tregs that lacked both CD27 and OX40 were defective in controlling inflammation, had reduced accumulation in both skin and SDLNs, and expressed high levels of IL-17A, as well as the master Th17 transcription factor, RORγt. Finally, we found that CD27 expression was inversely correlated with Treg IL-17 production in skin of psoriasis and hidradenitis suppurativa patients. Taken together, our results suggest that TNFRSF members play both redundant and unique roles in regulating Treg plasticity.

We further explored how CD27 influences $CD4^+$ effector (Teff) cells in inflamed tissues. Utilizing a murine model of inducible self-antigen expression in the epidermis, we elucidated the functional role of CD27 on auto-reactive Teff cells. Expression of CD27 on antigen-specific Teff cells resulted in markedly enhanced skin inflammation when compared to CD27-deficient Teff

cells. CD27 signaling promoted the accumulation of cytokine-producing T cells in a cell-intrinsic fashion. Surprisingly, this costimulatory pathway had minimal effect on early T cell activation and proliferation. Instead, signaling through CD27 resulted in the progressive survival of Teff cells during the autoimmune response. CD27 did not appear to affect the intrinsic (mitochondrial) apoptosis pathway. Instead, CD27-deficient Teff cells expressed higher levels of active-caspase 8. Taken together, these results suggest that CD27 does not promote Teff cell survival by increasing expression of anti-apoptotic BCL2 family members but instead acts by preferentially suppressing the cell-extrinsic apoptosis pathway, highlighting a previously un-identified role for CD27 in augmenting autoreactive Teff cell responses.

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Chapter I: Introduction

Regulatory T Cells

Regulatory T cells, characterized by the expression of the lineage defining transcription factor FOXP3, are the major immune subset responsible for maintaining immune homeostasis and preventing excessive inflammation.[1,2] The necessity of these cells is illustrated in both mice and humans that lack functional Tregs. Patients with immunodysregulation polyendocrinopathy X-linked syndrome (IPEX) suffer from a lethal, multi-organ autoimmunity due to mutations in FOXP3.[3] Furthermore, scurfy mice that have germline mutation in FOXP3 lack suppressive Tregs and also succumb to a multi-organ autoimmunity.[1] Because Tregs play such a critical role in maintaining immune homeostasis and are potent suppressors of inflammation, it is of great interest to understand how these cells can be therapeutically manipulated in disease.

Tregs were originally believed to be a relatively homogenous population derived exclusively from the thymus. Later studies revealed that Teffs could be induced to become Tregs outside of the thymus, and this peripherally induced population were classified as "peripheral" Tregs, or pTregs. Emerging data suggests the existence of even more complexity, as multiple Treg subsets within different tissues are being defined with specialized functions and unique cell fates [4]. Tregs are thus a complex and heterogeneous population, and are believed to mediate the majority of their functions in the tissues in which they reside.[5] It is therefore of fundamental importance to define the mechanisms of generation, maintenance, stability and function of different Treg subsets *in vivo* in order to develop optimal therapeutic strategies to augment or inhibit their function in different human diseases.

Tissue-Resident Tregs

A well-characterized subset of tissue-resident Tregs are those that reside within the gut (reviewed in Tanoue, Atarashi, & Honda, 2016). Unlike Tregs in other tissues, a significant proportion of Tregs within the intestines appear to be induced by dietary and commensal antigens. TCRs utilized by colonic Tregs are specific to antigens derived from commensal bacteria and different from TCRs isolated from Tregs in lymphoid tissues which tend to be specific for self-antigens.[7,8] There are also reduced frequencies of Tregs within the gut of germ free mice compared to mice raised in a specific pathogen free environment, which further suggests that intestinal Tregs are induced, and/or maintained by the intestinal microbiome.[9] Microbially induced Tregs within the intestine are unique compared to other tissues in part due to their expression of RORγt, the master transcription factor of the Th17 lineage.[10] RORγt +Tregs are dependent on STAT3 for development, and are the main producers of IL-10 in the colon.[11] Gut resident Tregs are particularly dependent on IL-10 for their suppressive function within this tissue. Notably, conditional deletion of IL-10 from Tregs results in spontaneous colitis and lung inflammation, but other organs appear to remain relatively unaffected without pushing the system.[12] Tissue-resident Tregs therefore appear to utilize different mechanisms to suppress inflammation within different tissues.

In addition to directly regulating inflammation, it is becoming clear that tissue resident Tregs have adapted alternative functions to promote tissue integrity and function. The first example of such a Treg with alternative functions was found within the visceral adipose tissue.[13] VAT-resident Tregs have a distinct transcriptional profile as compared to Tregs in lymphoid organs and express the adipose-associated transcription factor PPARγ. Treg-specific deletion of PPARγ results in decreased accumulation of Tregs within the VAT. Further experiments with

these conditional knockout mice demonstrated that VAT-resident Tregs were necessary for restoring insulin sensitivity via the anti-diabetic drug pioglitazone and provided the first evidence that Tregs can exhibit alternative functions within tissues.

In the muscle, Tregs play an important role in mediating repair in both settings of injury and a model of muscular dystrophy.[14,15] Unlike the gut, few Tregs reside within the muscle at steady state. Upon injury, Tregs accumulate within the muscle and express the growth factor amphiregulin (Areg) which promoted muscle repair via increasing the differentiation of muscle satellite cells. Within the lung, Treg expression of amphiregulin also plays a non-redundant role in directly promoting tissue repair during lung injury, independent of suppression.[16]

The skin is home to a large population of tissue-resident Tregs.[17] Our lab has done extensive work defining the development and functional roles of both murine and human skin-resident Tregs. During development, a population of highly activated Tregs migrate into the skin a CCR6-CCL20 dependent manner in response to commensal colonization and hair follicle morphogenesis.[18,19] At the steady state, skin-resident Tregs preferentially reside around hair follicle.[17,20,21] This observation led the lab to explore the role of Tregs in hair morphogenesis. Our lab has now demonstrated that skin-resident Tregs express high levels of the Notch ligand Jagged 1 (Jag1) and promote hair follicle stem cell function and regeneration.[22] Our lab has also demonstrated that Tregs play a critical role in promoting tissue repair after wounding in part though utilization of the EGFR pathway.[23] Skin-resident Tregs therefore play a diverse array of roles in skin biology, from regulating inflammation to promoting tissue homeostasis at both the steady state and after barrier breach.

Because Tregs play critical roles in both regulating inflammatory responses as well as promoting tissue function, it is of fundamental importance to understand the underlying biology

behind Tregs that reside in different tissues. It is also of interest to understand how various tissue Tregs might be therapeutically manipulated in various disease settings. In order to develop effective therapeutic strategies to augment or inhibit the function of tissue-specific Tregs, it is of fundamental importance to define the function of these cells within different tissues.

The Role of Th17 in Disease

Th17 cells are critical players in immunity to extracellular pathogens, however dysregulation of the Th17 pathway has been implicated in multiple autoimmune diseases(reviewed in [24]). IL-17A signals through IL-17RA and IL-17RC and induces the expression of a variety of pro-inflammatory cytokines including IL-1, IL6, and TNF, promoting neutrophil recruitment and activation and amplifying inflammation.[25] Th17 cells are classically defined by the expression of the cytokine IL-17A. Mice deficient in IL-17A are severely susceptible to extracellular or fungal infections.[26,27] Humans that have genetic defects in genes related to the Th17 pathway have an increased susceptibility to develop chronic or recurrent infections with *c. albicans*, illustrating the importance of this cell type in host immunity.[28]

Th17 differentiation is dependent on activation of the transcription factor STAT3, which increases expression of RORγt, the lineage defining transcription factor of Th17 cells.[29] Both RORγt and STAT3 cooperate to turn on the Th17 program and upregulate the expression of IL-17A.[30] The transcription factor RORα plays a partially redundant role with RORγt in promoting Th17 differentiation, and mice deficient in both RORγt and RORα cannot generate Th17 cells and are completely protected from EAE.[31] Nearly all IL-17 producing cells found in humans also express the chemokine receptor CCR6.[32,33] The pathogenic capacity of Th17 cells is controlled by the cytokine IL-23 which signals through IL-23R, its receptor. IL23/IL-23R-signaling critical

for stabilizing Th17 differentiation and this pathway is strongly associated with multiple human diseases.[34]

Multiple single nucleotide polymorphisms (SNPs) within IL-23R have been associated with multiple human autoimmune diseases, including psoriasis, psoriatic arthritis, rheumatoid arthritis, and Crohn's.[24] While the mechanism by which SNPs within IL23R influences the development of these diseases has yet to be elucidated, it strongly implicates Th17 cells as key orchestrators of pathology. IL-17A and IL-17R blockade have had incredible success in treating psoriasis, with up to 90% of patients responding with a PASI of 75 or greater in large phase three trials, illustrating the importance of this cytokine in this particular autoimmune disease.[35] Surprisingly, IL-17 blockade has not had much efficacy in Crohn's disease, and treatment actually resulted in greater adverse effects compared to placebo [36] Cua et. al. has now demonstrated that IL-17 plays a critical role in maintaining epithelial barrier integrity and function within the gut. [37] IL-17 signaling mediated these protective effects through the IL-17R adaptor protein Act-1 and regulated the localization of the protein occludin, important for tight junctions. Thus IL-17 can play roles in tissue repair as well as augmenting immunity. It is unclear whether IL-17 plays a protective role within the skin, although it seems that its pathogenic properties dominate as IL-17 blockade has such success in psoriasis.

Recent studies have suggested that Th17 cells are not a uniform population as once believed, and are instead highly heterogeneous and diverse in terms of function and cytokine producing potential.[38] Two dominant flavors of Th17 cells have been reported: non-pathogenic and pathogenic. Th17 cells differentiated *in vitro* (with IL-6 and TGFb) are poor inducers of EAE and have a more regulatory phenotype. [39] These "non-pathogenic" Th17 cells are similar to human Th17 cells that are specific to *s. aureus* antigens and co-produce IL-17 and IL-10 [40]. In

contrast, differentiating with IL1b+IL6+IL23 induces cells with a pathogenic transcriptional profile that potently drive EAE disease.[34,39] These "pathogenic" cells appear are similar to human *c. albicans* specific Th17 cells that express both IL-17A and IFNγ.[40] Single cell RNA-sequencing has revealed that a spectrum of transcriptional states can span across "pathogenic" to "non-pathogenic" Th17 fates [41]. How these different states influence immune responses to pathogens, barrier function, and autoimmunity are still being interrogated. Furthermore, whether therapies can be developed that tune the pathogenicity of Th17 cells has yet to be assessed.

Cytokine Production from Regulatory T cells in Health and Disease

In addition to tissue heterogeneity, Tregs can further subset by their use of T-helper associated transcription factors. Tregs utilize the transcription factors Tbet, IRF4, and STAT3 to control Th1, Th2 and Th17 responses respectively.[42–44] While Tregs require Teff-associated transcription factors for their suppressive function, excessive Treg plasticity may lead to the loss of suppressive function and/or acquisition of pro-inflammatory traits.[45] Optimal Treg function therefore requires tuning to acquire enough effector-associated traits for suppression while preventing excessive plasticity which can lead to instability.

Tregs within inflamed tissue often produce inflammatory cytokines characteristic of Teff cells (reviewed in [45]). In patients with psoriasis, FOXP3+ cells lesional skin biopsies produce more IL-17 compared to FOXP3+ cells within healthy control tissue.[17,46] Lamina isolated from patients with Crohn's disease also exhibits an increase in IL-17 producing FOXP3+ cells compared to healthy controls.[47,48] Diabetic patients have an increased in FOXP3+ IFNγ+ Tregs within the peripheral blood compared to healthy controls.[49] While there are now many reports of

the presence cytokine producing Tregs within inflamed tissues, the role of such Tregs in human disease remains unclear.

Studies in mice have allowed insight into the pathogenic potential of cytokine producing Tregs. Multiple murine models of chronic inflammatory diseases have demonstrated that cytokine producing Tregs, or at least cells that once expressed FOXP3, can contribute to disease pathology. Utilizing FOXP3-GFP lineage-tracing reporter mice, Zhou and colleagues demonstrated that in the inflammatory environment of NOD driven diabetes, there is a large percentage of CD4 cells that previously expressed FOXP3, referred to as exTregs.[50] These cells expressed high levels of IFNγ and were able to transfer diabetes when sorted and transferred into healthy mice. In murine models of asthma and chronic arthritis, Treg instability can lead to the loss of FOXP3 expression, acquisition of pro-inflammatory Th17 cytokines, and drive disease pathogenesis.[51,52] The mechanisms that lead to Treg instability are not well understood. Furthermore, whether an "exTreg" that has lost FOXP3 expression can be therapeutically targeted to re-express FOXP3 and regain suppressive function has yet to be determined.

In most human diseases, it remains unresolved whether cytokine producing Tregs originate from bona fide suppressor Tregs which transiently express cytokine but retain suppressive function, whether they are induced Tregs that have lost suppressive function, or whether they are exTregs that have converted into pathogenic cells. Several studies have demonstrated that cytokine producing FOXP3+ cells retain their suppressive function in *in vitro*.[47,53] However *in vitro* suppression assays are not always an accurate readout of suppressive activity *in vivo*. Despite these unknowns, it can be concluded that in such chronic inflammatory diseases, Tregs are either unstable and contributing to inflammation, or they are unable to overcome ongoing inflammation and mediate their suppressive effects. Either scenario leaves a

window for therapeutic intervention, by either correcting dysfunctional Tregs or augmenting their suppressive potential to resolve inflammation.

Treg and Th17 Plasticity

Tregs and Th17 cells in particular share a fair amount of phenotypic plasticity.[54] Both populations utilize TGFb in part for their differentiation. [55,56] Tregs and Th17 cells utilize the chemokine receptor CCR6 and are often found in similar anatomical compartments.[57–60] Both Tregs and Th17 cells critically depend on STAT3 for their function. When STAT3 is deleted from T cells using conditional knockout mice, these mice are completely deficient in their ability to mount Th17 responses and are completely protected from EAE.[30] Mice that lack STAT3 specifically within Tregs succumb to a fatal intestinal inflammation, as STAT3 is required by Tregs to control Th17 inflammation.[44]

A distinct population of microbially induced Tregs exists within the gut that co-expresses both FOXP3 and RORγt.[61] This population is microbially induced, and has a distinct transcriptional profile from other Tregs. Despite expressing RORγt, these Tregs retain suppressive function and protect against experimental colitis. [61,62] RORγt + Tregs can also be induced following immunization, and this population was shown to have increased suppressive function compared to RORγt - Tregs in a mode of EAE.[63] In certain contexts, Tregs can thus acquire Th17-like properties while retaining their suppressive function.

Not only can Tregs acquire Th17-like properties, but Th17 also exhibit plasticity and can lose their pathogenic potential and become more “regulatory-like”. Using fate reporter mice,

Gagliani and colleagues demonstrated that Th17 cells can lose IL-17A expression (becoming exTh17 cells) and gain the expression of IL-10 and LAG-3, genes characteristic of Tr1 cells.[64,65]. These exTh17Tr1 cells were found at low percentages within the small intestine at the steady state but increased in frequency during the resolution phases of various models of inflammation and infection, including EAE and *nippostrongylus brasiliensis* infection. While these cells expressed little FOXP3, they had significant regulatory function as they were protective in a model of Th17-mediated colitis. Thus, Th17 cells are also highly plastic and gain regulatory function during the resolution of inflammation.

In addition to the extensive plasticity between Tregs and Th17 cells, recent studies utilizing both *in vitro* and *in vivo* models have now demonstrated that Tregs can actually support Th17 development.[66] One study has shown that *in vitro*, Treg production of TGFb supported Th17 development in the absence of exogenous cytokine.[67] Whether this also occurs *in vivo* has yet to be assessed. Likely the major mechanism by which Tregs can support Th17 differentiation is through consumption of IL-2.[68] IL-2 and STAT5 signaling are potent inhibitors of Th17 differentiation, as STAT5 and STAT3 act in direct competition to bind to the IL-17 locus.[69,70] Tregs are potent consumers of IL-2 due to the expression of the high affinity chain of the IL2 receptor, CD25, and IL-2 consumption is a key mechanism of Treg suppression.[71,72] Pandiyan *et. al*. demonstrated that IL-2 consumption by Tregs could strongly promote Th17 development both *in vitro* and in response to *c. albicans infection*, and Tregs were required for *c. albicans* clearance.[68] Tregs also play a critical role for Th17 differentiation in the setting of *chlamydia muridarum* infection are required for clearance.[73] Therefore, Tregs can both support Th17 development and can exhibit plasticity towards Th17 differentiation themselves. Whether there

are mechanisms that limit Treg plasticity towards a Th17-like phenotype and/or limit the ability of Tregs to support Th17 differentiation remain to be determined.

TNFRSF Receptors in T Cell Function

Members of the tumor necrosis factor receptor superfamily (TNFRSF) play important roles in shaping the quality and magnitude of T cell response.[74] This family of costimulatory receptors consists of 30 receptors and 19 ligands, and these receptors can be grouped into two general classes, those that are inhibitory (such as Fas, TRAIL, and TNFR1), and those that are those that are stimulatory (including OX40, 4-1BB, CD27 and GITR). Ligation of inhibitory TNFRSF receptors typically results in cell-extrinsic apoptosis through FADD/TRADD-driven caspase pathways which ultimately results in caspase 8 activation.[75] In contrast, the co-stimulatory members of the TNFR super family lack death domains and instead tend to promote T cell responses by enhancing survival, accumulation and/ or differentiation.[76]

The roles of individual costimulatory TNFRSF receptors, including CD27, OX40, 4-1BB and GITR have primarily been studied in the context of their function on conventional CD4 and CD8 T cells. In these subsets, TNFR super family members tend to influence function by either 1) promoting the frequency of effector and/or memory cells or 2) controlling T cell functional differentiation and production of various pro-inflammatory cytokines.[74] While, many of these receptors have been reported to exhibit similar functional outcome on T cell activity (such as promoting T cell survival/expansion), most studies to date have looked at individual family members rather than assessing multiple TNFRSF members side-by-side. The relative

contributions of individual receptors and whether they play unique or redundant roles during an immune response has yet to be addressed.

While TNFRSF members have primarily been studied in effector CD4 and CD8 responses, many of these receptors are expressed (often preferentially and constitutively) on Tregs. The roles of these receptors in Treg function are beginning to be appreciated. It is now known that TNFRSF receptors play overlapping roles in promoting Treg development within the thymus.[77] However, the role of these receptors in promoting or inhibiting Treg function within the periphery remains less clear. In some studies, signaling through TNFRSF receptors has been reported to augment Treg function, yet in others, signaling through the same receptor can inhibit Treg function.[78] Ultimately, the roles of these receptors in peripheral Treg function are still being elucidated.

Many TNFRSF members are being evaluated as targets for immunotherapy. Because these receptors are widely expressed throughout the immune system, understanding the effects of different receptors on different cell types is of fundamental importance. The ultimate success of a therapy that agonizes or inhibits a given TNFRSF member will depend on the balance of its function on Tregs and Teffs as well as other immune cell types.

CD27 in T Cell Responses

One particular TNFRSF family member, CD27, has been shown to play important roles in Teff survival, function, and memory responses.[79] CD27 is expressed on T cells, B cells, NK cells, and other hematopoietic cell types. The only known ligand for CD27 is CD70, a TNF family member ligand expressed on activated B cells, dendritic cells (DCs) and other subsets of antigen presenting cells (APCs).[80–83] Engagement of CD27 by CD70 induces the recruitment of

the TRAF2 and TRAF5 adapter proteins to activate canonical and non-canonical NFkB signaling pathways.[84] This costimulatory pathway has been implicated in multiple autoimmune diseases including systemic lupus erythematous, rheumatoid arthritis, inflammatory bowel disease, and psoriasis.[85] CD27 signaling is particularly important for the clearance of EBV, as patients that lack functional CD27 or CD70 present with increased susceptibility infection, particularly to EBV-associated Hodgkin lymphoma.[86–88] CD27 signaling can also promote anti-tumor immunity and an agonists against CD27 are currently being evaluated as novel immunotherapies in cancer.[89]

In T cells, CD27 has mainly been reported to act as a costimulatory molecule, influencing T cell activation, proliferation and differentiation. CD27 was originally shown to be important for the generation of productive CD4 and CD8 memory responses, and mice deficient in CD27 have impaired immune responses to secondary challenge with influenza.[90] A recurring theme in the CD27 literature is a role for this receptor in promoting survival. In human and mouse CD8+ T cells, CD27 can prevent apoptosis by increasing the expression of anti-apoptotic proteins including BCL-XL and MCL-1 as well is through increasing expression of Pim-kinase.[91,92] Other mechanisms by which CD27 has been reported to promote survival in CD8 cells is by increasing the expression of IL-7R and promoting autocrine IL-2 production.[93,94] CD27 can also promote survival via the extrinsic apoptosis pathway by downregulating the expression of FasL on CD4+ T cells.[95] These mechanisms have primarily been defined in promoting survival and expansion of CD8+ T cells. The role of this receptor in CD4 survival (both on Teffs as well as Tregs) is less clear.

In CD4 cells, CD27 signaling plays a role in the development of Th1 responses. During immunization, CD27 engagement increases the frequency of Il-2 and IFNγ producing CD4+ cells

and promotes the acquisition of a Th1 profile at the transcriptional level.[96] Co-stimulation through CD27 can promote Teff expression of T-bet and in turn, lead to the upregulation of the IL-12Rbeta2 chain, making Teffs more sensitive to IL-12 induced Th1 differentiation[97]. The role of CD27 in regulating Th2 responses it not as well studied, but some evidence suggests that this pathway is not as important in Th2 responses, as it does not appear to play a role in a murine model of asthma[98]. Recent studies have demonstrated that CD27-CD70 signaling appears to negatively regulate Th17 differentiation/ IL-17 production from both CD4 and γδ T cell subsets.[99–101] In γδ T cells, CD27 is a thymic determinant of the balance between IL-17 and IFNγ producing γδ T cells subsets.[100,101] Later, CD27 signaling in CD4 Teff cells was shown to attenuate Th17 differentiation via epigenetic silencing of the IL-17A and CCR6 loci, in a mechanism independent of RORγt.[99] CD27 signaling therefore appears to promote Th1 while suppressing Th17 differentiation.

The role of this costimulatory pathway in Tregs is less well understood. Elucidating the role of CD27 specifically on Tregs has been challenging due to the lack of a conditional knockout. However, some studies have attempted to elucidate the role of this receptor on Tregs using *in vitro* experiments and utilizing the germline knockout mice. CD27 expression has been shown to correlate with increased suppressive function within Tregs, however appeared to be independent of CD27 signaling.[102] CD27KO mice have reduced frequencies of Tregs within both the thymus and peripheral lymphoid organs due to a role for this receptor in promoting Treg survival during thymic development.[103] Whether CD27 influences Treg suppressive function is less clear. CD27 did not appear to influence Treg suppressive function in an *in vitro* assay of Treg suppression, however such assays are not a physiological readout of suppressive function *in vivo*.[103] CD27 expression by Tregs may influence their ability to regulate Th1 responses. In one

study, Tregs utilized CD27 to downregulate CD70 expression on DCs, making CD70 less available for Teffs, resulting in reduced Th1 responses.[104] The role of CD27 signaling in peripheral Treg function within various inflammatory and tissue contexts remains to be elucidated.

OX40 in T cell Responses

OX40 is another costimulatory TNFRSF member that plays important roles in T cell function. Manipulation of the OX40 pathway is also being explored as an avenue for therapeutic targeting in both autoimmunity and cancer.[105,106] Unlike CD27, which is expressed constitutively on naïve T cells, OX40 is not expressed on resting cells. Instead, OX40 is expressed conventional T cells only upon activation.[107,108] OX40 has also been reported to be expressed by NK cells and neutrophils, yet the role of this receptors on these cell types is not well characterized.[105] The ligand for OX40 is OX40L, and is expressed on multiple type of antigen-presenting cells, including but not limited to B cells, macrophages, and dendritic cells.[109–111] Activated CD4 and CD8 cells can also express OX40L.[112,113]

OX40 can promote CD4 function in multiple models of inflammation and immunization. OX40 appears to play a limited role in early Teff activation, perhaps due to the low expression of this receptor at rest and dependence on activation for expression.[107,114] Instead, OX40 is important for IL-2 production by Teffs and enhances proliferation and survival at later timepoints after immunization and plays a critical role in generating an productive memory responses. OX40 also appears to influence antigen-specific CD8 memory generation to influenza and vaccinia virus infection.[115,116]

Similar to CD27, OX40 appears to also regulate Teff differentiation into the various T helper lineages. In several conditions/models that drive Th1 responses, OX40 can promote the expansion and survival of Th1 cells, potentially through increasing the expression of IL-2.[107,117,118] Unlike CD27, the OX40 pathway can also promote the development of productive Th2 responses. *In vitro*, OX40 signaling enhances the production of IL-4 from Teffs and promotes their differentiation into the Th2 lineage.[119] Studies utilizing a model of *leishmania major* demonstrated that OX40 promoted Th2 differentiation *in vivo.*[120,121] Mice that received a blocking antibody against OX40L were able to successfully control the infection due to a decrease in Th2 response, while mice that constitutively expressed OX40L on T cells were more susceptible to *l. major* infection due to an increase in Th2 responses. In contrast, OX40 has recently been reported to attenuate Th17 differentiation and exhibits a protective function in murine EAE.[122] In this study the authors demonstrated that OX40 activated the NFkB family member RelB, which in turn activated the histone methyltransferases G9a and SETDB1which deposited repressive chromatin marks at the IL17A and IL-17F loci to silence gene expression, again independent of RORγt. OX40 therefore appears to promote Th1 and Th2 differentiation, but limits Th17 differentiation.

While OX40 is only expressed on Teffs upon activation, this receptor is constitutively expressed by Tregs.[108] Multiple studies have now demonstrated that OX40 signaling can influence Treg development as well as suppressive function. Mice deficient in OX40 have significantly reduced numbers and percentages of Tregs within the thymus and peripheral lymphoid organs.[108] Conversely, mice that constitutively express OX40 have increased Treg percentages in these compartments.[108] During thymic development, OX40 as well as other TNFRSF members (including GITR and TNFR2) appear to play semi-redundant roles in

enhancing thymic Treg generation.[77] Furthermore, OX40 is important in Treg proliferation/ homeostatic expansion, as Tregs deficient in OX40 display decreased expansion after adoptive transfer into lympho-deficient recipients compared to WT Tregs, and are unable to control T-cell mediated colitis.[108,123,124] In these models, OX40 seems to promote Treg survival by either protecting them from activation-induced cell death and/or augment Treg sensitivity to IL-2.[123,124] While OX40 appears to promote Treg expansion, some studies have suggested that this pathway can also block Treg suppressive function. In a tumor model, the administration of an OX40 agonizing antibody was shown to induce tumor rejection, in part by limiting Treg suppressive function.[125] OX40 has also been shown to inhibit Treg suppression of GVHD.[126] The role of this receptor in Treg function is therefore highly complex, and how this receptor influences Treg function within different contexts remains to be addressed.

Overview of Thesis Work

In this dissertation, I sought to elucidate the role of TNFRSF receptors in cutaneous immune responses. Chapter 2 is focus on elucidating the individual and combined roles of CD27 and OX40 Treg function during Th17 responses. In this study, we began with the observation that in steady state adult mice, skin-resident Tregs expressed lower levels of RORγt compared to those within the colon. However, these skin-resident Tregs expressed many cytokine receptors and transcription factors associated with Th17 differentiation, suggesting that they were primed to respond to Th17-driven inflammation. Indeed, upon infection with c. albicans, a potent Th17-driving pathogen, skin-resident Tregs upregulated their expression of RORγt and also expressed IL-17A. We therefore sought to understand the mechanisms that limited Treg plasticity into a

Th17-like fate. We found that the TNFRSF members, CD27 and OX40, were preferentially expressed on Tregs relative to Teffs within the skin. Furthermore, both CD27 and OX40 signaling suppressed the expression of Th17-associated genes (including RORγt, IL-17A, and CCR6) from Tregs in a cell-intrinsic manner both *in vitro* as well as *in vivo*. Notably, only OX40 played a non-redundant role in promoting Treg survival, as OX40-deficient Tregs accumulated at significantly lower frequencies within both the SDLN and skin after transfer into lymphodeficient mice. In an adoptive transfer model of *c. albicans* infection, Tregs that lacked both OX40 and CD27 expression were defective in their suppressive capacity, displayed reduced accumulation in both the skin, and SDLNs, and expressed high levels of IL-17A and RORγt. Finally, we found that CD27 expression was inversely correlated with Treg IL-17 production in two human diseases: psoriasis and hidradenitis suppurativa. Taken together, this chapter illustrates that TNFRSF members can have both redundant and unique roles in regulating Treg plasticity during inflammation.

In Chapter 3, we sought to explore how CD27 influences Teff responses in a model of inducible cutaneous antigen expression. In this study, we found that CD27 expression by antigen-specific T cells promoted skin inflammation, as mice that received CD27-deficient Teffs were protected from inflammation. We found that CD27 signaling promoted the accumulation of IFNγ and IL-2 producing T cells in the skin draining lymph nodes in a cell intrinsic fashion. CD27 did not appear to affect early T cell activation or proliferation, but instead appeared to promote T cell survival. We utilized BH3 profiling to test whether CD27 promoted survival via regulating the intrinsic (or mitochondrial) apoptosis pathway. Surprisingly, we found that both CD27-sufficent and CD27-deficient Teffs were equally sensitive to mitochondrial outer membrane permeabilization upon exposure to either BH3 activator or sensitizer peptides,

suggesting that CD27 did not regulate Teff survival via the intrinsic apoptotic pathway. Instead, CD27-deficient Teffs expressed higher levels of active-caspase 8, suggesting that CD27 might promote Teff survival by limiting activation of the extrinsic (or death receptor mediated) apoptotic pathway.

Chapter II. The TNFRSF Members, CD27 and OX40, Coordinately Limit Th17 Differentiation in Regulatory T Cells

Abstract

Regulatory T cells (Tregs) are closely related to Th17 cells and utilize aspects of the Th17-differentiation program for optimal immune regulation. In several chronic inflammatory human diseases, Tregs express IL-17A, suggesting that dysregulation of Th17-associated pathways in Tregs may result in either loss of suppressive function and/or conversion into pathogenic cells. The pathways that regulate the Th17 program in Tregs are poorly understood. We have identified two TNFRSF members, CD27 and OX40, to be preferentially expressed by skin-resident Tregs. Both CD27 and OX40 signaling suppressed the expression of Th17-associated genes from Tregs in a cell intrinsic manner *in vitro* and *in vivo*. However, only OX40 played a non-redundant role in promoting Treg survival. Tregs that lacked both CD27 and OX40 were defective in controlling inflammation, had reduced accumulation in both skin and SDLNs, and expressed high levels of IL-17A, as well as the master Th17 transcription factor, RORγt. Finally, we found that CD27 expression was inversely correlated with Treg IL-17 production in skin of psoriasis and hidradenitis suppurativa patients. Taken together, our results suggest that TNFRSF members play both redundant and unique roles in regulating Treg plasticity.

Introduction

Regulatory T cells (Tregs) are critical for maintaining immune homeostasis and mitigating tissue damage caused by excessive inflammation [127]. It is becoming increasingly appreciated that Tregs adapt to the local inflammatory environment by acquiring specific programs that facilitate optimal immune regulation. In this capacity, Tregs utilize effector T (Teff) cell associated transcription factors Tbet, Irf4, and Stat3 to regulate Th1, Th2, and Th17 responses, respectively [42–44]. Expression of these 'master' helper T (Th) cell lineage transcription factors imparts Tregs with the ability to co-localize with Th cells driving a given immune response through expression of Th-specific chemokine receptors [128]. However, Tregs only acquire aspects of Th cell programs and generally fail to secrete cytokines characteristic of these cells within healthy tissue [45]. Thus, Tregs maintain a delicate balance between expression of transcriptional programs that mediate immune regulation and expression of specific pathways associated with Th cell differentiation. The molecular mechanisms that regulate Th cell associated pathways in Tregs are currently unknown.

In multiple human diseases, Tregs within inflamed tissue produce appreciable amounts of inflammatory cytokines. In psoriasis, a cutaneous inflammatory disease driven by dysregulated Th17 responses, Tregs in lesional skin produce increased IL-17 compared to Tregs in non-lesional skin and skin from normal healthy individuals [17,46]. Similarly, both lamina propria and PBMCs isolated from patients with Crohn's disease have increased IL-17 producing Tregs relative to healthy controls [47,48]. In support of the latter, dysregulation of the Th17 program within Tregs has been shown to contribute to disease pathogenesis. In murine models of severe

asthma and chronic arthritis, Treg instability lead to acquisition of pro-inflammatory Th17 cytokines, loss of FOXP3 expression and increased disease severity [51,52]. Thus, dysregulation of Th cell programs within Tregs can negatively impact their ability to control inflammation.

The tumor necrosis factor receptor superfamily (TNFRSF) of costimulatory receptors (including, but not limited to CD27, OX40, GITR, and 4-1BB) play critical roles in both the development and regulation of productive immune responses [129]. While TNFRSF members mediate similar cellular processes, comprehensive studies comparing redundant and unique effects of specific receptors are lacking. In addition, many of these receptors were originally studied in the context of Teff cell function; however, it is now evident that many TNFRSF members are expressed by Tregs [78]. The function of many of these receptors on Tregs is poorly understood.

We have found that CD27 is highly expressed on Tregs that stably reside in human skin [17]. CD27 plays important roles in Teff cell priming, expansion, survival, and differentiation [79]. The only known ligand for CD27 is CD70, which is transiently expressed on DCs, B cells, T cells and NK cells after activation [81,130,131]. CD27 engagement by CD70 induces the recruitment of the TRAF2 and TRAF5 adaptor proteins, which activate the JNK and NFkB signaling pathways [132]. In γδ T cells, CD27 signaling is a thymic determinant of IFNg- vs. IL-17-producing subsets [100]. In CD4+ Teff cells, CD27 promotes the development of productive Th1 responses [96,97]. More recently, CD27 signaling has been reported to attenuate Th17 responses *via* epigenetic silencing of IL-17A and CCR6 in Th17 cells [99]. On Tregs, high levels of CD27 expression correlates with increased survival and suppressive function, and also plays a minor role in thymic development [102,103,133,134]. However, the function of this TNFRSF member on Tregs within peripheral tissues is currently unknown.

OX40 is another member of the TNFRSF that is highly expressed on Tregs [108]. Signaling through OX40 occurs after engagement with OX40L, which is broadly expressed by multiple immune lineages [105]. Similar to CD27, OX40 signaling promotes Teff cell survival, differentiation, thymic Treg development, and can attenuate Th17 differentiation [77,122,135,136]. However, the effects of OX40 signaling on Treg biology is poorly understood.

Given that CD27 and OX40 are highly expressed on Tregs and limit Th17 differentiation in Teff cells, we hypothesized that signaling through these receptors would play a role in regulating the Th17 program in Tregs in inflamed tissues. In addition, we speculated that these pathways would have completely overlapping and redundant roles in Tregs. Consistent with our hypothesis, we found that both CD27 and OX40 limit expression of Th17-associated genes in Tregs; however, signaling through OX40 resulted in enhanced Treg accumulation when compared to the CD27 pathway. Our results elucidate unique, partially redundant roles for members of TNFRSF in limiting Th17 differentiation in Tregs and promoting their accumulation in peripheral tissues.

Results

Tregs in skin are poised to respond to Th17 inflammation

Within the gastrointestinal tract, IL-17 producing cells play a major role in maintaining barrier homeostasis, in part by promoting epithelial integrity [137,138]. A defined subset Tregs within the gut express the Th17-associated transcription factor, RORgt, which allows them to regulate Th17-responses within this tissue [10,11,62]. In contrast, the skin contains relatively few IL-17 producing cells in the steady-state and increased numbers of these cells are observed during pathologic skin inflammation [139,140]. Because Tregs in the gut are constantly regulating Th17 responses and Tregs in the skin need to regulate these responses only in specific inflammatory contexts, we hypothesized that gut-resident Tregs would be more 'Th17 skewed' than skin-resident Tregs. To test this hypothesis, we compared expression of the master Th17 transcription factor, RORgt, in Tregs within the colonic lamina propria, skin and skin draining lymph nodes (SDLN) in healthy adult mice. Compared to the gut where >40% of Tregs express RORgt, only ~6% of Tregs within both murine skin and SDLNs expressed this transcription factor (Fig. 1.1A). RNA-sequencing of Tregs and Teff cells sorted from healthy murine and human skin confirmed that Tregs express very low Rorc transcript levels (Fig. 1.1B and fig. S1.1). Interestingly, skin-resident Tregs expressed high levels of other transcription factors involved in Th17 polarization (including Stat3, Rora, Irf4, and Batf), as well as receptors for Th17-polarizing cytokines (including IL-6R, IL-6ST and IL-21R) (Fig. 1.1B). However, skin-resident Tregs expressed little to no Th17-associated cytokines, including IL-17a, IL-17f, IL-18, IL-22, IL-21 as well as low levels of the pro-inflammatory cytokine receptor IL-23R (Fig. 1.1B and fig. S1.1).

These results suggest that Tregs in skin are not 'Th17 skewed' in the steady-state, but instead are poised to be able to respond to Th17-inducing stimuli.

To test whether Tregs can respond to Th17 inflammation, we infected WT mice cutaneously with *candida albicans (c. albicans)*, a pathogen known to drive potent Th17 responses [141]. Tregs in skin expressed increasing levels of RORgt with time after infection (Fig. 1.1C), which coincided with an increase in skin Treg production of IL-17A (Fig. 1.1D). These results suggest that Tregs in skin induce expression of RORgt and begin to secrete appreciable levels of IL-17a in response to Th17 inflammation in this tissue.

The TNFRSF members CD27 and OX40 are preferentially expressed on Tregs in skin

Tregs in tissues can express components of Th17 differentiation and aberrant regulation of this process is thought to contribute to human disease pathology [10,17,47,51,62,142]. Mechanisms that regulate Th17 differentiation in Tregs are poorly understood. Recently, two members of the TNFRSF, CD27 and OX40, have been reported attenuate Teff cell differentiation into the Th17 lineage [99,122]. TNFRSF members are often expressed by Tregs in addition to Teff cells; however, it is currently unknown if these receptors influence Treg function in tissues. We hypothesized that CD27 and OX40 limit Th17 differentiation in Tregs during tissue inflammation. To begin to test this hypothesis, we first quantified the expression of CD27 and OX40 on skin-resident Tregs. CD27 is expressed on both Tregs and Teff cells in SDLNs (Fig. 1.2A). However, CD27 is preferentially expressed on skin-resident Tregs relative to skin-resident Teffs. In contrast, OX40 was preferentially expressed by Tregs in both skin and SDLNs (Fig. 1.2B). We observed that CD27 and OX40 were co-expressed by Tregs within both the skin and SDLN (fig. S1.2). High

expression of both CD27 and OX40 on skin-resident Tregs suggests that these costimulatory receptors might preferentially influence Treg function in this tissue.

We next assessed the relationship between the expression of these TNFRSF receptors and IL-17 production before and after cutaneous *c. albicans* infection (fig. S1.3). Both Tregs and Teffs within the skin expressed increased levels of IL-17A after *c. albicans* infection. Skin-resident Tregs maintained preferential expression of both CD27 and OX40 relative to skin-resident Teffs after inflammation. Furthermore, expression of these receptors was inversely correlated with Treg IL-17 production, as IL-17A producing Tregs expressed lower levels of both CD27 (fig. S1.3A) and OX40 (fig. S1.3B) compared to the IL-17 non-producing cells. Notably, skin-resident Teffs expressed low levels of CD27 and OX40 before and after infection, and Teff IL-17 production was not correlated with CD27 or OX40 expression. Taken together, these results suggested that CD27 and OX40 might influence Treg function during Th17-driven inflammation.

CD27 and OX40 signaling regulate Th17 differentiation in Tregs in vitro

To test whether signaling through CD27 and OX40 attenuates Th17 differentiation in Tregs, we developed an *in vitro* assay in which expression of Th17-associated genes could be induced in these cells. Tregs (CD4+CD25+GFP+) were sorted from FOXP3-GFP reporter mice [143] to >97% purity and cultured with anti-CD3/CD28 activator beads with the Th17-polarizing cytokines IL-6, IL-1B and IL-23. When compared to Tregs cultured with IL-2 alone, Tregs cultured in Th17 promoting conditions expressed increased levels of the Th17-associated cytokines IL-17A and IL-17F after PMA-ionomcyin re-stimulation (Fig. 1.3A and B). No

difference was observed for the Th1 cytokine, IFNg (Fig. 1.3C). Treg culture in Th17-polarizing conditions also resulted in increased expression of the Th17-associated chemokine receptor CCR6 as well as RORgt (Fig. 1.3D and E).

Using this culture system, we assessed whether stimulation through CD27 and/or OX40 could influence the expression of Th17 or Th1-associated proteins. The addition of FcCD70, a recombinant Fc-tagged CD70 that can engage and agonize CD27 signaling [144], or OX86, an agonistic antibody against OX40 [125], were able to suppress Treg expression of IL-17A, IL-17F, CCR6 and RORgt (Fig. 1.3A, B, D & E). Neither of these agonists influenced IFNg expression (Fig. 1.3C). Addition of either FcCD70 or OX86 did not affect Treg FOXP3 or CD25 expression, as assessed by MFI (Figure 1.3F & G).

We observed a trend towards an additive suppressive effect when both FcCD70 and OX86 were added in combination. Notably, OX86 treatment induced greater reduction in expression of the Th17-assocaited molecules compared to FcCD70. Antibody-mediated crosslinking of receptors can result in increased receptor oligomerization and activation compared to recombinant ligands [145,146]. Therefore, discrepancy between FcCD70 and OX86 may be due to fundamental differences in activating TNF receptors using recombinant ligands compared to agonistic antibodies approaches.

As CD27 and OX40 signaling have been implicated in promoting Th1 differentiation, we also explored the roles of these receptors on Treg plasticity during Th1 polarization. Compared to polarizing Tregs in Th17 conditions, polarizing Tregs in Th1 conditions increased the expression of IFNγ and Tbet from Tregs (fig. S1.4A & B). Conversely, Tregs polarized in Th1 conditions expressed significantly less IL-17A and RORγt compared to Th17 conditions (fig.

S1.4C&D). While IFNγ expression from Tregs was subtly reduced in Th1 polarizing conditions when FcCD70 was added to the culture, no differences were observed when OX86 was added, nor was there a difference when both OX86 and FcCD70 were added in combination (fig. S1.4A). Interestingly, Tbet expression was reduced when FcCD70 and/or OX86 were added to the cultures (fig. S1.4B). No differences were observed after FcCD70 and/or OX86 treatment in terms of the percentages of IL-17A expressing Tregs or RORγt MFI (fig. S1.4C-D). These results suggest that unlike in Teffs where CD27 and OX40 can promote Th1 differentiation, in Tregs signaling through these receptors might suppress plasticity in Th1 like cells.

To validate our findings with CD27 and OX40 agonists, we cultured Tregs in the presence of expanded and matured bone-marrow derived dendritic cells (BMDCs) as a source of natural CD70 and OX40 ligands (fig. S1.5A). Compared to culturing Tregs in Th0 conditions, culturing Tregs in Th17 conditions induced the expression of IL-17A (fig. S1.5B), but had no effect on IFNg (fig. S1.5C). FACS-sorted WT, CD27$^{-/-}$ or OX40$^{-/-}$ Tregs were cultured in the presence of BMDCs in Th17-promoting conditions. Compared to WT Tregs, both CD27$^{-/-}$ and OX40$^{-/-}$ Tregs expressed higher levels of IL-17A when cultured with matured BMDCs (fig. S1.5D and F). No difference was observed in IFNg production between the groups (fig. S1.5E and G).

To assess how CD27 engagement on Tregs effects Th17-associated gene expression, we performed whole transcriptome RNA sequencing (RNAseq). Tregs were cultured under Th17 polarizing conditions in the presence or absence of Fc-CD70. After 6 days of culture, RNA was isolated for RNAseq. Strikingly, over four replicate experiments there were only 18 differentially expressed genes between FcCD70-treated and untreated groups that had a false discovery rate (FDR) of less than 0.05 (fig. S1.6A and B). Consistent with our results at the protein level,

transcripts for both IL-17A and RORgt were significantly reduced after FcCD70 treatment. Gene set enrichment analysis (GSEA) revealed that Th17-associated genes [147] were significantly enriched in untreated compared to FcCD70-treated samples (fig. S1.6C). Because CD27 signaling resulted in a decrease in RORgt at both the transcript and protein levels, we also utilized GSEA to determine whether the RORgt-regulated signature was attenuated after FcCD70 treatment. RORgt-driven genes [148] were significantly enriched in untreated compared to FcCD70-treated samples (fig. S1.6D). At the RNA level, Tregs expressed low levels of other Th17-assicated cytokines such as GMCSF, IL22 and IL21 and no difference was detected upon FcCD70 treatment (Supplementary Table 1.1). Consistent with our findings at the protein level (Figure 1.3F & G), we did not see major differences in Treg associated genes, including FOXP3, CD25, and CTLA4 (Supplementary Table 1.2). This again suggested that CD27 engagement did not attenuate the Th17 program through increasing the expression of FOXP3 or other Treg-associated genes. Taken together, these results suggest that signaling through CD27 and OX40 regulate the Th17 differentiation program in Tregs, and that engagement of CD27 most markedly effects RORgt and IL-17 expression in this assay.

CD27 and OX40 attenuate Th17 differentiation in Tregs in vivo

To determine if the CD27 and OX40 pathways attenuate Th17 differentiation in Tregs in a cell-intrinsic fashion *in vivo*, we developed a Treg adoptive transfer model of cutaneous *c. albicans* infection (Fig. 1.4A). WT CD45.1^{+} Tregs and either CD27$^{-/-}$ or OX40$^{-/-}$ CD45.2^{+} Tregs were sort-purified and combined with sort-purified CD45.1.2^{+} WT Teff cells at a 1:1:2 ratio and adoptively transferred into RAG2-deficient (RAG2$^{-/-}$) recipients. Both Tregs and Teff cells homeostatically proliferate in RAG2$^{-/-}$ hosts, with defined populations readily detected in skin

two weeks after transfer (data not shown). At this time point, mice were cutaneously infected with *c. albicans* according to a well-established model known to elicit potent Th17 responses [149,150]. Skin and SDLNs were harvested 7 days after infection (*i.e.,* 21 days after transfer) and Treg accumulation and cytokine production was quantified by flow cytometry. In animals that received WT and CD27$^{-/-}$ Tregs, both of these populations were present at equal percentages and numbers in SDLNs, and FOXP3 MFIs were equivalent between the two groups (Fig. 1.4B and C). To determine if CD27 suppressed Th17 differentiation in Tregs, we quantified RORgt expression and intracellular IL-17 production. Compared to WT Tregs, CD27$^{-/-}$ Tregs had increased expression of RORgt (Fig. 1.4D). In addition, CD27$^{-/-}$ Tregs produced increased levels of IL-17A, but not IFNg (Fig. 1.4E & F). Taken together, this data suggests that CD27 expression on Tregs limits RORgt and IL-17 expression in a cell-intrinsic fashion *in vivo*, with no obvious effect on cell accumulation.

To assess the role of OX40 signaling on Tregs, we co-transferred WT CD45.1+ Tregs and OX40$^{-/-}$CD45.2^{+} Tregs with WT Teff cells (identical to above) into the same RAG$^{-/-}$ recipients followed by *c. albicans* infection. In contrast to CD27$^{-/-}$ Tregs, OX40$^{-/-}$ Tregs exhibited a severe defect in their ability to accumulate in the SDLN relative to WT Tregs (Fig. 1.4F). However, there was no difference in FOXP3 MFI, suggesting that OX40 does not affect Treg stability (Figure 1.4I). Similar to CD27$^{-/-}$ Tregs, the remaining OX40$^{-/-}$ Tregs expressed higher levels of RORgt and IL-17A when compared to WT Tregs, with no difference in IFNg expression (Fig. 1.4J-L).

To definitively determine when OX40 influenced Treg survival, we assessed the effect of OX40 on Treg accumulation in the absence of *c. albicans* infection. WT or OX40KO Tregs were transferred into RAGKO recipients and the percentages of Tregs within the SDLN were

assessed at days 5 and 15 after transfer (fig. S1.7). In this experiment, OX40KO Tregs accumulated within the SDLN at lower frequencies compared to WT Tregs, suggesting that this accumulation defect in the absence of OX40 signaling is not dependent on *c. albicans* infection. This finding was consistent with previous reports demonstrating that OX40 is required for Treg survival during inflammation, and OX40$^{-/-}$ Tregs fail to protect against an adoptive transfer model of colitis [123,124].

Taken together, these results demonstrate that on Tregs, signaling through either CD27 or OX40 resulted in cell-intrinsic suppression of IL-17A and RORgt expression. However, in contrast to CD27, OX40 played a non-redundant role in promoting Treg accumulation.

The CD27 and OX40 pathways synergize to attenuate Th17 differentiation in Tregs in vivo

To determine if the CD27 and OX40 pathways have an additive effect in attenuating Th17 differentiation in Tregs *in vivo,* we crossed CD27$^{-/-}$ and OX40$^{-/-}$ mice to generate CD27$^{-/-}$/OX40$^{-/-}$ (DKO) animals. WT CD45.2^{+} or DKO CD45.2^{+} Tregs were sort-purified, combined 1:1 with sort-purified WT CD45.1^{+} Teffs, and adoptively transferred into RAG$^{-/-}$ recipients, followed by cutaneous *c. albicans* infection as described above (Fig. 1.5A). Unlike results observed in mice receiving Tregs deficient in CD27 or OX40 (fig. S1.8), mice that received Tregs deficient in both of these receptors (*i.e.,* DKO Tregs) had significantly more skin inflammation, as measured clinically by increased scaling and erythema and histologically by more pronounced epidermal hyperplasia and mononucelar cell infiltrate (Fig. 1.5B-D). Compared to mice that received WT Tregs, mice that received DKO Tregs had significantly increased expansion of

lymphocytes in SDLNs (Fig. 1.5E), suggesting that DKO Tregs had a reduced capacity to regulate inflammation.

Similar to our findings using $OX40^{-/-}$ Tregs, DKO Tregs were present at significantly lower frequencies and absolute numbers in the SDLNs with a similar trend observed in skin (Fig. 1.5F and G). Of the transferred $CD45.2^{+}$ Tregs that remained, DKO Tregs expressed more than double the amount of RORgt in both the skin and SDLN when compared to WT Tregs (Fig. 1.5H and I). DKO Tregs also produced more than double the amount of IL-17A with no difference in IFNg production (Fig. 1.5J and M). These results suggest that the CD27 and OX40 pathways synergize to attenuate Th17 differentiation in Tregs, and that Tregs that are deficient in both of these TNFRSF members are not capable of effectively controlling skin inflammation.

CD27 expression inversely correlates with Treg production of IL-17 in diseased human skin

Similar to Tregs in murine skin, CD27 and OX40 are preferentially and highly expressed on Tregs in healthy human skin relative to Teffs (Fig. 1.6A and fig. S1.9). In multiple human chronic inflammatory diseases, Tregs within inflamed tissue have been reported to express increase levels of IL-17A compared to healthy controls [17,46–48]. Furthermore, SNPs within CD27 have recently been identified as susceptibility loci in psoriasis [151]. Thus, we explored the relationship between IL-17 production and CD27 expression on tissue Tregs in two inflammatory skin diseases, psoriasis (PSO) and hidradenitis suppurativa (HS).

In inflammatory conditions, recently activated Teffs can transiently express FOXP3 [152], yet the levels FOXP3 expression tends to be lower compared to bona fide suppressor Tregs [153]. We therefore compared percentages and MFI of FOXP3 in healthy and diseased skin (fig.

S1.10). There was a greater percentage of FOXP3+ cells within PSO skin compared to healthy, and the MFI of FOXP3 was also increased (fig. S1.10A). We also did not observe a difference in the core Treg signature genes as assessed by transcriptional profiling of skin-resident Tregs from healthy vs psoriatic tissue (Lowe *et. al.,* manuscript in preparation). In HS, both FOXP3 percentages and MFIs were unchanged between lesional and adjacent non-lesional tissue (fig. S1.10B). Taken together, this data suggests that FOXP3+ cells within these diseases are most likely suppressor cells rather than Teffs transiently upregulating FOXP3.

Consistent with previous studies, Tregs within lesional PSO skin biopsies express higher levels of IL-17A compared to Tregs in healthy skin (Fig. 1.6B). The majority of Tregs within PSO skin expressed CD27 (Fig. 1.6C) and we confirmed that these cells are *bona fide* Tregs by whole transcriptome RNAseq analysis (data not shown). Interestingly, Treg expression of IL-17 was inversely correlated with CD27 expression levels as measured by mean fluorescent intensity (MFI) of staining (Fig. 1.6C).

Hidradenitis suppurativa is a highly inflammatory disease of the skin characterized by painful intertriginous follicular abscesses and fibrous tracts. Recent studies have shown that lesional biopsies from HS patients have increased frequencies of Th17 cells compared to healthy controls [154,155]. Whether Tregs within inflamed HS tissue produce inflammatory cytokines has not been assessed. Thus, we quantified IL-17 production from Tregs in lesional and adjacent non-lesional skin obtained from the same HS patient. When compared to non-lesional skin, Tregs within lesional HS skin produced significantly more IL-17A (Fig. 1.6D). Consistent with results observed in PSO skin, Treg expression of IL-17 was inversely correlated with CD27 expression in HS skin (Fig. 1.6E). Taken together, these results demonstrate that Tregs produce

IL-17 in inflamed human skin and that expression of this cytokine inversely correlates with CD27 expression on these cells.

Discussion

It is becoming increasingly clear that Tregs co-opt Teff cell transcriptional programs for their optimal suppressive function.[42–44] Tregs and Th17 cells share a fair amount of both phenotypic and functional plasticity and excessive expression of Th17-associated genes in Tregs can impact disease pathogenesis.[51,52,66] Here, we demonstrate that two TNFRSF members, CD27 and OX40, are highly expressed by skin-resident Tregs. Utilizing both *in vitro* and *in vivo* approaches, we demonstrate that both CD27 and OX40 synergize to limit Th17 differentiation in these cells. Notably, OX40, but not CD27, influenced Treg survival and accumulation *in vivo*. Thus, our results reveal both overlapping and non-redundant roles for TNFRSF receptors in influencing Treg function.

TNFRSF receptors have been shown to play a variety of roles in T cell biology, influencing cell survival, expansion and differentiation. More recently, both CD27 and OX40 have been reported to regulate immune responses by attenuating the pathogenicity of Th17 cells.[99,122,129] Despite these studies, the roles that these receptors play in peripheral Treg function is poorly understood. Because TNFRSF receptors play such diverse roles in T cell biology and Tregs are functionally distinct from Teff cells, it cannot be assumed that these receptors will affect Tregs in the same way that they affect other T cell subsets. In the context of cutaneous Th17 inflammation, OX40 and CD27 signaling in Tregs attenuates the expression of Th17-associated genes. Notably, skin-resident Teff cells express significantly lower levels of CD27 and OX40 compared to Tregs in the steady-state. It is interesting to speculate that lack of CD27 and OX40 expression enables Teff cells to be poised to rapidly mount a Th17 response upon

barrier breach or infection. In contrast, high expression of these receptors on skin-resident Tregs inhibits these cells from converting into IL-17 producing 'Th17-like' cells in the context of Th17-mediated tissue inflammation. In addition, in contrast to CD27, OX40 has the added benefit of promoting Treg survival.

One of the major roles for TNFRSF receptors is promoting T cell survival. OX40 had a significant effect on Treg accumulation *in vivo*. Compared to WT Tregs, OX40-deficient Tregs accumulated at much lower frequencies in both the SDLN and skin after *c. albicans* infection. These findings are consistent with previous studies demonstrating that OX40-deficient Tregs exhibit defective survival *in vivo*.[123,124,156] Because Tregs utilize OX40 for thymic development[77,124], it is possible that defects in Treg accumulation in peripheral tissues are secondary to a cell-intrinsic developmental defect, resulting in less 'biologically fit' cells exiting the thymus of these mice. When both OX40 and CD27 were deleted from Tregs, these cells had lower accumulation in both the skin and SDLNs (Fig. 1.5F & G). This is most likely secondary to lack of OX40 signaling. Interestingly, a much more pronounced survival defect was observed when only OX40 was deleted in Tregs (Fig. 1.4G). However, these mice failed to develop signs of heightened inflammation compared to mice given WT Tregs (Fig. S1.8). Thus, our data suggests that the pronounced increase in skin inflammation observed when Tregs lacked both CD27 and OX40 cannot be solely attributed to reduced Treg survival, and that this is most-likely secondary to the pronounced Th17 skewing observed in these cells in addition to reduced numbers.

In contrast to OX40, we observed that CD27 had no role in Treg survival. This finding suggests that there are fundamental differences between CD27 and OX40 signaling on Tregs. This was somewhat surprising given that there are studies demonstrating that CD27 promotes

Teff survival.[91,93,94] Furthermore, similar to OX40, CD27 also plays a minor role in promoting survival during Treg development within the thymus[77,103,124]. Rather than ruling out a role for CD27 Treg survival, our findings further illustrate that the role of any given TNFRSF receptor is highly contextual and highlight the complexity of these pathways in T cell biology.

Previous studies have demonstrated that CD27 and OX40 attenuate Th17 differentiation in Teff cells *via* epigenetic silencing of the IL-17A/F and CCR6 loci, independent of RORγt.[99,122] In contrast, we found that CD27 and OX40 signaling on Tregs resulted in decreased RORγt expression. In addition, GSEA analysis of our RNAseq dataset comparing Tregs in Th17-polarizing conditions, with or without engagement of CD27, revealed that RORγt response genes were significantly reduced upon signaling through this receptor. Thus, our results suggest that engagement of the CD27 pathway in Tregs reduces both RORγt levels and the expression of genes driven by this transcription factor, revealing what may be a fundamental difference between CD27-mediated signaling in Tregs compared to Teff cells.

While CD27 and OX40 are expressed constitutively by skin-resident Tregs, signaling through these receptors is entirely dependent on engagement by their ligands, CD70 and OX40L. Both CD70 and OX40L can be expressed by a variety of immune cell types.[105,157] In the gut, there is a population of antigen presenting cells that constitutively express CD70[83], but it is unclear whether an analogous population of APCs exists in skin. Langerhans cells that reside in the epidermis can promote Treg function and also play a critical role in Th17 responses to *c. albicans*.[141,158,159] Langerhans cells have been shown to express CD70 after viral infection and OX40L in response to UVB irradiation.[160,161] It is currently unknown whether CD70 and OX40L are expressed on Langerhans cells, or other APC populations, during Th17-mediated skin

inflammation. Future studies are necessary to elucidate where and when these ligands are expressed and how they are available for Treg engagement in tissues.

We found that CD27 expression was inversely correlated with Treg IL-17 production in lesional skin biopsies from patients with PSO and HS. This inverse correlation has also been observed for Teffs and γδ T cells in mice.[100,162] It has also been reported that human Tregs that differentiate into IL-17 producing cells express lower levels of CD27.[163] In psoriasis, genome wide association studies have identified CD27 as a susceptibility loci.[151] Interestingly, CD27 is not expressed by the majority of skin-infiltrating Teffs, the major inflammatory mediators of this disease. It is interesting to speculate that defective signaling through CD27 in Tregs plays a role in human disease by failing to control the Th17 differentiation pathway in these cells. It remains unclear whether CD27 is downregulated on a subset of Tregs during skin inflammation or whether a pre-existing population of cells that express low levels of CD27 exists, rendering this population more susceptible to differentiate into 'Th17-like' cells.

A major outstanding question is whether cytokine-producing Tregs in inflamed tissues contribute to the pathogenesis of human disease. Do these cells retain suppressive capacity and are simply unable to control robust tissue inflammation? Are they unable to regulate because of cell-intrinsic defects in their suppressive function or do they actively contribute to disease by secreting cytokines known to mediate pathology? The answers to these questions are currently unknown and very difficult to discern in humans. Nevertheless, the results presented herein demonstrate that the TNFRSF receptors CD27 and OX40 can synergize to limit Treg differentiation into IL-17-producing cells. Future studies will be needed to definitively elucidate the roles of these cells in disease pathogenesis and evaluate whether targeting TNFRSF receptor

pathways therapeutically can enhance Treg function in the setting of chronic tissue inflammation.

Materials and Methods

Animals

All mice were housed and bred at UCSF in compliance with institutional guidelines. $CD27^{-/-}$ mice were generously provided by Stephen Schoenberger (with permission from Jannie Borst). C57BL/6 (WT), FOXP3-GFP, CD45.1, $RAG2^{-/-}$, and $OX40^{-/-}$ strains were purchased from Jackson Laboratories. DKO ($CD27^{-/-}$ /$OX40^{-/-}$ double knockout) mice were generated by crossing $CD27^{-/-}$ mice to $OX40^{-/-}$ mice. All animals were socially housed in a 12 hour light/dark cycle. Animals 6-12 weeks of age were used in experiments, and all experiments were performed with sex and age-matched controls.

Adoptive Transfer Model of c. albicans *infection*

Candida Albicans (Calb-Ag) was generously donated by Dan Kaplan and protocols were approved by the UCSF Institutional Animal Care and Use Committee. In some experiments, 14 days prior to infection, 8-12-week-old $RAG^{-/-}$ mice were reconstituted with sort-purified Tregs ($3x10^5$) and Teffs ($3x10^5$). Cutaneous infection was performed as previously described.[141] The day before infection, mice were anesthetized, dorsal skin was shaved with an electric razor, and depilatory cream was applied to the shaved area for 30 seconds before wiping clean. *C. albicans* was grown in YPAD medium at 30°C in a shaking incubator until the OD600 was 1.5-2.0. *C. albicans* was washed in sterile PBS and resuspended at $4x10^9$/mL. Upon infection, the stratum corneum was removed with 15 strokes of 220 grit sandpaper (3M) and 50uL ($2x10^8$ *c. albicans*)

was evenly applied to the skin with a sterile, pre-wetted cotton swab. Mice were harvested 7 days post infection. Infected mice were housed in a UCSF BSL2 facility according to NIH guidelines.

Human skin specimens

Studies using human tissue were approved by the UCSF Committee on Human Research and by the IRB of UCSF. Normal human skin was obtained from patients at UCSF undergoing elective surgery and was discarded a routine procedure. Psoriatic skin biopsies were obtained from patients with active clinical disease (study number 10-02830). Hidradenitis suppurativa skin biopsies were obtained from patients with active clinical disease and matching non-lesional skin was taken from 10cm away from active lesions (study number 16-19770). Skin samples were digested and processed as described previously.[17] All patients provided written informed consent prior to biopsies.

FACS sorting

Spleen and lymph nodes (mesenteric and SDLN) were harvested from 6-10-week-old mice. CD4+ T cells were enriched by negative selection using the EasySep CD4+ T Cell Isolation kit (Stemcell Technologies, Catalog #19852). Enriched cells were then stained for surface antigens. Tregs (Live CD4+CD25+) and Teffs (Live CD4+CD25-) were sorted (>97% purity) on a FACSAriaII (BD Biosciences). In some experiments, Tregs were sorted from FOXP3-GFP reporter mice[143] (Live CD4+CD25+GFP+).

Tissue Processing for Flow Cytometry

Skin and SDLNs were harvested for flow cytometry analysis. SDLNs (harvested from the axillary, brachial, and inguinal lymph nodes) were isolated and mashed over a sterile wire mesh to generate a single cell suspension. Skin was harvested, lightly defatted, minced with scissors, and resuspended in a digestion mix with 2mg/mL collagenase XI (Sigma-Aldrich, cat #C9407), 0.5mg/mL hyaluronidase (Sigma-Aldrich, cat #H3506), and 0.1mg/mL DNase (Sigma-Aldrich, cat #DN25) in RPMI with 2% FCS and 1% penicillin- streptomycin. Skin was digested for 45 minutes in a 37°C shaking incubator at 225 RPM. Digested skin was washed and vortexed for 15 seconds before filtering through a 100uM strainer. Cell counts were performed using a Nucleocounter NC-200 (Chemometec). Cells were restimulated *ex vivo* with Cell Stimulation Cocktail (Tonbo Biosciences, cat #TNB-4975) for 4 hours before staining for FACS analysis.

Antibodies and Flow Cytometry

Cells were stained for surface antigens and a live/dead marker (Ghost Dye™ Violet 510, Tonbo Biosciences) in FACS buffer (PBS with 2% Calf Serum and 1% penicillin-streptomycin) for 30 minutes at 4°C. To stain for intracellular markers, cells were fixed and permeabilized using the FOXP3-staining buffer kit (eBioscience). Antibodies used are listed in Supplementary Table 1. Samples were run on a Fortessa (BD Biosciences) in the UCSF Flow Cytometry Core. FlowJo software (FlowJo, LLC) was used to analyze flow cytometry data.

In vitro Th17 and Th1 cultures

FACS sorted Tregs (1x10^5) were cultured in 96-well flat bottom plates in the presence of 1x10^5 Mouse T-Activator CD3/CD28 Dynalbeads (Thermo Fisher Scientific, Cat#11456D). Tregs were cultured in Th0 conditions in the presence of 100U/mL IL-2. Polarization in Th17 conditions occurred with IL-6 (25ng/mL), TGFb (2ng/mL), anti- IFNγ (5ug/mL) and anti-IL-4(5ug/mL) continually in culture with IL-1B (10ng/mL) and IL-23 (10ng//mL) added at day 3. Tregs were cultured in Th1 conditions in the presence of IL-12 (10ng/mL). Cells were cultured in the presence, absence, or combination of FcCD70 (0.5 mg/mL, Sino Biological, cat #51129-M04H) and/ or OX86(5ug/mL, Bio-X-Cell, cat #BE0031). Cells were restimulated on day 6 for cytokine analysis by FACS. In some experiments, Tregs were sorted (CD4+CD25+FOXP3-GFP+) on day 6 and cell pellets were flash frozen in liquid nitrogen for RNA-sequencing.

RNA-sequencing

Frozen pellets were sent to Expression Analysis, Quintiles (Morrisville, NC). All sample preparation and processing for sequencing was performed by Expression Analysis. RNA was isolated using QIAGEN RNeasy Spin Columns and quality was checked using an Agilent Bioanalyzer Pico Chip. cDNA libraries were converted using the Illumina TruSeq Stranded mRNA sample preparation kit. Using an Illumina RNASeq, cDNA was sequenced to a 25M read depth. Reads were aligned to Ensembl mg GRCm38.p4 reference genome using TopHat (v. 2. 0. 12). After alignment, SAM files were generated using SAMtools. Htseq-count (0. 6. 1p1, with union option) was used to obtain read counts. Differentially expressed genes between paired samples was determined using the R/Bioconductor package DESeq2.

Statistics

Statistics were performed using GraphPad Prism Software. Significance between two groups was determined using either two-tailed unpaired (for measuring differences between separate groups), paired (for measuring significance between populations within the same patient) Student's t test. Significance comparing 3 or more groups was determined using a one-way ANOVA multiple comparisons test. All in vivo experiments were performed with at least two to three independent experimental cohorts. The number of mice per group is annotated in figure legends, and mean values are visually depicted with error bars representing the standard error of the mean (SEM). P-values correlate with symbols as follows: ns = not significant = $p>0.05$, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p < 0.0001$. No animals were excluded from statistical analysis.

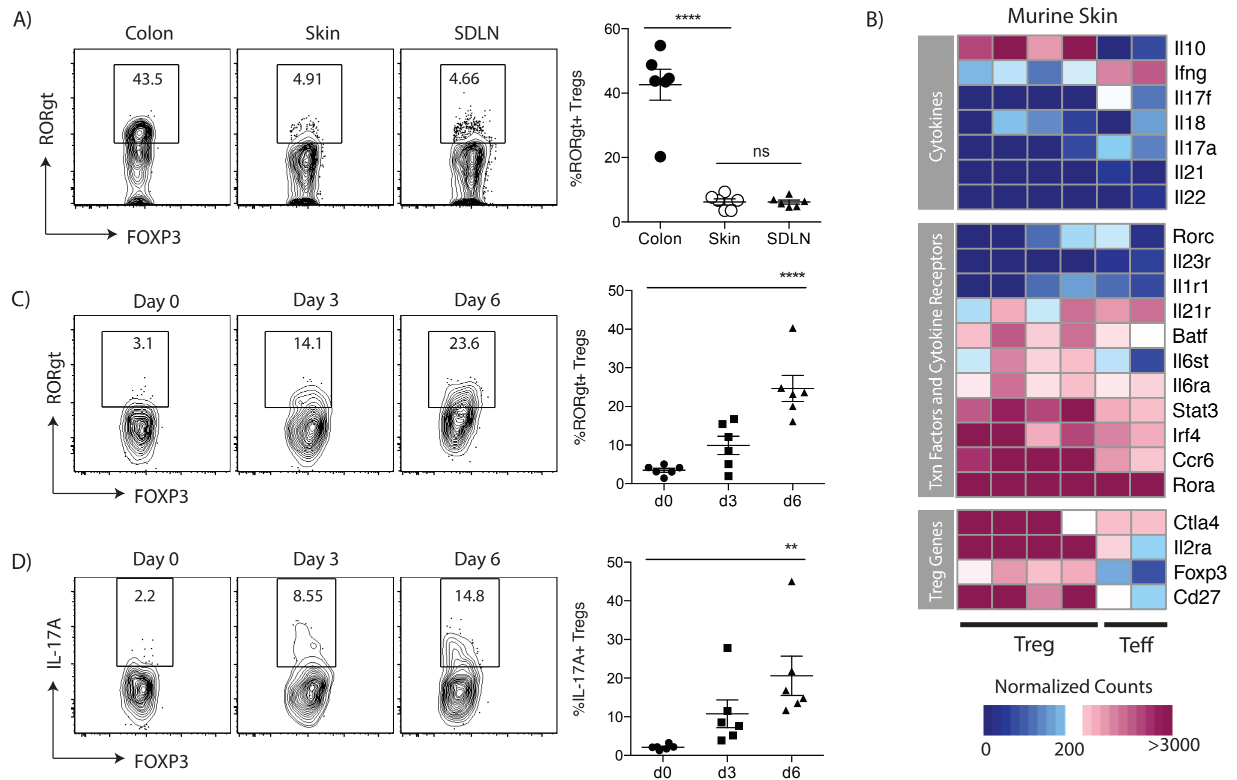


Figure 1.1: Skin-resident Tregs differentiate towards IL-17 producing cells during Th17-mediated tissue inflammation.
*(A) Percentage of RORγt expressing Tregs (gated on Live CD45+CD4+FOXP3+ cells) in the colon, skin, and SDLN of WT mice as quantified by flow cytometry. Results are from 1 experiment with n=6 mice per group. (B) Tregs and Teff cells were sort purified from normal healthy skin of FOXP3-GFP mice and gene expression quantified by whole transcriptome RNAseq. Heatmap of cytokines (top), transcription factors and cytokine receptors (middle), and Treg-specific genes (bottom) are shown. (C-D) The percentage of (C) RORγt and (D) IL-17A expressing Tregs in the skin of WT mice on day 0, 3, and 7 after cutaneous c. albicans infection was quantified by flow cytometry. Results are from 2 replicate experiments with n=2-6 mice per group. Data are mean ± SEM. p-values are determined using a one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001.*

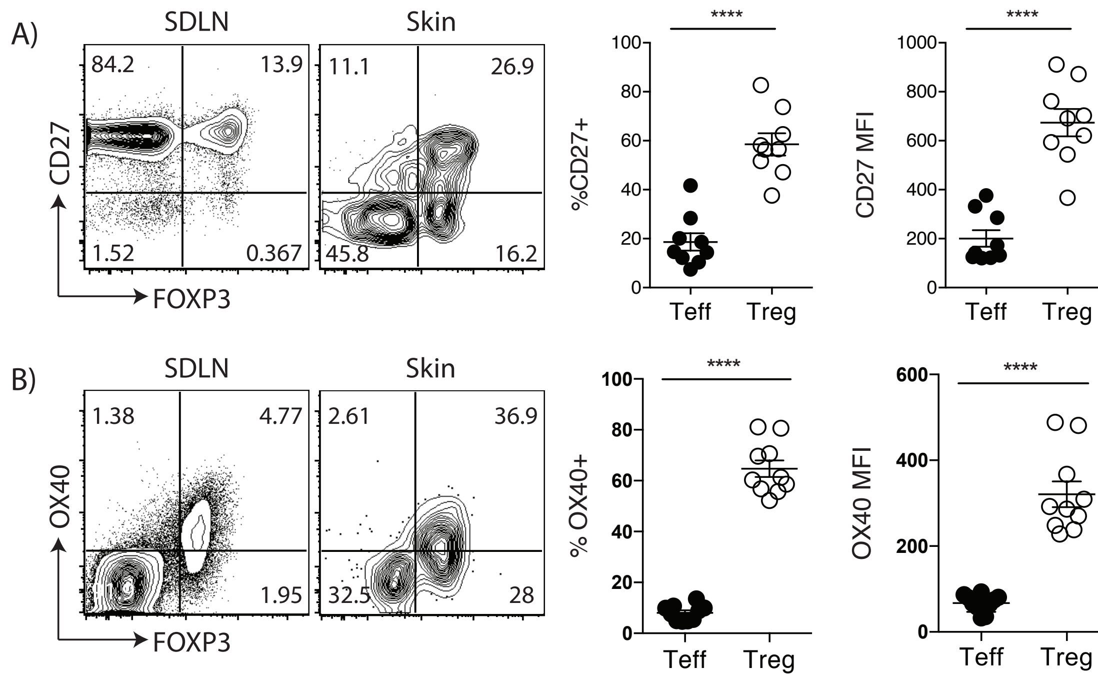


Figure 1.2: The TNFR family members CD27 and OX40 are preferentially expressed by skin-resident Tregs.

*Representative FACS plots and quantification of percentages and mean fluorescence intensity (MFI) of (A) CD27 and (B) OX40 expression on Tregs and Teffs cells in healthy murine skin. Populations are pre-gated on LiveCD45+CD4+ cells. Results are pooled from 3 independent experiments with n = 9-10 mice/experiment. Data are mean ± SEM. p-values are determined using paired students t-test. ****p<0.0001*

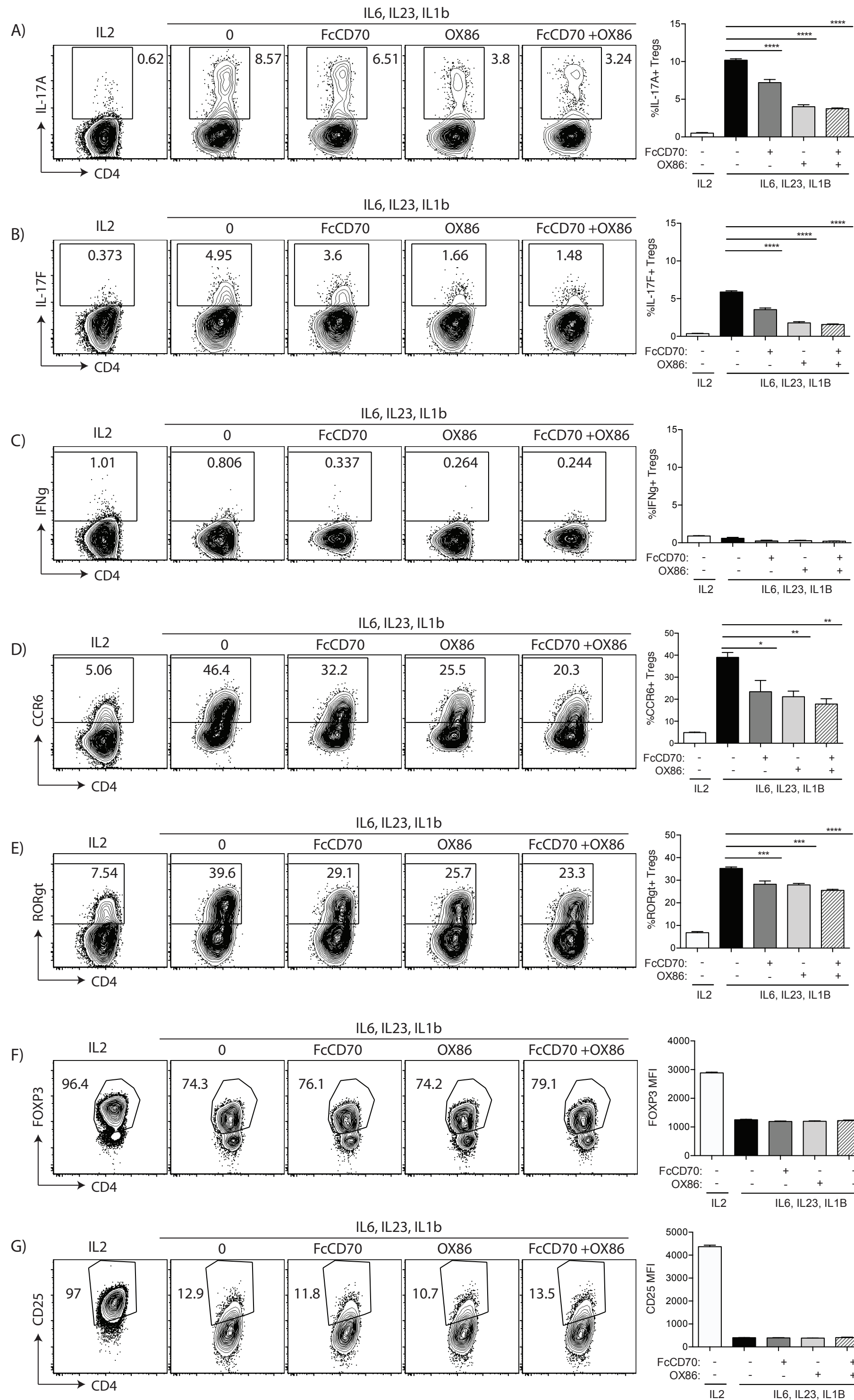


Figure 1.3: CD27 and OX40 signaling attenuate Th17 differentiation in Tregs *in vitro*. *Tregs were sort purified from FOXP3-GFP reporter mice and cultured with anti-CD3/anti-CD28 coated Dynalbeads in either Th0 or Th17-polarizing conditions in the presence or absence of FcCD70 and/or an agonistic anti-OX40 monoclonal antibody (OX86). On day 6, Tregs were re-stimulated with PMA and ionomycin. Representative FACS plots of* ***(A)*** *IL-17A,* ***(B)*** *IL-17F,*

(C) *IFNγ,* ***(D)*** *CCR6,* ***(E)*** *RORγt,* ***(F)*** *FOXP3, and* ***(G)*** *CD25 after gating on Live+CD4+FOXP3+ cells are shown and either percentages (A-D) or MFIs (F-G) are quantified. Data is representative of >3 independent experiments with technical replicates and graphs depict mean ± SEM. p-values are determined using a one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=p>0.05.*

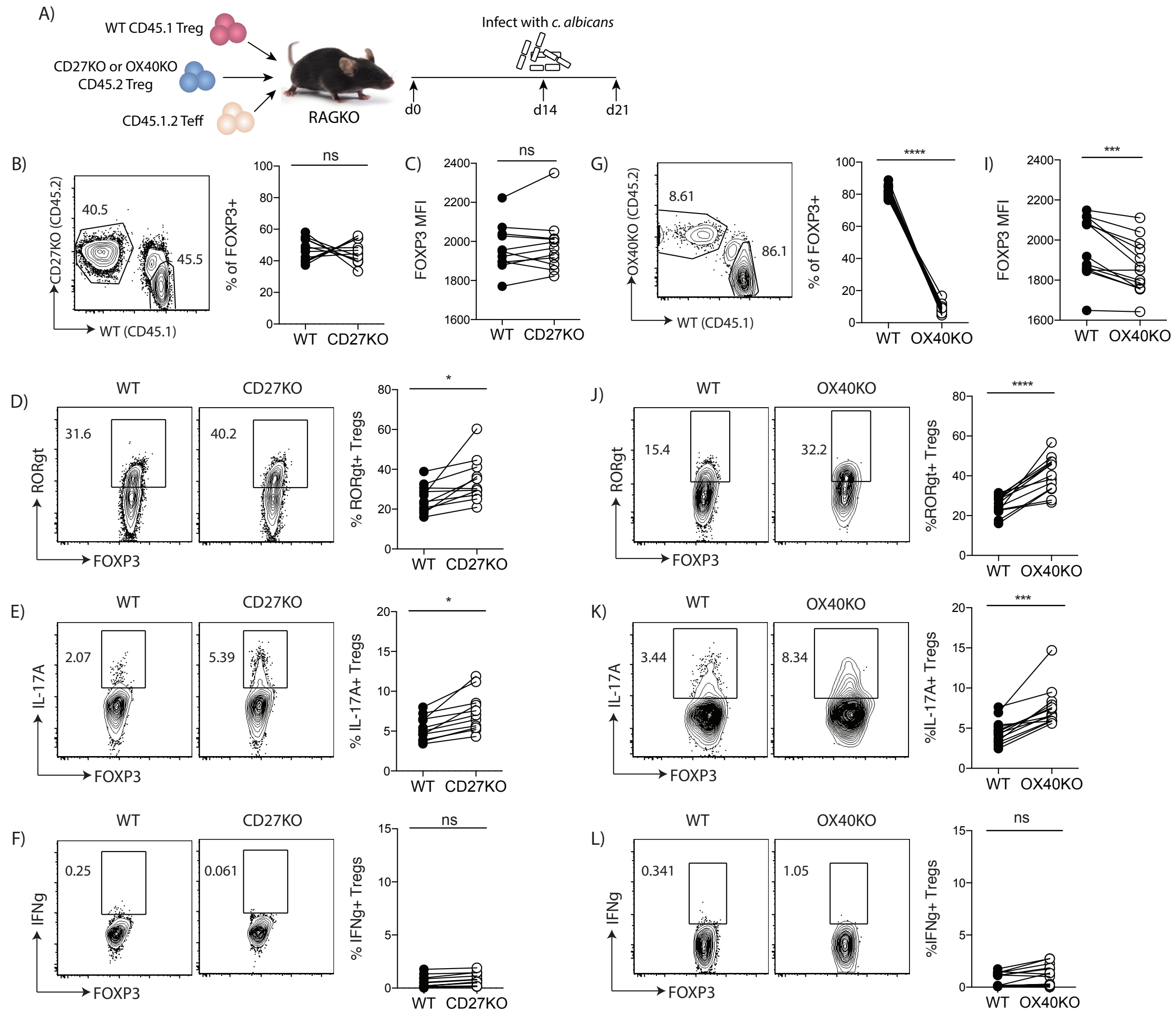
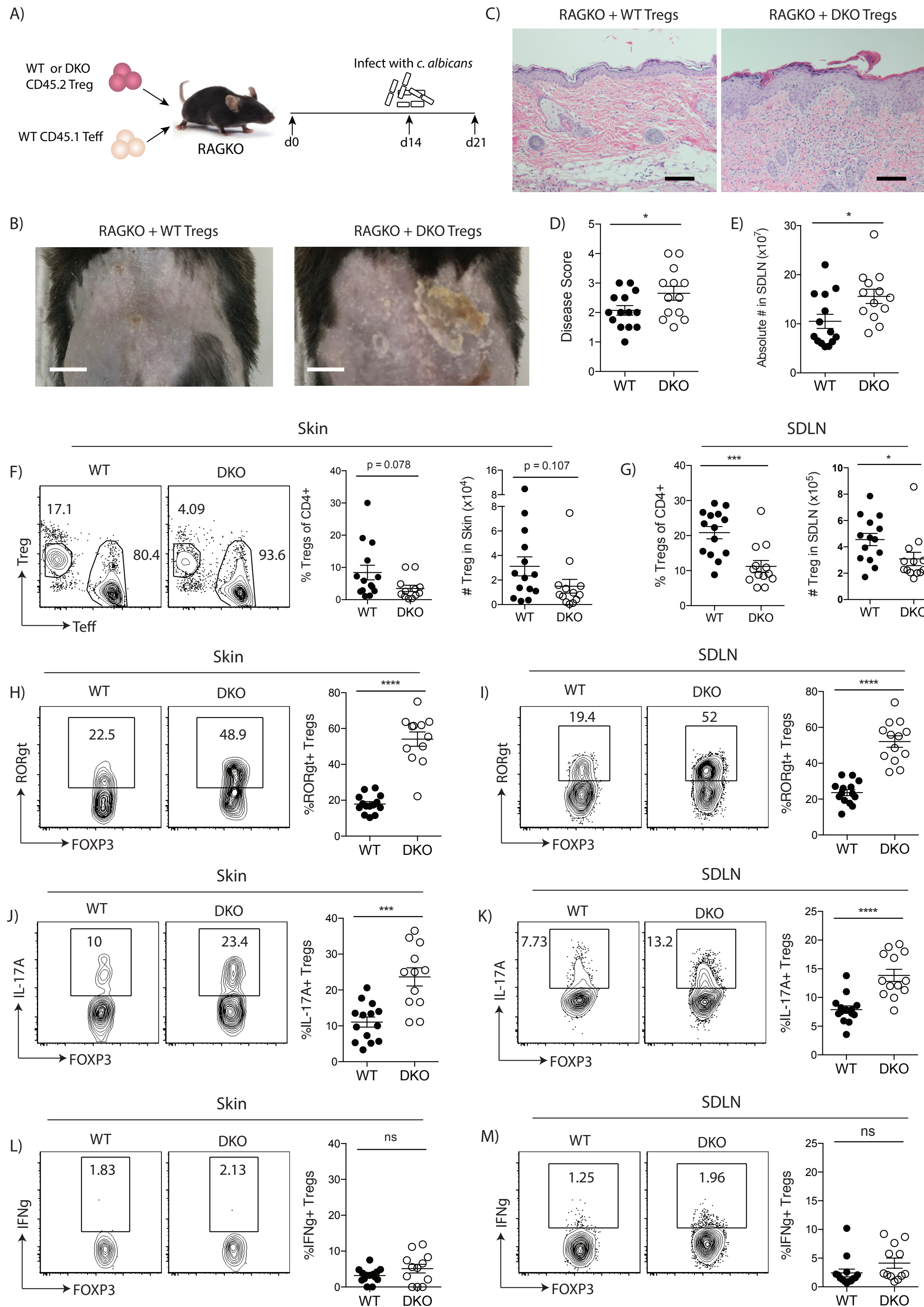


Figure 1.4: CD27 and OX40 attenuate Th17 differentiation in Tregs in a cell-intrinsic manner *in vivo*.

(A) *CD45.1.2 Teffs, WT CD45.1 Tregs and CD27$^{-/-}$ or OX40$^{-/-}$ CD45.2 Tregs were sort purified and co-transferred at a 1:1:2 ratio into RAG$^{-/-}$ recipients. Fourteen days later, mice were infected with c. albicans and skin and SDLNs were harvested 7 days after infection.* ***(B)*** *Representative FACS plots and quantification of percentages of WT (CD45.1) and CD27$^{-/-}$ (CD45.2) Tregs in the SDLN after gating on Live+CD4+TCRb+FOXP3+ cells.* ***(C)*** *Quantification of FOXP3 MFI in WT and CD27$^{-/-}$ Tregs.* ***(D-F)*** *Representative FACS plots of and quantification of (D) RORγt, (E) IL-17A, and (F) IFNγ expression by WT or CD27$^{-/-}$ Tregs in the SDLN.* ***(G)*** *Representative FACS plots and quantification of percentages of WT (CD45.1) and OX40$^{-/-}$ (CD45.2) Tregs in the SDLN after gating on Live+CD4+TCRb+FOXP3+ cells.* ***(I)*** *Quantification of FOXP3 MFI in WT and OX40$^{-/-}$ Tregs.* ***(J-L)*** *Representative FACS plots and quantification of (J) RORγt, (K) IL-17A, and (L) IFNγ expression by WT or OX40$^{-/-}$ Tregs in the SDLN after. Data is compiled from 2 independent experiments with n = 11-13 mice per group.*

*Graphs depict mean ± SEM. p-values are determined using a paired students t-test ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=p>0.05.*



A)
WT or DKO CD45.2 Treg
WT CD45.1 Teff
RAGKO
Infect with c. albicans
d0
d14
d21
C)
RAGKO + WT Tregs
RAGKO + DKO Tregs
B)
RAGKO + WT Tregs
RAGKO + DKO Tregs
D)
Disease Score
WT
DKO
E)
Absolute # in SDLN (x10^7)
WT
DKO
Skin
SDLN
F)
WT
DKO
17.1
80.4
4.09
93.6
Treg
Teff
% Tregs of CD4+
p = 0.078
# Treg in Skin (x10^4)
p = 0.107
G)
% Tregs of CD4+
***
Treg in SDLN (x10^5)
*
Skin
SDLN
H)
22.5
48.9
RORgt
FOXP3
%RORgt+ Tregs
****
I)
19.4
52
J)
10
23.4
IL-17A
%IL-17A+ Tregs
***
K)
7.73
13.2
****
L)
1.83
2.13
IFNg
%IFNg+ Tregs
ns
M)
1.25
1.96

Figure 1.5: Signaling through CD27 and OX40 synergize to attenuate Th17 differentiation in Tregs *in vivo*.

*(A) Model of cutaneous c. albicans infection. WT or CD27-/-OX40-/- double knockout (DKO) CD45.2 Tregs were sort purified and adoptively transferred with CD45.1 Teffs into RAG-/- recipients. On day 14 after transfer, mice were infected cutaneously with c. albicans. (B) Representative clinical images of dorsal skin inflammation 7 days after infection. (C) Representative histology of dorsal skin 7 days after infection. (D) Quantification of disease based on blinded histologic scoring of epidermal hyperplasia and mononuclear cell infiltrate. (E) Absolute numbers of SDLN cells as measured by hemocytometer. (F-G) Quantification of percentages and absolute numbers of CD45.2+ (Tregs) and CD45.1+ (Teff) cells (gated on Live CD4+TCRb+) in (F) skin and (G) SDLN by flow cytometry. WT or DKO Treg expression of (H-I) RORγt, (J-K) IL-17A, and (L-M) IFNγ (gated on CD4+CD45.2+FOXP3+ cells) in skin and SDLN as quantified by flow cytometry. Data is combined from 3 independent experiments with n = 13 -14 mice per group, and graphs depict mean ± SEM. p-values are determined using unpaired students t-test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ns=$p>0.05$.*

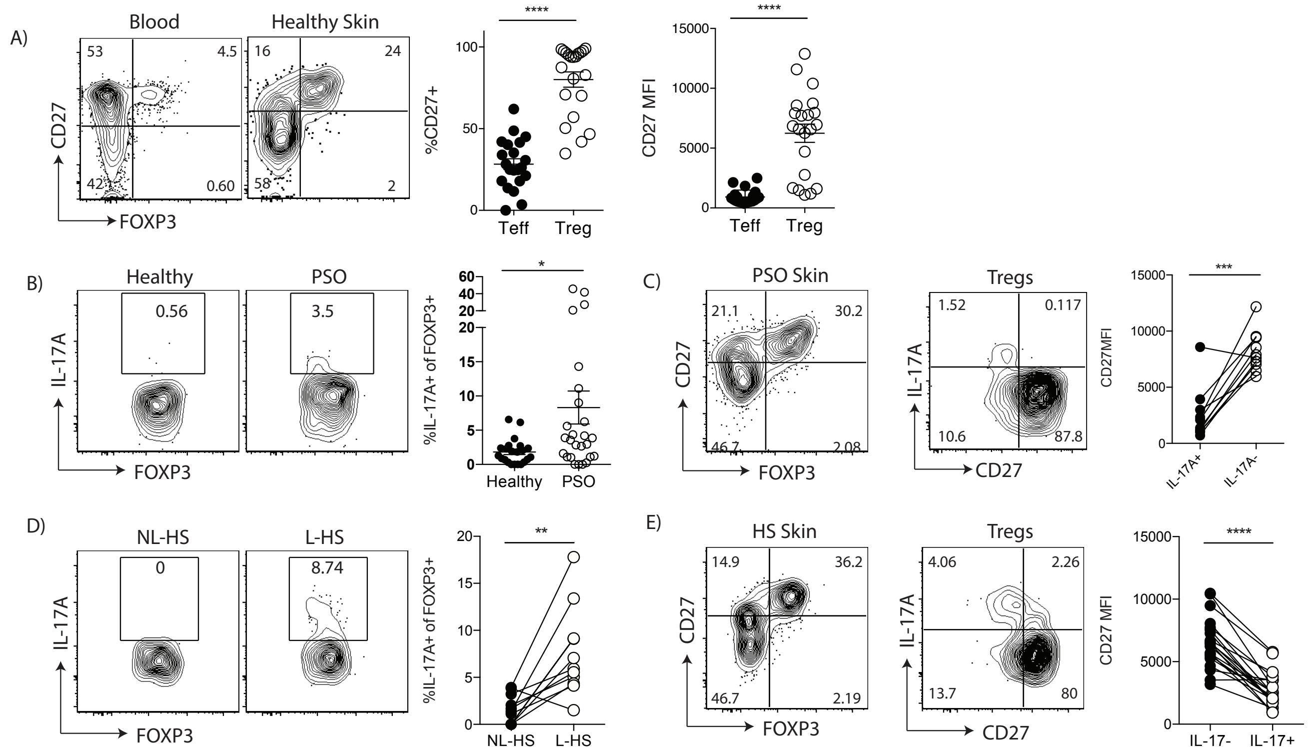
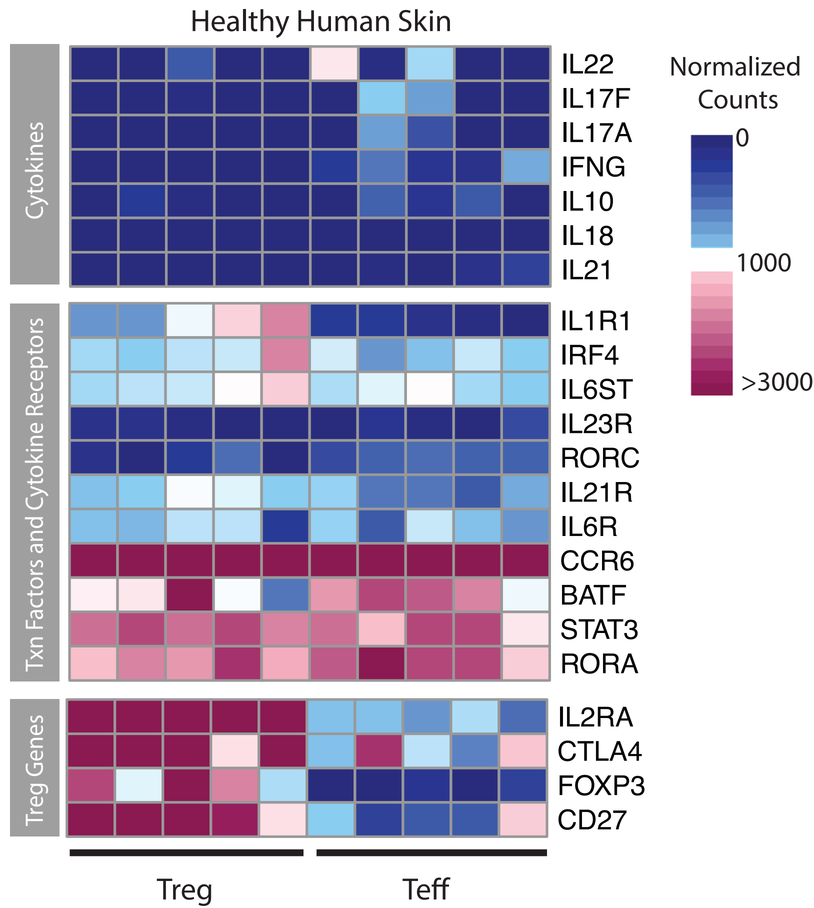


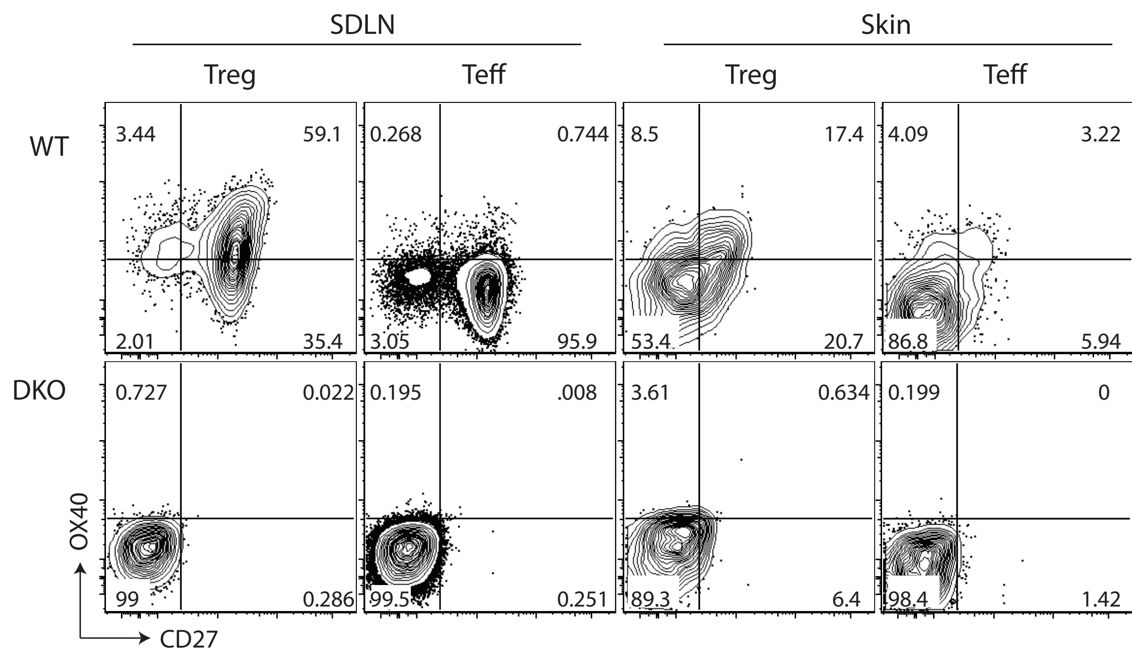
Figure 1.6: CD27 expression inversely correlates with Treg production of IL-17 in diseased human skin.

*__(A)__ Representative FACS plots and quantification of percentages and MFI of CD27 on Tregs and Teff cells (gated on Live CD45+CD3+CD4+) within healthy human skin. Results are pooled data from n = 21 healthy human skin biopsies. __(B)__ Treg IL-17 production in healthy or lesional psoriatic (PSO) skin was quantified by flow cytometry after PMA/ionomycin re-stimulation. Results are pooled from 22 healthy controls and 26 PSO patients. __(C)__ Representative FACS plot of CD27 expression on Tregs and Teffs in PSO skin is shown. Correlation between Treg IL-17 production and CD27 MFI in lesional PSO skin was quantified by flow cytometry. Lines represent paired data from the same sample. __(D)__ Treg IL-17 production in biopsies from paired non-lesional (NL-HS) and lesional (L-HS) skin of hidradenitis suppurativa patients was quantified by flow cytometry (gated on Live CD45+CD3+CD4+FOXP3+ cells). Lines represent paired data from a single patient. Results are combined from 6 HS patients. __(E)__ Representative FACS plot of CD27 expression on Tregs and Teffs in lesional HS skin. Correlation between Treg IL-17 production and CD27 MFI in lesional skin biopsies from HS patients as quantified by flow cytometry. Lines represent paired data from the same sample. p-values are determined using unpaired students t-test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ns=$p>0.05$.*



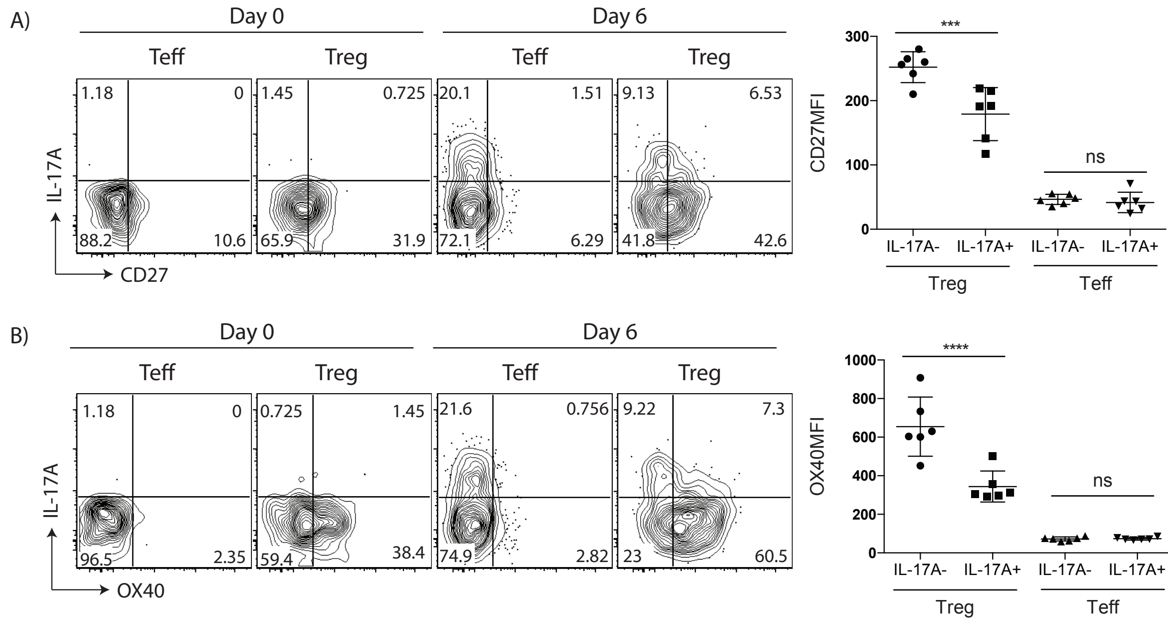
Supplementary Figure 1.1: Human skin-resident Treg Expression of Th17-associated genes.

Tregs and Teff cells were sort purified from healthy human skin for whole transcriptome gene expression analysis by RNAseq. Heatmap of Th17-associated cytokines (top), transcription factors and cytokine receptors (middle), and Treg-specific genes (bottom) are shown.



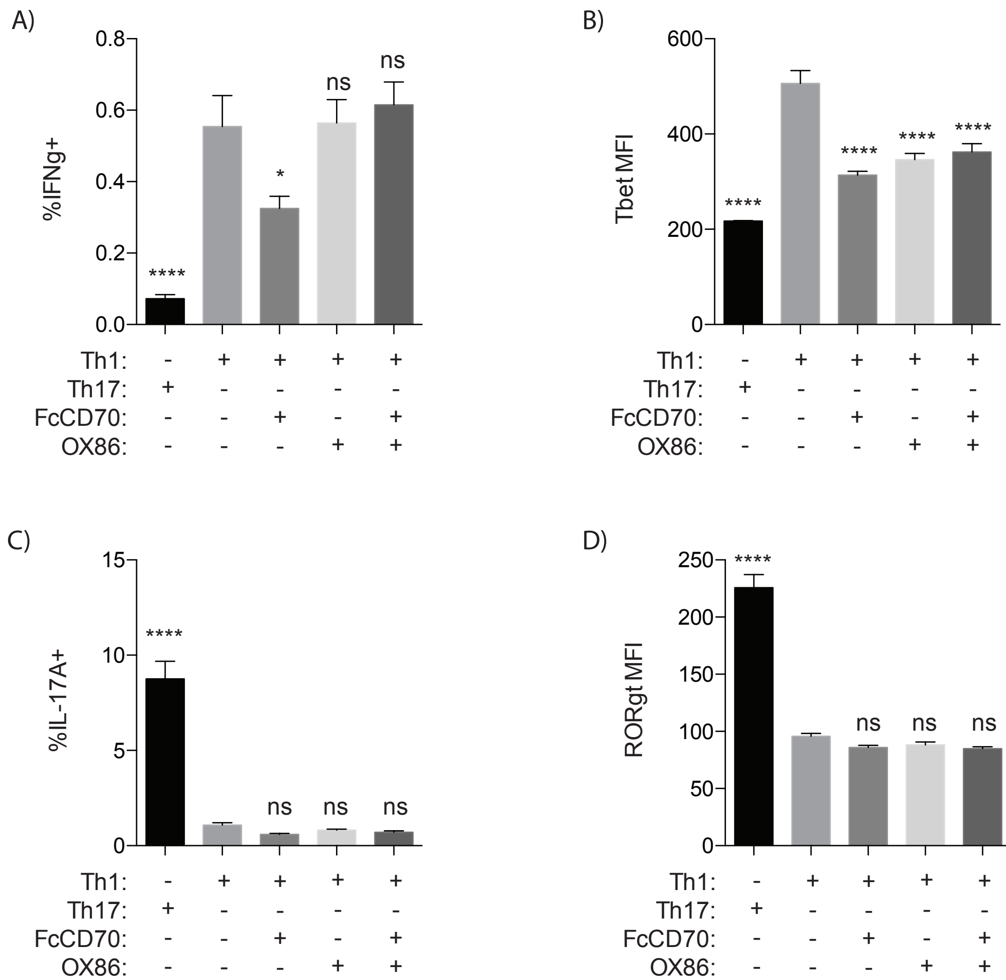
Supplementary Figure 1.2: CD27 and OX40 Co-Expression on Tregs

Representative FACS plots of CD27 and OX40 expression on Tregs (FOXP3+) and Teffs (FOXP3-) cells in healthy murine skin and SDLN. Top panel represents staining in WT mice, bottom panels represent staining from CD27-/-OX40-/- (DKO) mice. Populations are pre-gated on Live CD45+CD4+ cells. Results are representative of n=6 mice.

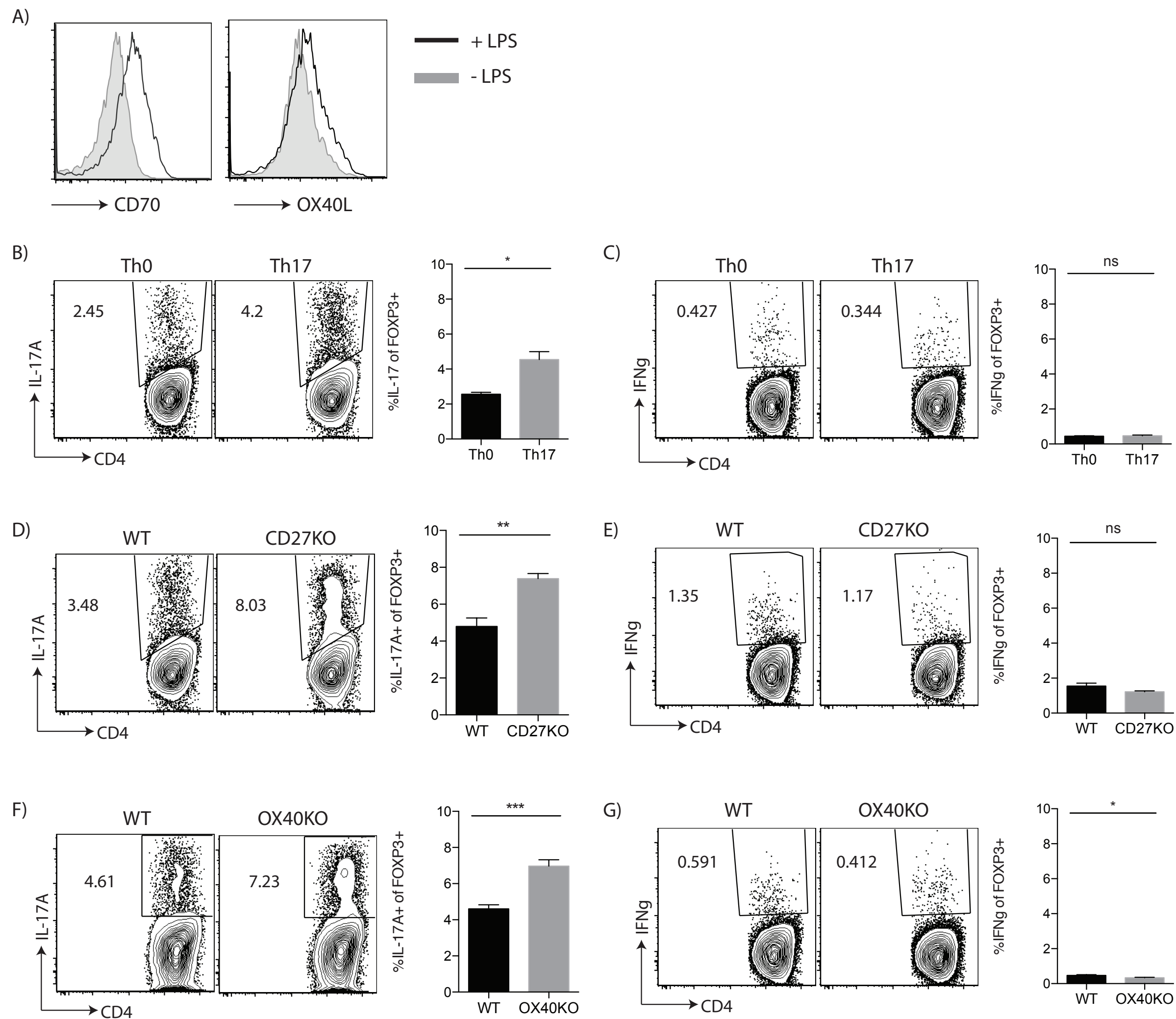


Supplementary Figure 1.3: Treg IL-17 expression after c. albicans infection is inversely correlated with OX40 and CD27 expression.

*Representative FACS plots comparing IL-17 and either (A) CD27 and (B) OX40 on Tregs (FOXP3+) and Teffs (FOXP3-) in the skin of WT mice on days 0 and 6 after cutaneous c. albicans infection. Populations are pre-gated on Live CD45+CD4+ cells. Results are representative of 2 independent experiments with n=6 mice and graphs depict mean ± SEM. p-values are determined using a one-way ANOVA multiple comparisons test. ***p<0.001, ****p<0.0001, ns=p>0.05.*

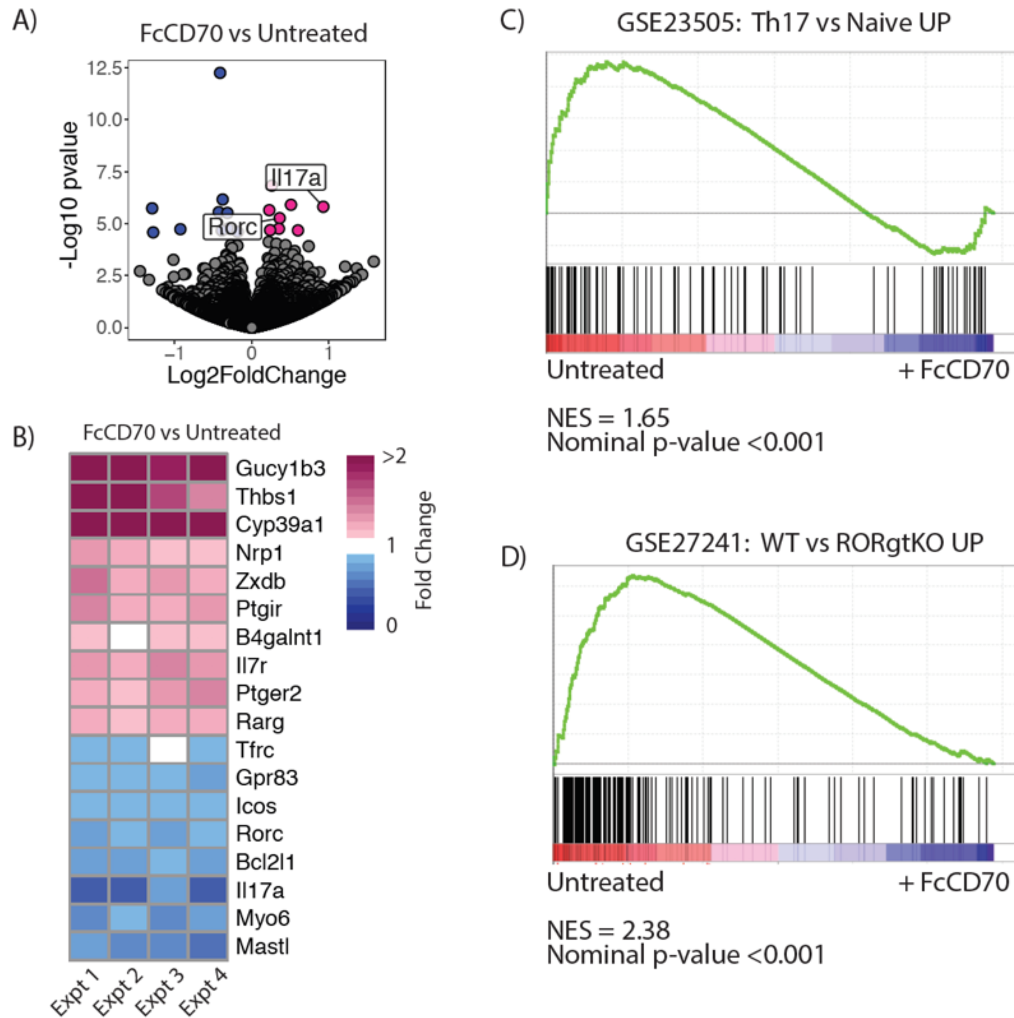


Supplementary Figure 1.4: Effect of CD27 and OX40 signaling on Th1 differentiation *in vitro*.
*WT Tregs were sort purified from FOXP3-GFP reporter mice and cultured with anti-CD3/anti-CD28 coated Dynalbeads in either Th1 or Th17-polarizing conditions in the presence or absence of FcCD70 and/or an agonistic anti-OX40 monoclonal antibody (OX86). Cultures were restimulated on day 6 with PMA and ionomycin. (A) IFNγ, (B) Tbet, (C) IL-17A, and (D) RORγt expression was quantified by FACS in each condition. Data is representative of 2 independent experiments with technical replicates and graphs depict mean ± SEM. p-values are determined using a one-way ANOVA multiple comparisons test and compared to Th1 conditions without FcCD70 or OX86 added. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=p>0.05.*



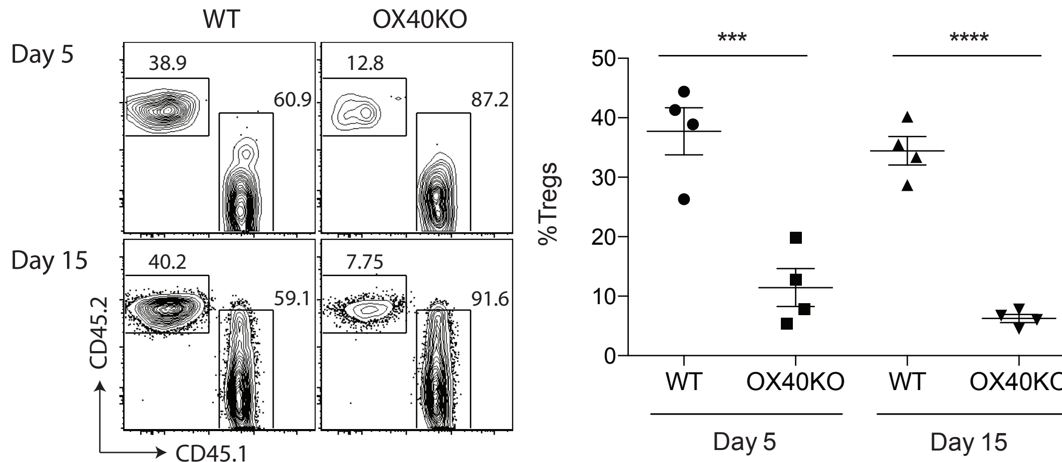
Supplementary Figure 1.5: Deletion of CD27 or OX40 on Tregs results in increased IL-17 expression *in vitro*.

*WT, CD27$^{-/-}$, or OX40$^{-/-}$ Tregs were FACS sorted to >95% purity and cultured in the presence of WT BMDCs in either Th0 (IL-2) or Th17 (IL-6 and TGFb) conditions. Cultures were re-stimulated with PMA/ionomycin on day 6. (A) Representative histogram FACS plots of CD70 and OX40L expression on LPS-matured compared to immature (no LPS) BMDCs (pre-gated on CD45+CD11b+CD11c+MHCII+). Representative FACS plots and quantification of IL-17 and IFNγ between (B-C) WT Tregs cultured in either Th0 or Th17 promoting conditions, (D-E) WT and CD27KO Tregs cultured in Th17 promoting conditions, (F-G) WT and OX40KO Tregs cultured in Th17 promoting conditions. Data is representative of 2-3 independent experiments with technical replicates, graphs depict mean ± SEM. p-values are determined using unpaired students t-test. *p<0.05, **p<0.01, ***p<0.001, ns=p>0.05.*



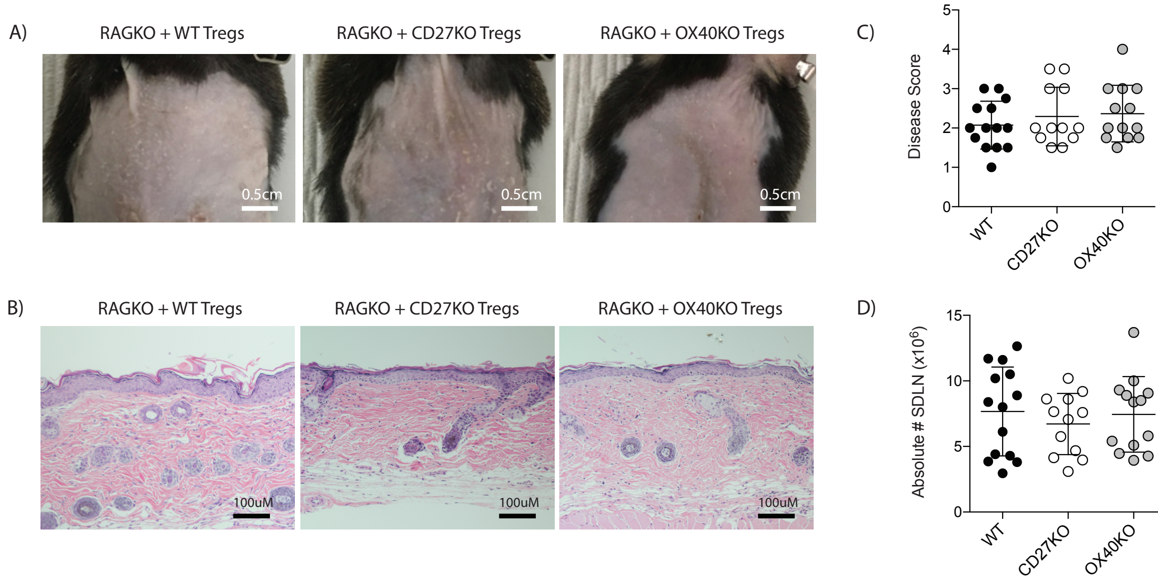
Supplementary Figure 1.6: CD27 signaling suppresses Treg expression of Th17-associated genes.

Tregs were sort purified from FOXP3-GFP reporter mice and cultured with anti-CD3/anti-CD28 coated Dynalbeads in either Th0 or Th17-polarizing conditions in the presence or absence of FcCD70. On day 6 we performed whole transcriptome RNAseq on purified cells. ***(A)*** *Volcano plot comparing gene expression of either FcCD70-treated or untreated Tregs after in vitro polarization in Th17 conditions.* ***(B)*** *Heatmap of top 18 differentially expressed genes with a false discovery rate of <0.05.* ***(C-D)*** *GSEA enrichment plot of Th17-upregulated (TOP) and RORγt -driven (BOTTOM) genes in untreated vs FcCD70-treated Tregs under Th17-polarizing conditions.* ***(C)*** *Th17-upregulated genes (GSE23505) and* ***(D)*** *RORγt -driven genes (from GSE27241) were obtained from MSigDB. NES= normalized enrichment score. Data are combined from 4 independent experiments.*



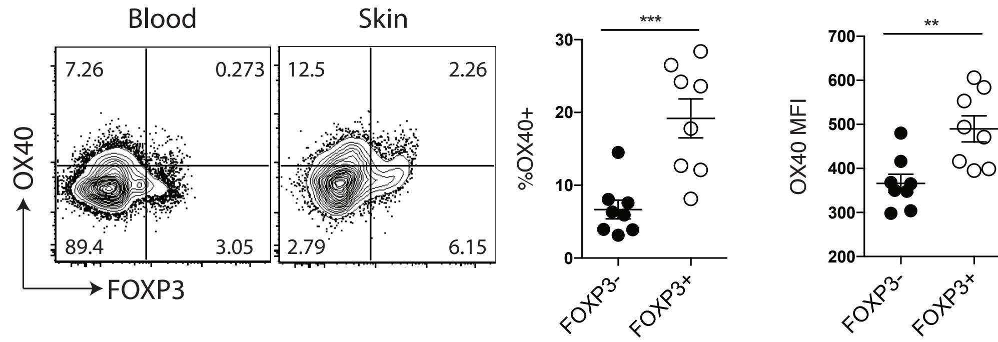
Supplementary Figure 1.7: OX40 is important for Treg accumulation in adoptive transfer model before c. albicans infection.

*WT FOXP3-GFP or OX40$^{-/-}$ FOXP3-GFP knockout CD45.2 Tregs were sort purified, combined at a 1:1 ratio and adoptively transferred with CD45.1 Teffs into RAG$^{-/-}$ recipients. On days 5 and 15 after transfer, the percentages of Tregs (CD45.2+) and Teffs (CD45.1+) were assessed in the SDLNs (gated on Live CD4+TCRb+). Data is representative of 1(day 5) or 2 (day 15) independent experiments with n=4 mice per group, graphs depict mean ± SEM. p-values are determined using a one-way ANOVA. ***p<0.001, ****p<0.0001*



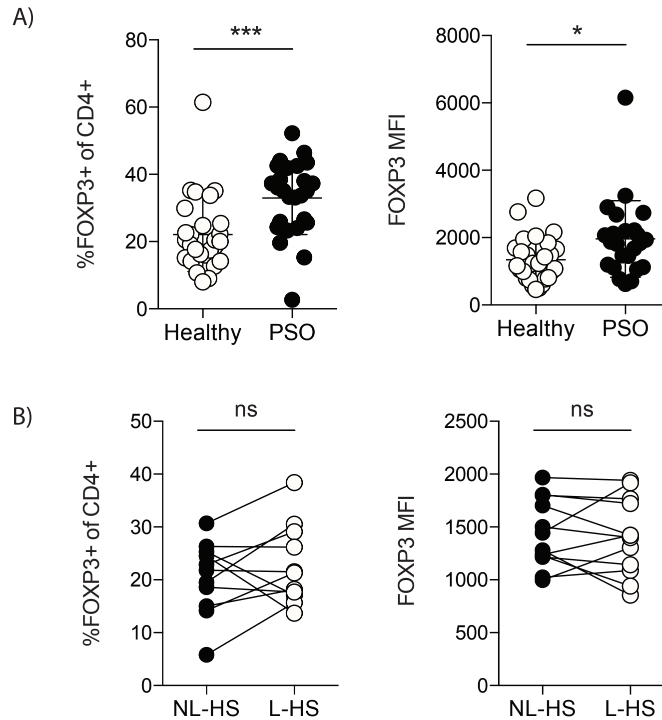
Supplementary Figure 1.8: Deletion of either CD27 or OX40 in Tregs has minimal effect on skin inflammation.

WT, CD27$^{-/-}$, or OX40$^{-/-}$ CD45.2 Tregs were sort purified and adoptively transferred with CD45.1 Teff cells into RAG$^{-/-}$ recipients. On day 14 after transfer, mice were infected cutaneously with c. albicans and inflammation was quantified 7 days after infection. ***(A)*** *Representative clinical images of dorsal skin, 7 days after infection.* ***(B)*** *Representative H&E of dorsal skin, 7 days after infection.* ***(C)*** *Quantification of histologic disease based on blinded scoring of epidermal hyperplasia and mononuclear cell infiltrate.* ***(D)*** *Absolute numbers of cells in the SDLNs as quantified by hemocytometry. Data is combined from 3 independent experiments, and graphs depict mean ± SEM.*



Supplementary Figure 1.9: OX40 is preferentially expressed on Tregs in healthy human skin.

*Representative FACS plots from the skin and PBMCs of healthy patients. Percentages and MFI of CD27 on Tregs and Teff cells (gated on Live CD45+CD3+CD4+) within healthy human skin are quantified. Results are pooled data from n = 8 healthy human skin biopsies. p-values are determined using an unpaired students t-test. **p<0.01, ***p<0.001.*



Supplementary Figure 1.10: FOXP3% and MFI of Tregs in human disease.
*(A) Quantification of percentages and MFI of FOXP3+ cells in healthy or psoriatic skin biopsies as assessed by flow cytometry (Gated on Live CD45+CD3+CD4). Results are combined from n=27 healthy and n=26 psoriatic biopsies. (B) Quantification of percentages and MFI of FOXP3+ cells in paired non-lesional and lesional HS biopsies. Lines represent paired data from a single patient. Results are combined from 11 patients. p-values are determined using either an unpaired (A) or paired (B) students t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=p>0.05.*

Supplementary Table 1.1: Th17 Cytokines

Gene Name	baseMean	log2FoldChange	lfcSE	pvalue	padj
Il17a	85.18692763	0.927851812	0.193070641	1.54E-06	0.006680398
Il21	123.9508384	0.405441265	0.216915864	0.061606451	0.960606336
Il22	1.722768321	-0.426634626	0.423289914	0.313501653	0.960606336
Csf2	25.75856287	0.291088084	0.411032782	0.478829121	0.960606336
Ifng	10.00533278	0.310045531	0.482519994	0.52051303	0.960606336
Il17f	305.5442741	1.025560412	0.32924315	NA	NA

Supplementary Table 1.2: Treg Associated Genes

Gene Name	baseMean	log2FoldChange	lfcSE	pvalue	padj
Tigit	682.0419693	-0.332034759	0.092118005	0.000312814	0.202565759
Il2ra	17959.28688	0.241536451	0.086707816	0.005342275	0.778101151
Ctla4	15127.34363	0.109883314	0.061009687	0.071690304	0.960606336
Ebi3	357.3230928	-0.216957043	0.135100107	0.10829624	0.960606336
Entpd1	330.4939134	0.270103691	0.187963058	0.15071682	0.960606336
Lag3	797.0920346	-0.120801892	0.086833709	0.164168853	0.960606336
Gzma	73.07402965	-0.384457059	0.307360755	0.210995312	0.960606336
Tgfb2	1.199667096	-0.322250168	0.379554213	0.395868624	0.960606336
Tnfrsf18	3346.363622	-0.056985718	0.098062707	0.561162894	0.960606336
Lrrc32	8181.927244	-0.070479987	0.137362549	0.607885121	0.964852981
Ikzf2	5533.566751	-0.044136456	0.119012507	0.710745081	0.982867276
Tnfrsf4	2679.327421	-0.03839592	0.112580614	0.733063924	0.985342144
Ikzf4	2161.262098	-0.037640071	0.115060695	0.743567815	0.985645112
Cd27	6519.635361	-0.016432138	0.055314528	0.766415121	0.988663519
Il10	65.06340868	0.061074591	0.341293109	0.857976527	0.995639966
Tgfb3	38.99724029	0.046034498	0.358579233	0.897848046	0.999901957
Adcy9	74.25219954	0.017689061	0.324120468	0.956476597	0.999901957
Gzmb	1420.232093	-0.003537661	0.162495699	0.98263079	0.999901957
Havcr2	93.81270945	0.004627851	0.246988133	0.985050802	0.999901957
Foxp3	5229.220494	0.201238122	0.221162894	NA	NA

Supplementary Table 1.3 Antibodies

Antibody	Source	Catalog Number	Clone	Color
Anti-Mouse RORγt	BD	562684	Q31-378	PE-CF594
Anti-Mouse IFNγ	BioLegend	505831	XMG1.2	BV650
Anti-Mouse IFNγ	BD Biosciences	557998	XMG1.2	Alexa Fluor 700
Anti-Mouse CD4	BD Horizon	563747	RM4-5	BV650
Anti-Mouse CD4	BD Horizon	563152	53-6.7	BV605
Anti-Mouse CD4	BioLegend	100540	RM4-5	PerCP Cy 5.5
Anti-Mouse IL-17A	BioLegend	506922	TC11-18H10.1	PE-Cy 7
Anti-Mouse IL-17F	BD Biosciences	561656	079-289	PE
Anti-Mouse CD196	BD Pharmigen	561753	140706	A647
Anti-Mouse CD45.2	eBioscience	47-0454-82	104	A780
Anti-Mouse CD45.1	BioLegend	110723	A20	Alexa Fluor 700
Anti-Mouse/Rat FOXP3	eBioscience	48-5773-82	FJK-16s	eFluor 450
Anti-Mouse CD27	BD Pharmigen	561785	LG.3A10	PE
Anti-Mouse CD134	BioLegend	119410	ACT35	PE
Anti-Mouse CD45	eBioscience	56-0451-82	30-F11	Alexa Fluor 700
GHOST	Tonbo	13-0870-T100		Aqua
Anti-Mouse CD25	eBioscience	17-0251-82	PC61.5	APC
Anti-Human CD27	eBioscience	46-0271-80	LG.7F9	PerCP-eFluor 710
Anti-Human CD4	eBioscience	MHCD0417	S3.5	PE-Texas Red
Anti-Human CD45	eBioscience	8047-9459-120	2D1	APC-eFluor780
Anti-Human IL-17A	eBioscience	50-7178-41	eBio64CAP17	e660
Anti-Human FOXP3	eBioscience	48-4776-41	PCH101	eFluor 450

Chapter III. CD27 Promotes Effector T Cell Survival by Suppressing the Cell-extrinsic Apoptosis Pathway

Abstract

Signaling through CD27 plays a role in T cell activation and memory. However, it is currently unknown how this costimulatory receptor influences $CD4^+$ effector (Teff) cells in inflamed tissues. In the current study, we utilized a murine model of inducible self-antigen expression in the epidermis to elucidate the functional role of CD27 on auto-reactive Teff cells. Expression of CD27 on antigen-specific Teff cells resulted in markedly enhanced skin inflammation when compared to CD27-deficient Teff cells. CD27 signaling promoted the accumulation of IFNγ and IL-2-producing T cells in skin draining lymph nodes in a cell-intrinsic fashion. Surprisingly, this costimulatory pathway had minimal effect on early T cell activation and proliferation. Instead, signaling through CD27 resulted in the progressive survival of Teff cells during the autoimmune response. Utilizing BH3 profiling to assess mitochondrial cell priming, we found that CD27-deficient cells were equally as sensitive as CD27-sufficient cells to mitochondrial outer membrane polarization upon exposure to either BH3 activator or sensitizer peptides. In contrast, CD27-deficient Teff cells expressed higher levels of active-caspase 8. Taken together, these results suggest that CD27 does not promote Teff cell survival by increasing expression of anti-apoptotic BCL2 family members but instead acts by preferentially suppressing the cell-extrinsic apoptosis pathway, highlighting a previously un-identified role for CD27 in augmenting autoreactive Teff cell responses.

Introduction

Costimulatory receptors shape the initiation, magnitude and quality of an immune response. The prototypical costimulatory receptor is CD28 and stimulation through this receptor is required for optimal T cell activation and productive immune responses [164]. Additional costimulatory receptors include the TNFR super family (TNFRSF), which includes OX40, CD30, 4-1BB and CD27 [129]. These TNFR family members lack pro-apoptotic death domains and signaling upon engagement with their respective ligands induces the activation of the NFkB and JNK pathways. While the functions of these receptors can be complementary, the outcome of signaling through any given receptor is highly context dependent. It is therefore of interest to understand how individual TNFRSF receptors influence immune responses within different tissues and inflammatory contexts.

One particular TNFR family member, CD27, is constitutively expressed by naïve and memory T cells in secondary lymphoid organs as well as on activated B cells [165]. The only known ligand for CD27 is CD70. Expression of CD70 is tightly regulated and only transiently expressed on DCs, B cells, T cells and NK Cells after immune activation [81,130,131]. Engagement of CD27 by CD70 induces the recruitment of the TRAF2 and TRAF5 adaptor proteins which in turn activate either the JNK or NFkB signaling pathways [132]. This costimulatory pathway influences T cell function in several disease models [79]. In settings of viral infection, CD27 contributes to the effective generation of both primary and memory CD8+ T cell responses [90,115,166,167]. CD27 can also promote T cell responses in the context of productive immunization [93,96,168,169]. Finally, constitutive expression of CD70 on DCs result in autoimmunity [170]. The

CD27 pathway is therefore a promising target for therapeutic intervention to either augment immune responses to infections and tumors or to attenuate excessive inflammation in the setting of autoimmunity.

Signaling through CD27 can augment T cell responses, in part by promoting cell survival. In specific contexts, engagement of CD27 can prevent apoptosis in both human and mouse CD8+ T cells by increasing expression of anti-apoptotic BCL2 family members (including BCL-XL) [91,92,97]. CD27 can also promote survival by inducing the downregulation of FasL and suppressing extrinsic (or death-receptor mediated) apoptosis [95]. In addition to regulating the expression of anti-apoptotic proteins, CD27 may also promote T cell survival and maintenance by modulating the expression of both cytokines and cytokine receptors. During viral infection, CD27 can induce IL-2 expression and promote CD8+ T cell survival through autocrine IL-2 signaling [94]. CD27 signaling has also been implicated in increasing the frequency of IL-7 receptor expressing memory precursors [93]. The mechanisms by which CD27 promotes T cell survival therefore appear to be highly complex and contextually dependent.

The molecular mechanisms responsible for CD27-mediated influence of T cell function have predominately been defined in CD8+ T cells. The role of CD27 signaling in CD4+ T cell responses remains poorly understood. In addition, how the CD27 pathway influences T cell responses outside of secondary lymphoid organs (*i.e.,* in peripheral tissues such as the skin) remains to be defined. In the current study, we use a well-established model of cutaneous autoimmunity to study the role of CD27 signaling in CD4+ Teff cell responses to tissue antigen [171].

Results

CD27 expression by antigen-specific CD4+ Teff cells promotes skin inflammation

To study the role of CD27 in CD4+ T cell-mediated autoimmunity, we utilized a model of inducible antigen expression within the epidermis [21,171,172]. This model involved two transgenes, one which has a cytokeratin 5 (K5) promoter driving the expression of a tetracycline trans-activator protein and the other transgene drives the expression of the model antigen ovalbumin (OVA) under the control of a tetracycline-response element (TGO). Mice with both K5 and TGO transgenes enable expression of OVA to be stringently induced in the basal layer of the epidermis upon treatment with doxycycline. The transfer of ovalbumin-specific CD4+ T cells (OTII) into K5/TGO recipients treated with doxycycline induces a robust inflammatory dermatitis.

We used the K5/TGO model to elucidate the role of the CD27 pathway on autoreactive CD4+ Teff cells specific for a tissue antigen. CD27-/- mice were crossed to OTII-TCR transgenic mice, and resultant mice were used as a source of ova-specific T cells that lacked CD27 expression. CD4+ T cells were isolated from either WT OTII or CD27-/- OTII mice and transferred into K5/TGO recipients. Mice were then treated with doxycycline to induce antigen expression in skin. Recipient mice that received WT OTII cells developed a robust inflammatory dermatitis that progressively worsened over the course of 11 days (Figure 1A-B). This dermatitis was accompanied by an increase in percentages and absolute numbers of neutrophils (CD45+CD11b+Ly6G+) and inflammatory macrophages (CD45+CD4-CD8-MHCII+CD11b+Ly6Chi) in skin (Figure 1C-D). In contrast, mice receiving CD27-/- OTII cells

had attenuated skin disease, reduced scaling, and decreased neutrophil and inflammatory macrophage influx in skin. These results demonstrate that CD27 expression on antigen-specific CD4+ Teff cells promotes autoimmunity to tissue antigen.

CD27 promotes the accumulation of IFNγ- *and IL-2-producing autoreactive CD4+ Teff cells.*

We next evaluated how CD27 expression influenced the accumulation of antigen-specific CD4+ T cells in skin and SDLN upon antigen induction. WT OTII or CD27-/- OTII cells were adoptively transferred into K5/TGO recipients and mice were treated with doxycycline. After 9 days of antigen induction, skin and SDLNs were harvested and the accumulation of antigen-specific T cells was quantified. In the SDLN, CD27-/- OTII cells accumulated at lower percentages and absolute numbers compared to WT OTII cells (Figure 2A) and a similar trend was seen in skin, although this was not statistically significant (Figure 2B). We have previously shown that in this model, ova-specific T cells almost exclusively express IFNγ [172]. We therefore quantified expression of this cytokine *via* intracellular cytokine staining by flow cytometry. When compared to WT OTII cells, CD27-/- OTII cells produced significantly less IFNγ and IL-2 in SDLNs (Figure 2C-D). Taken together, these results suggest that CD27 expression promotes the accumulation of cytokine-producing autoreactive CD4+ Teff cells in the SDLN in response to autoantigen expression in skin.

CD27 promotes autoreactive Teff cell accumulation in a cell-intrinsic manner.

To determine if CD27 signaling promotes autoreactive CD4+ T cell accumulation in a cell-intrinsic or cell-extrinsic fashion, WT OTII and CD27-/- OTII cells were combined at a 1:1

ratio and adoptively transferred into K5/TGO recipients. Accumulation of WT and CD27-/- OTII cells was quantified at days 3, 5, 7 and 10 after antigen induction (Figure 3). On days 3 and 5 after antigen induction, both WT and CD27-/- OTII cells were present at equivalent percentages, suggesting that CD27 does not markedly influence the early CD4+ T cell response to tissue antigen. However, by day 7, WT OTII cells were present at approximately 20-fold higher levels compared to CD27-/- OTII cells and this difference was maintained at day 10. These results suggest that CD27 signaling promotes autoreactive Teff cell accumulation in a cell-intrinsic fashion and this effect is most pronounced approximately 7-10 days after antigen presentation.

CD27 promotes autoreactive Teff cell survival but not proliferation.

To determine the mechanism by which CD27 promotes the accumulation of antigen-specific CD4+ T cells in our adoptive transfer model, we first quantified proliferation. WT OTII CD45.1 or CD27-/- OTII CD45.2 cells were CFSE labeled, combined at a 1:1 ratio and co-transferred into K5/TGO recipients. Recipients were then placed on doxycycline to induce antigen expression. SDLNs were harvested 4 days after transfer and CFSE dilution quantified by flow cytometry. WT and CD27-/- OTII cells displayed similar levels of proliferation with no differences was observed in either the proliferative or division index of each population (Figure 4A). We also quantified Ki67 expression as a metric of cell cycling. Both WT and CD27-/- cells expressed similar levels of Ki67 (Figure 4B). Consistent with these results, both WT and CD27-/- cells expressed similar levels of CD69, a robust marker of early T cell activation (Figure 4C). These results suggest that signaling through CD27 does not promote Teff cell accumulation by augmenting early T cell activation, entry into the cell cycle or cumulative cell proliferation.

We next assayed whether signaling through CD27 affected Teff cell survival in our model. Similar to above, both WT OTII CD45.1 and CD27-/- OTII CD45.2 cells were adoptively transferred at a 1:1 ratio into K5/TGO recipients followed by antigen induction. Survival was assessed at days 5 and 7 after transfer by quantifying the percentage of dead and dying cells, using Ghost dye (an amine reactive viability dye) and Annexin V staining (Figure 4D). A significant increase in dead or dying Teff cells (GHOST+Annexin V+) was observed in the CD27-/- OTII population compared to WT, and this trend increased with time after antigen induction (Figure 4D). Taken together, these results suggest that CD27 augments the accumulation of autoreactive CD4+ T cell responding to tissue antigen by promoting cell survival rather than activation or proliferation.

CD27 signaling does not promote survival via the intrinsic (mitochondrial) apoptosis pathway.

One mechanism by which multiple TNFRSF receptors promote survival is through the induction of anti-apoptotic BCL2 family members [92,135,173–175]. These anti-apoptotic proteins suppress cell death by antagonizing both multidomain pro-apoptotic proteins (BAX and BAK) as well as activator BH3-only proteins (BID and BIM). Is this manner, anti-apoptotic BCL2 family members can prevent mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome *c* [176]. Opposing anti-apoptotic proteins are sensitizer BH3-only proteins. This group of BH3 family members bind to anti-apoptotic proteins, inhibiting their function and allowing for the release of activators and eventual activation of BAX and BAK. This process is referred to as the mitochondrial or intrinsic apoptotic pathway, and whether a cell undergoes apoptosis is ultimately regulated by the balance of pro-apoptotic, anti- apoptotic, and sensitizer BH3-only family members (reviewed in [176]). CD27 has been reported to induce the expression of

anti-apoptotic proteins including BCL-XL and MCL-1[91,92,97]. Thus, we hypothesized that signaling through CD27 promotes Teff cell survival by suppressing MOMP through the induction of anti-apoptotic proteins. To test this hypothesis, we utilized BH3 profiling. BH3 profiling is a powerful technique that can be utilized to assess mitochondrial priming [177]. This technique involves exposing cells to peptides that mimic the BH3 domains of endogenous activator or sensitizer BH3-only proteins and then measuring the degree of cytochrome c release from mitochondria. Peptide mimics of global sensitizers BIM and PUMA will inhibit all members of the anti-apoptotic family. BIM peptide mimics will also directly interact with BAX and BAK, whereas PUMA cannot. This technique can therefore be used to compare differential sensitivity of individual populations to mitochondrial apoptosis by measuring cytochrome c release in response to various BH3-only peptides [178]. If a certain population of cells is more primed for mitochondrial apoptosis, such a population should release more cytochrome c in response to exposure to an activator BH3 peptide such as BIM. Differences in response to treatment with PUMA further illustrate that that a population exhibits a dependence on anti-apoptotic family members for survival.

We performed BH3 profiling on WT and CD27-/- OTII cells isolated from the SDLNs of recipient K5/TGO mice 4 days after antigen induction. Isolated cells were exposed to various concentrations of peptides or vehicle controls, then fixed and stained for mitochondrial cytochrome c. Assay validation confirmed that both BIM and PUMA peptide exposure induced a dose-dependent release of mitochondrial cytochrome c (Figure 5). Strikingly, in contrast to our hypothesis, both BIM and PUMA induced equal cytochrome c release in both WT and CD27-/- OTII cells at all concentrations tested, suggesting that these populations are equally primed for

MOMP (Figure 5). Thus, differences in survival between autoreactive WT and CD27-/- OTII cells in our model cannot be attributed to activity of anti-apoptotic BCL2 family members.

Signaling through CD27 suppresses the extrinsic cell death pathway to promote autoreactive Teff cell survival.

The other major mechanism that influences survival is the extrinsic, or death receptor mediated apoptosis pathway. The ligation of death receptors, such as TNFR, CD95, or TRAIL1 can induce the activation of caspase-8 *via* proteolytic cleavage (reviewed in [179]). Caspase-8 activation leads to the downstream caspase cascade that ultimately results in apoptosis. To determine if CD27 expression suppressed the extrinsic apoptosis pathway, we quantified active-caspase 8 by flow cytometry. At day 4 post-antigen induction, CD27-/- OTII cells expressed significantly higher levels of active caspase 8 compared to WT controls (Figure 5C). These results suggest that CD27 promotes Teff cell survival by suppressing the extrinsic apoptosis pathway.

Discussion

Developing therapeutics that target the immune system are of great interest to treat both autoimmunity and cancer. The latest generation of cancer immunotherapies targeting immunoregulatory receptors on T cells (*i.e.,* checkpoint inhibitor therapy) have had enhanced response rates in many tumors compared to conventional therapies. Notably, when checkpoint inhibitors are used in combination, response rates are higher than either therapy alone [180]. In addition to "removing the brakes" from the immune response, it is also of great interest to understand how to "add fuel to the fire" and augment T cell responses. One way to achieve this is through targeting costimulatory receptors, including those of the TNFRSF [181]. TNFRSF receptors play crucial roles in augmenting T cell responses by promoting activation, expansion, survival, differentiation and memory. It is therefore of fundamental importance to understand how these receptors function in different disease settings and within different tissues. In current study, we demonstrate that one TNFRSF member, CD27, enhances cutaneous immune responses by promoting autoreactive CD4+ Teff cell survival by suppressing the extrinsic (death receptor-mediated) apoptosis pathway.

Our findings are consistent with prior studies demonstrating that CD27 plays a major role in promoting T cell survival [91–94,97]. Several studies have demonstrated that this pathway increases the expression of anti-apoptotic BCL-2 family members in CD4 and CD8 T cells, including BCL-2 and BCL-XL [91,92,97]. We utilized BH3 profiling to test whether CD27 signaling suppressed sensitivity to mitochondrial induced cell death by increasing the expression of anti-apoptotic BCL2 proteins in autoreactive CD4+ Teff cells responding to tissue self-antigen [177]. If

increasing the expression of anti-apoptotic proteins was the major mechanism by which CD27 promoted Teff survival, CD27-deficient cells would be more sensitive to cytochrome c release upon treatment with either BIM (activator) or PUMA (sensitizer) peptides. Notably, we did not observe any difference in priming between CD27-sufficient or CD27-deficient cells. While our study does not necessarily rule out a role for CD27 in promoting survival through increasing the expression of anti-apoptotic proteins, our results strongly suggests that this is not the major mechanism in CD4+ Teff cells. This is consistent with previous work demonstrating that forced over-expression of BCL2 family members in CD8+ T cells alone or in combination was not enough to compensate for CD27-deficiency [91], suggesting that CD27 can promote survival through multiple mechanisms.

We found that CD27 expression on CD4+ Teff cells promotes survival *via* suppressing the extrinsic apoptosis pathway. Death-receptor ligation results activation of caspase-8 which in turn initiates the downstream caspase cascade which ultimately leads to apoptosis. CD27-sufficent cells expressed lower levels of active-caspase 8 compared to CD27-deficient cells. This finding, combined with our results demonstrating that antigen-specific Teff cells are equally sensitive to BH3-peptide induced MOMP with and without CD27, strongly suggests that CD27 promotes survival by suppressing activation of the extrinsic apoptosis pathway.

Our results are consistent with other models of tissue autoimmunity, demonstrating that blocking CD27 suppresses experimental colitis and lung allergy [182,183]. In contrast to these studies, it has been shown that CD27 deficiency augments autoimmune disease in experimental autoimmune encephalomyelitis (EAE) [99]. Although these studies appear to be contradictory, CD27 is now known to play different roles in CD4+ T cell function depending on the inflammatory context. Multiple studies have demonstrated that while CD27 augment Th1

responses[96,97,100,184,185], signaling through this pathway attenuates Th17 responses [99,100]. The K5/TGO model used in this study induces a strong Th1 response, as defined by IFNγ and IL-2 production from the transferred antigen specific Teff cells, with minimal IL-17 production [172]. We observed a decrease in IFNγ and IL-2 production from CD27-deficient cells after antigen induction, with no difference in IL-17 (data not shown). Thus, our results support the current literature, demonstrating that CD27 enhances Th1 responses. The functional consequence of CD27 signaling in Th17-driven cutaneous inflammatory responses (such as psoriasiform inflammation or *c. albicans* infection) remain to be determined.

In conclusion, in this study we demonstrate that CD27 plays a critical role in augmenting cutaneous autoimmunity by promoting autoreactive CD4+ T cell survival. These results suggest that manipulating the CD27 pathway may be a promising therapeutic approach in autoimmune or malignant diseases of the skin.

Materials and Methods

Animals

All mice were bred and maintained in specific pathogen free facilities at UCSF according to NIH guidelines and experiments were approved by the Institutional Animal Care and Use Committee of UCSF. K5/TGO BALB/C [171] mice were backcrossed to C57BL/6 mice for at least 10 generations and crossed to CD45.1 mice purchased from Jackson Laboratories to generate K5/TGO/CD45.1.2 recipients. CD27-/- [90] mice were generously donated by Jannie Borst and crossed to OTII mice. Control donors were generated by crossing OTII to the CD45.1 strain.

Adoptive Transfer of T cells and Disease Development

Spleens and lymph nodes of donor WT OTII CD45.1 and CD27-/- OTII CD45.2 mice were harvested and processed. Single cell suspensions underwent TAC lysis to remove red blood cells followed by a magnetic CD4+ isolation (STEMCELL). Between $2x10^5$ and $3x10^6$ OTII cells (as noted in figure legends) were adoptively transferred *i.v.* into gender matched K5/TGO/CD45.1.2 recipients. Recipient mice were placed on a 1mg/kg doxycycline chow diet to turn on antigen in the skin. In experiments where skin disease was scored, mice were shaved and depilated in order to monitor disease development.

Skin Processing for Flow Cytometry

Skin was processed and digested as previously described. Briefly, trunk skin was shaved, harvested, lightly defatted and minced. Skin was then incubated in digestion media: RPMI with 2mg/mL collagenase XI (Sigma, Catalog #C9407), 0.5mg/mL hyaluronidase (Sigma, Catalog

#H3506) and 0.1 mg/mL DNase (ICN) for 45 minutes at 37C in a shaking incubator. Digested skin was then washed with RPMI, filtered, and then stained for flow cytometry.

Antibodies and Flow Cytometry:

Cells were stained for surface antigens and a live/dead marker (Ghost Dye™ Violet 510, Tonbo Biosciences) in PBS with 2% Calf Serum and 1% penicillin-streptomycin for 30 minutes at 4°C. To stain for intracellular cytokines, 4x10^6 cells were stimulated with Tonbo restimulation cocktail (Catalog #TNB-4975) in tissue culture media for 4 hours. Cells were then washed, and surface antigens were stained in PBS with 2% FBS and 1% penicillin/streptomycin. Intracellular staining was performed using the eBioscience FOXP3 staining kit (Catalog #00-5523-00). Antibodies used are listed in Supplementary Table 1. Samples were run on a Fortessa (BD Biosciences) in the UCSF Flow Cytometry Core. FlowJo software (FlowJo, LLC) was used to analyze flow cytometry data.

BH3 Profiling

BH3 profiling was performed as previously described [177]. Cells were isolated from the SDLNs of mice that received WT and CD27-/- OTII cells followed by CD4 enrichment via magnetic bead isolation (STEMCELL). Cells were then stained with surface markers in 2% FBS and 1% penicillin/ streptomycin. After the surface stain, cells were resuspended in MEB buffer at $2x10^6$ cells/mL. Cells were then treated with various peptide concentrations in the presence of 0.001% digitonin and incubated for 1 hour at room temperature. After peptide exposure, cells were fixed in 4% PFA for 10 minutes, followed by neutralization with N2 buffer (1.7M Tris, 1.25M Glycine pH 9.1). Cells were then stained with anti-cytochrome C A647 (BD) in

intracellular staining buffer (10%BSA, 2% Tween20 in PBS) at 4C overnight. Cells were analyzed by FACS the next day.

BIM and PUMA peptides were dose titrated and used at final concentrations from 1uM-0.01uM and 10um- 0.1uM respectively. DMSO was used as a vehicle control (maximal mitochondrial cytochrome C retention) while 15uM alamethycin was used as a positive control by directly permeabilizing the mitochondria to induce full cytochrome C release. Percentage of cytochrome c release for each sample was calculated as % cytochrome c release = 1- (MFI_{sample} – MFI_{ALA}) / (MFI_{DMSO} – MFI_{ALA}).

Caspase 8 Staining

SDLNs were processed into a single cell suspension and stained for 30 minutes with the CaspGLOW Fluorescein Active Caspase-8 (Cat# 88-7005) staining kit and protocol. Following active caspase staining, cells were stained with surface markers and analyzed by FACS.

Statistics

Statistics were performed using GraphPad Prism software. P-values were calculated using two-tailed paired or unpaired students t-tests.

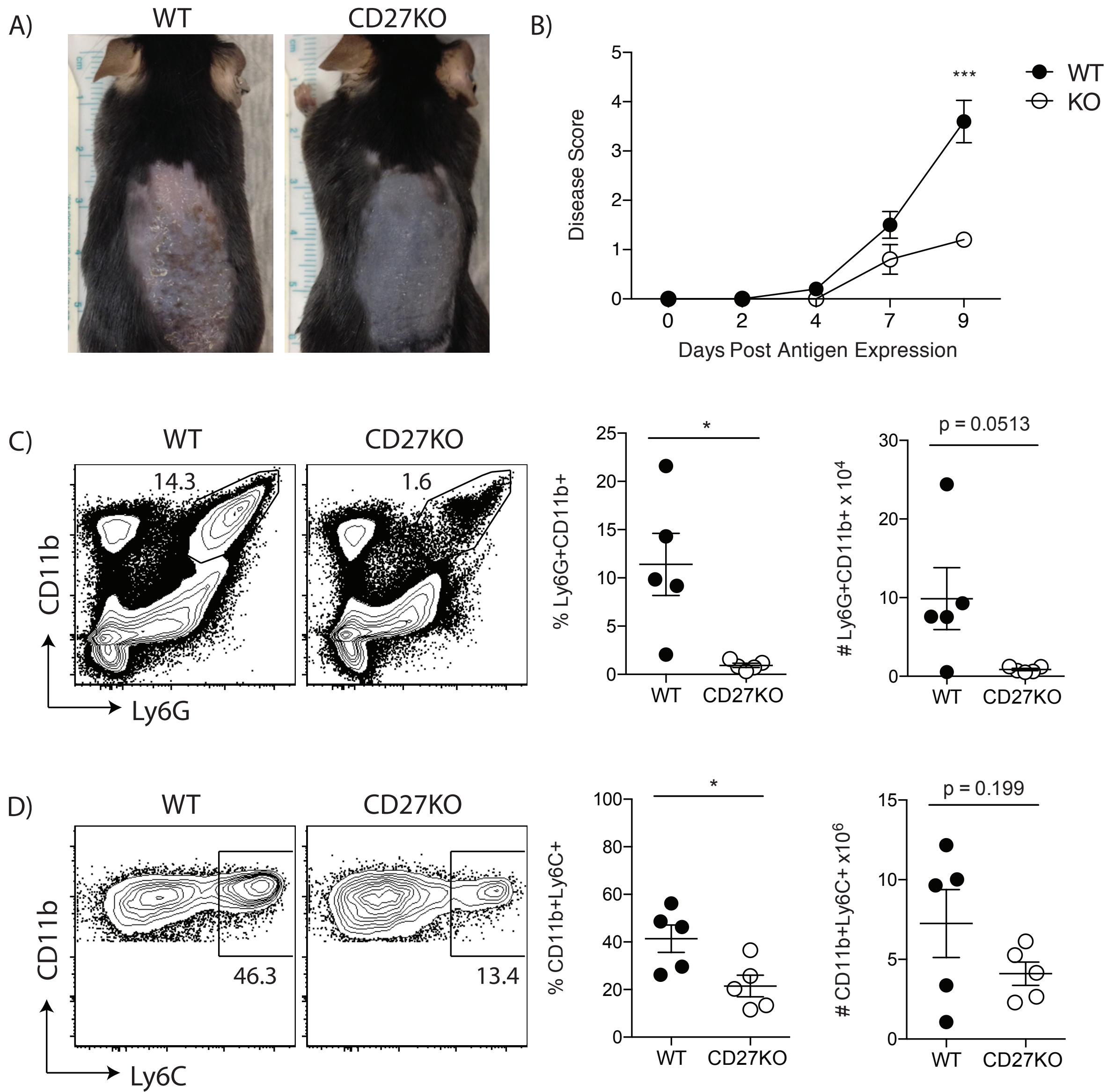


Figure 2.1. CD27 expression by CD4+ T cells promotes inflammation in response to cutaneous antigen.

3x106 WT OTII or CD27-/- OTII cells were adoptively transferred i.v. *into K5/TGO recipients. The day of transfer, mice were placed on doxycycline chow to induce antigen expression. (A) Representative photos of back skin of either WT or CD27-/- mice 9 days after antigen induction. (B) Clinical scores of disease after antigen induction. (C) Representative FACS plots and quantification of neutrophil (gated on CD45+CD11b+Ly6G+) percentages and absolute numbers within the skin. (D) Representative FACS plots and quantification of (E) percentages and (F) absolute number of M1 (Ly6C+) and M2 (Ly6C-) macrophages (gated on Live CD45+ CD4-CD8-CD11b+MHCIIhi) within the skin. Data is representative of 2 independent*

experiments with n=5 mice per group. P-values are determined using an unpaired students t-test.
*$p<0.05$, *** $p<0.001$, ns = $p>0.05$.

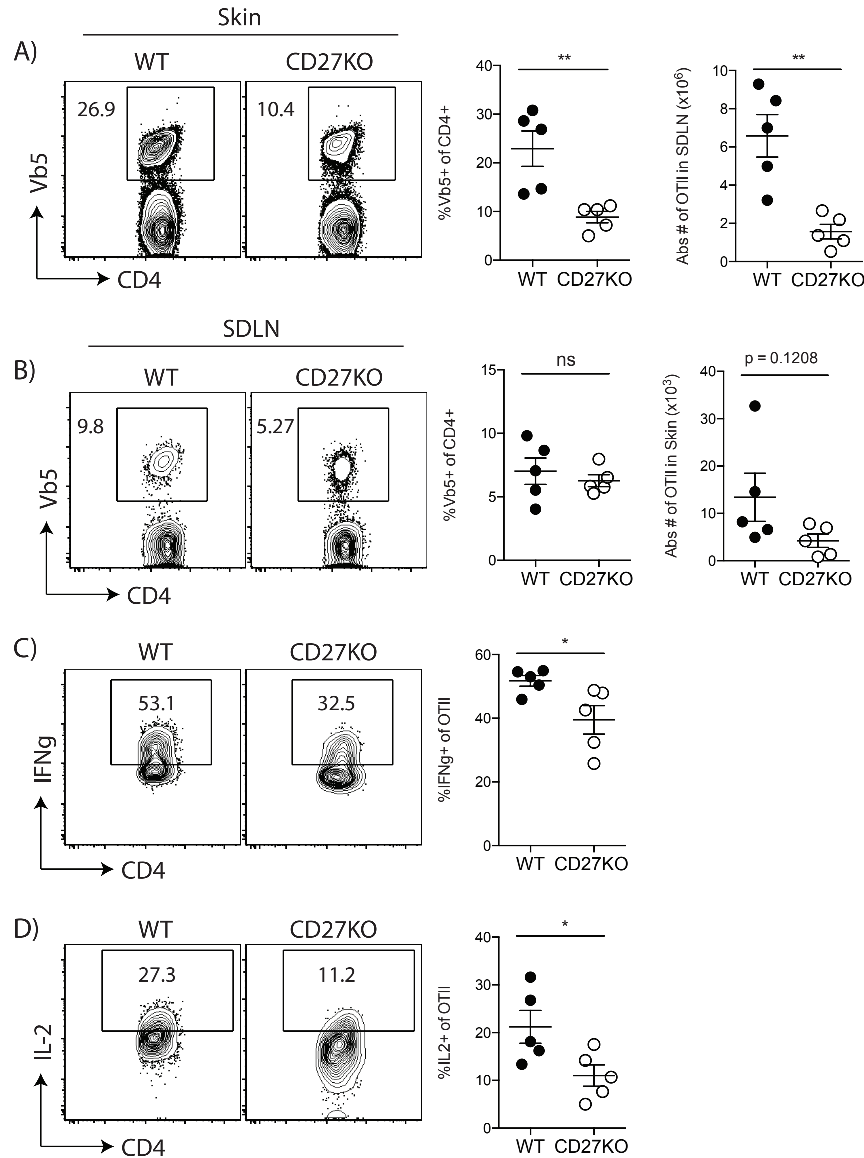


Figure 2.2: CD27 promotes accumulation of cytokine-producing antigen specific CD4+ T cells in the SDLN and Skin.

$3x10^6$ WT OTII CD45.1 or CD27-/- OTII CD45.2 cells were adoptively transferred i.v. *into K5/TGO CD45.1.2 recipients. The day of transfer, mice were placed on doxycycline chow to induce antigen expression. Skin and SDLN were harvested at day 9 to quantify the accumulation and cytokine production from adoptively transferred T cells. (A-B) Representative FACS plots (gated on Live CD4+ Vb5+ cells) and quantification of percentages and absolute numbers of transferred Vb5+ cells in the (A) SDLN and (B) skin. (C-D) Representative FACS plots and quantification of (C) IFNγ and (D) IL-2 producing OTII cells in the SDLN.*

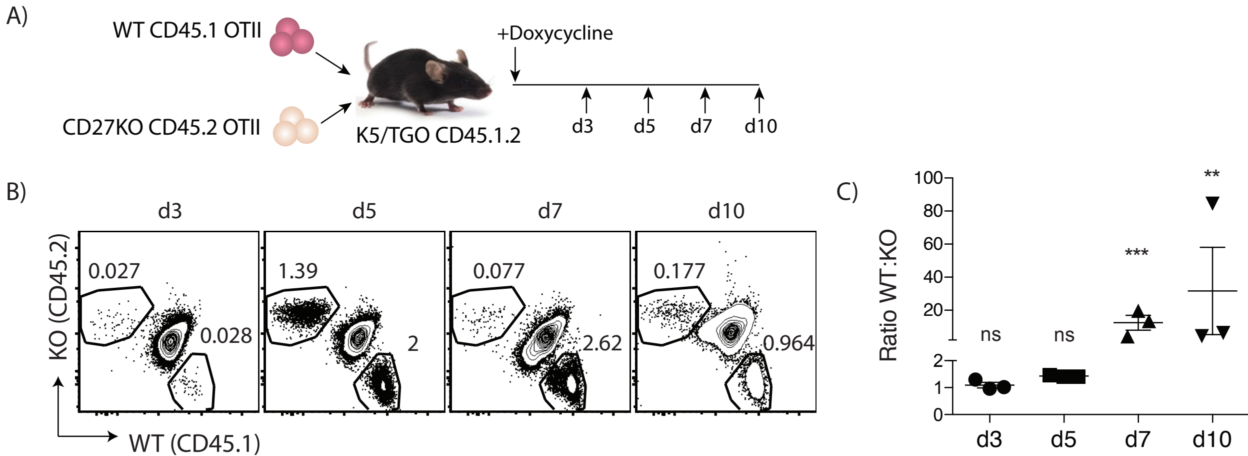


Figure 2.3: CD27 promotes CD4+ T cell accumulation into the SDLN in a cell-intrinsic manner.

*(A) Experimental model. WT OTII CD45.1 and CD27-/- OTII CD45.2 cells were combined at a 1:1 ratio and $4x10^5$ cells were adoptively transferred into K5/TGO CD45.1.2 recipients and placed on doxycycline chow. (B) Representative FACS plots of the percentages of WT and CD27-/- OTII cells at various timepoints after antigen induction (gated on Live CD4+). (C) Quantification and ratios of WT:CD27-/- cells in the SDLN at days 3, 5, 7 and 10 after antigen induction. Statistics calculated using an unpaired students T test to compare percentage of WT vs CD27-/- cells at each timepoint. Data is representative of 2 independent experiments with 3 mice per timepoint. Graphs depict mean ± SD and p-values are determined using an unpaired students t-test. **p<0.01, ***p<0.001, ns = p>0.05.*

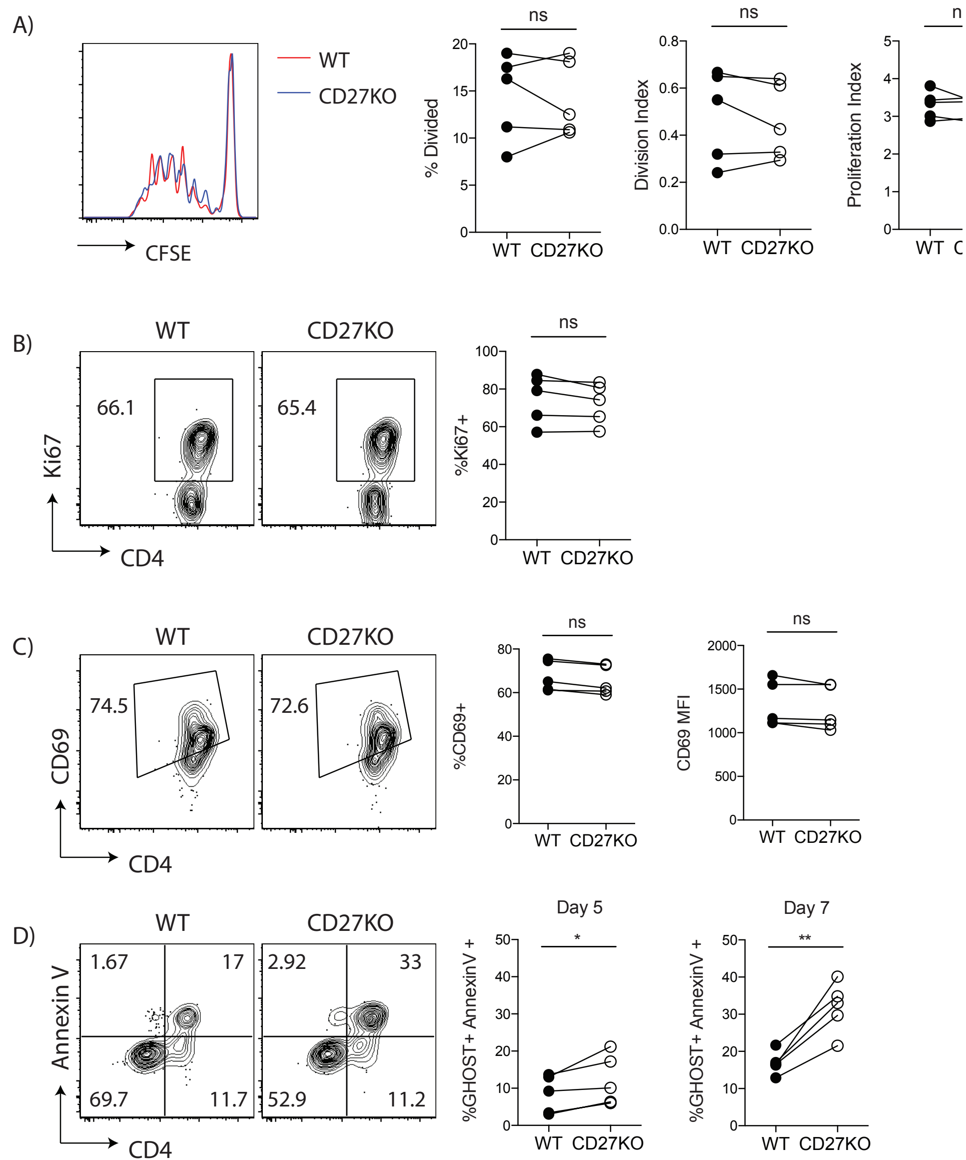


Figure 2.4: CD27 promotes T cell survival in response to antigen.
*(A) WT OTII CD45.1 and CD27-/- OTII CD45.2 cells were CFSE labeled, combined at a 1:1 ratio. $4x10^5$ cells were adoptively transferred into K5/TGO CD45.1.2 recipients and recipients were placed on doxycycline chow to induce antigen expression. 4 days after transfer, SDLN were harvested and CFSE dilution on the transferred WT and CD27-/- populations (gated on Live CD4+ then CD45.1 or CD45.2 respectively) was assessed. (B) Representative FACS plots and quantification of percentages of Ki67+ WT or CD27-/- OTII cells 4 days after transfer. (C) Representative FACS plots and quantification of percentages and MFI of CD69 in WT or CD27-/- OTII cells 4 days after transfer. (D) Representative FACS plots and quantification of cell death in WT or CD27-/- OTII cells as assessed by Annexin V and viability dye incorporation. Data is representative of at least two independent experiments with n=5 or more mice. P-values are determined using a paired students t-test. *p<0.05, **p<0.01, ns = p>0.05.*

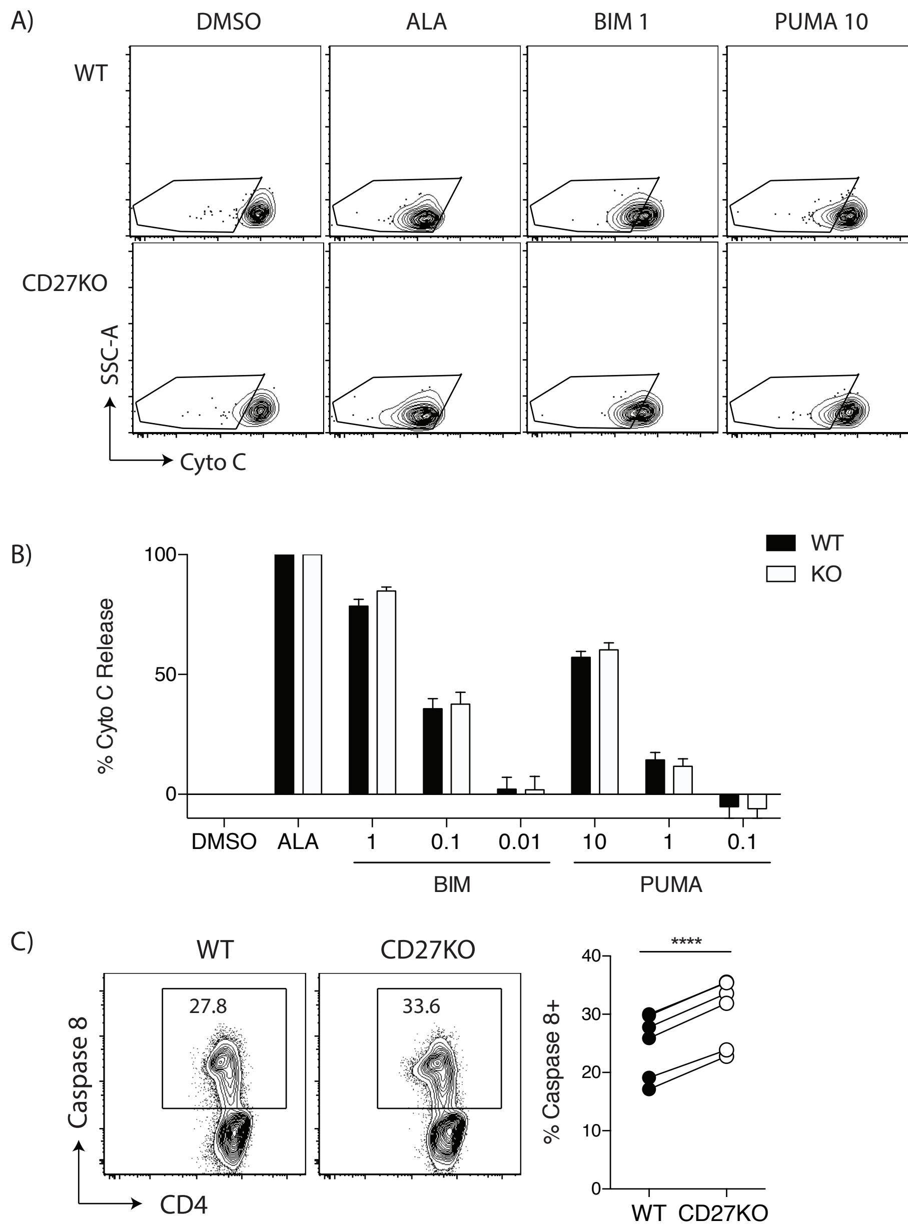


Figure 2.5: CD27 signaling on CD4+ T cells does not promote survival via the intrinsic (mitochondrial) apoptosis pathway, and instead inhibits the caspase 8 (extrinsic) apoptotic pathway.

WT CD45.1 and CD27-/- CD45.2 OTII cells were combined at a 1:1 ratio and $3x10^6$ cells were adoptively transferred into K5/TGO CD45.1.2 recipients followed by doxycycline administration. 4 days after antigen induction, SDLNs were harvested. After CD4 enrichment, BH3 profiling was performed using various concentrations (as indicated in figure) of BIM (activator) peptide, PUMA (sensitizer) peptide, DMSO (vehicle), or ALA (positive control). Cytochrome C release was then measured by FACS. (A) Representative FACS plots of cytochrome C staining in either WT (top) or CD27-/- (bottom) cells, gated on Live CD4+ then either CD45.1 or CD45.2

*respectively. (B) Quantification of percentage cytochrome c release after various peptide or vehicle treatments. (C) WT OTII CD45.1 and CD27-/- OTII CD45.2 cells were adoptively transferred into K5/TGO recipients, which were then placed on doxycycline chow to induce ova expression. 4 days after transfer, SDLNs were harvested and stained for active caspase 8 and analyzed by flow cytometry. Representative FACS plots of active caspase 8 staining in either WT (CD45.1+) or CD27-/- (CD45.2+) cells, after gating of Live CD4+Vb5+, are shown and quantified. Data is representative of 3 independent experiments with n=4 mice. Graphs depict mean ± SEM. And statistics in (C) were quantified using a paired students t test. ****$p<0.0001$*

Table 2.1 Antibodies

Antibody	Source	Catalog Number	Clone	Color
Anti-Mouse CD45.2	eBioscience	47-0454-82	104	APC-eFluor 780
Anti-Mouse CD45.1	BioLegend	110723	A20	Alexa Fluor 700
GHOST	Tonbo	13-0870-T100		Aqua
Anti-Mouse CD4	BioLegend	100540	RM4-5	PerCP Cy 5.5
Anti-Mouse CD4	BD Horizon	563747	RM4-5	BV650
Anti-Mouse CD4	BD Horizon	563152	53-6.7	BV605
Anti-Mouse TCR Vb5.1/2	BioLegend	139504	MR9-4	PE
Anti-Mouse Cytochrome C	BD Pharmigen	560263	6H2.B4	Alexa Fluor 488
Annexin V	BD Biosciences	556419		FITC
Anti-Mouse Ki67	BD Biosciences	561283	B56	Ki67
Anti-Mouse Ly6G	BioLegend	127618	1A8	PE-Cy7
Anti-Mouse CD11b	eBioscience	47-0112-82	M1/70	APC-eFluor 780
Anti-Mouse CD45	eBioscience	56-0451-82	30-F11	Alexa Fluor 700
Anti-Mouse Ly-6C	BD Horizon	563011	AL-21	BV605
Anti-Mouse MHC II	eBioscience	48-5321-82	M5/114.15.2	eFluor 450
Anti-Mouse IFNγ	eBioscience	11-7311-82	XMG1.2	FITC
Anti-Mouse IL-2	eBioscience	17-7021-82	JES6-5H4	APC
Anti-Mouse CD69	BioLegend	104529	H1.2F3	BV605

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