Multi-Stage Regulation of CD8+ T Lymphocyte Differentiation by TGF-Beta

Multi-stage regulation of $CD8^+$ T lymphocyte differentiation by TGF-β

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ABSTRACT

Epithelial resident memory CD8$^+$ T cells (eT_{RM}) have emerged as critical immune sentinels, playing a dominant role in protecting against barrier-invading pathogens and tumors, yet also mediating pathogenesis of autoimmune disorders. While their functional importance is established, determinants of eT_{RM} differentiation have yet to be fully elucidated. One critical cytokine in eT_{RM} differentiation is TGF-β, which is known to have a wide range of consequences on CD8$^+$ T cell fate. A major regulator of this pleiotropism is the context by which TGF-β is activated from its latent form- thought to be tightly regulated by αV-integrins expressed on many cell types, including epithelial and dendritic cells (DC). Therefore, we sought to investigate the control of TGF-β activation during eT_{RM} differentiation.

We initially hypothesized that DC in the skin activate TGF-β for eT_{RM} formation. Upon conditional deletion of TGF-β-activating αV-integrin in DC, we observed a profound deficiency in skin eT_{RM} that was not due to effects in the skin during terminal differentiation or priming. Instead, we found that at homeostasis, DC-activated TGF-β on naïve CD8$^+$ T cells epigenetically conditions them for eT_{RM} formation in lymph nodes through non-cognate interactions of migratory DC with naïve CD8$^+$ T cells. Collectively, our studies identify a novel stage of T cell differentiation, during which the pre-immune repertoire is actively conditioned for a specialized fate.

Although terminal eT_{RM} differentiation was independent of DC-activated TGF-β, other sources of TGF-β in the skin may regulate factors involved in long-term tissue retention. We

therefore studied spatiotemporal dynamics to understand contributions of local TGF-β in the skin to eT_{RM} differentiation. Through intravital microscopy of the skin, we found that TGF-β controls T cell migration into the epidermis via hair follicles by induction of chemokine receptor CCR6. This uncovers a new role for TGF-β in regulating spatial organization of cells within tissues. Finally, to extend our studies of spatiotemporal dynamics, we developed a GFP-Smad2 reporter system to further probe TGF-β signaling nuances in T cells *in vivo*.

Together, these studies provide novel insights into the cellular dynamics mediating eT_{RM} development and bolster our understanding of TGF-β signaling in the adaptive immune system.

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DEDICATION

“But a handful I have learned, still a universe remains.” – *Avvaiyyar*, Ancient Indian Philosopher

Dedicated to my late grandfathers, K.R. Subramaniyan and B. Mahalingam

CHAPTER 1: Introduction

OVERVIEW

The immune system is an ever-growing reflection of the co-evolution of species with their surrounding environments. From the evolution of basic pattern recognition to the incorporation of variable, somatically mutating receptors enabling more specific antigen recognition, the immune system takes in that which makes it stronger and more robust to create a system as resistant to pathology as possible. Broadly speaking, this function can be broken down into two major processes: the first being constant immunosurveillance at host-environment ("barrier") interfaces through broad recognition of patterns, structures and metabolites, and the second being the active engagement of cellular processes with increased specificity to instruct appropriate responses, as a function of this immunosurveillance. Particularly remarkable is the development of hundreds of billions of distinct antigen receptors, generated in a random fashion, that facilitate fine-tuned recognition and have the capacity to adopt a variety of functions that bolster immunity. How these billions of receptors are optimally appropriated to contribute to the functional diversity of the immune system remains a major driving question behind many studies of adaptive immunity.

$CD8^+$ T lymphocytes are one critical component of the adaptive immune response, using their enhanced cytotoxic and effector functions to protect against pathogens and tumors. $CD8^+$ T cells can be classified into two major bins, the first being short-lived effector cells that rapidly respond to an invading pathogen, and the second being a long-lived population of memory cells, that can provide protection against re-occurring pathogens over a lifetime. As $CD8^+$ T cells are activated by antigen presenting cells, they are exposed to cognate peptide antigens:MHC, a co-stimulatory signal and various cytokines that shape their differentiation into diverse functional

subsets of both effector and memory T cells that appropriately compartmentalize across tissues to provide optimal protection against pathogens.

One of the critical cytokines regulating the differentiation, expansion and function of $CD8^+$ T cells is TGF-β, exerting its pleiotropic effects across the life cycle of $CD8^+$ T cells. While TGF-β has traditionally been viewed as an immunosuppressive cytokine, halting the proliferation of cells and promoting the generation of suppressive immune populations, emerging literature indicates that TGF-β may also play a role in the differentiation and maintenance of memory responses. Unlike the majority of cytokines, there is a heavy context-dependency for the outcome of TGF-β signaling in a $CD8^+$ T cell, perhaps owing to the promiscuity of Smad transcription factors in associating with other master transcription factors to control cell fate. For example, in short-lived effector T cells, TGF-β can promote apoptosis by regulating pro- and anti-apoptotic proteins, while in memory T cells, it can enhance their survival in some tissues by up-regulation of IL-15 receptor.

TGF-β has in fact also emerged as one of the critical regulators of epithelial tissue resident memory cell (eT_{RM}) differentiation. T_{RM} have emerged as critical sentinels in barrier immunity, in some senses bridging the innate capacity for immunosurveillance of barrier tissues with enhanced adaptive immune functionality, in an antigen specific manner. Unlike the memory T cells that circulate through the body, eT_{RM} are thought to take up long-term residence in epithelial barrier tissues, and adopt not only general memory phenotypic profiles, but also tissue-specific programs that select for specific functionality and facilitate the long-term maintenance of these cells in barrier sites. While the functional importance of T_{RM} is well-established, the factors governing their differentiation, both in effector sites and even during the generation of precursors, has yet to be fully elucidated. TGF-β, in particular, has been implicated in many aspects of eT_{RM} generation, including the induction and maintenance of an

eT_{RM} program through regulation of signature integrin CD103. However, given the complexity of TGF-β signaling and the limited dissection thus far of its regulation across the spectrum of $CD8^+$ T cell differentiation, it remains unknown exactly which contexts of TGF-β signaling may actually optimize eT_{RM} differentiation and function. In this chapter, we detail foundational literature and motivations pertaining to the study of TGF-β in the differentiation of eT_{RM}.

I. CD8$^+$ T LYMPHOCYTES: A BRIEF OVERVIEW

The development and differentiation of CD8$^+$ T lymphocytes

Lymphoid progenitors entering the thymus from the bone marrow undergo V(D)J recombination in the thymus to generate a diverse repertoire of T cell receptors (TCRs), after which they reach the "double positive" (DP) stage where they express both CD4 and CD8. During positive selection, if T cells do not signal efficiently with CD4 as a co-receptor and up-regulate the transcription factor ThPOK, the cell can then commit to a CD8 lineage with productive signaling using CD8 as a co-receptor, up-regulating Runx3 as a master transcription factor. After a round of negative selection in the medulla against TCRs that react strongly towards self-antigens, mature CD8$^+$ T cells egress from the thymus and migrate to the secondary lymphoid organs (SLO), where they recirculate between the lymph nodes and spleen during homeostasis, maintained in a naïve state by IL-15 signaling and tonic MHC-I-dependent signals[1-3], prior to their activation by antigen presenting cells (APCs).

Upon infection or vaccination, APCs interact with naïve CD8$^+$ T cells[4,5] in the SLO to provide cognate peptide:MHC and co-stimulation that activates them, causing their proliferation and initial differentiation into what is now characterized as short-lived effector cells and memory precursor cells[6,7]. After activation, these short lived effector cells contract, while the memory cells continue to differentiate and are then maintained long-term in an ever-expanding niche[8,9]. While TCR and co-stimulation are known to be the critical "signal 1" and "signal 2" in T cell differentiation, the role of other cytokines and cellular interactions that may skew the fate of CD8$^+$ T cells during their proliferation and differentiation is now gaining increased appreciation. Shortly following activation, it is now well-established that significant transcriptional changes[10] are accompanied by long-lasting epigenetic changes[11,12], such as the promoter methylation and changes in chromatin accessibility and 3D architecture. These epigenetic changes, in turn, can

impact the plasticity of ongoing T cell differentiation[13]. Many of these nuances are known to be modulated by TCR signal strength[14] and the cytokine milieu established during priming, supplied for example by the activating APC or $CD4^+$ helper T cells, or even a result of the localization of cells within the SLO during priming. Efforts to boost vaccination to form memory T cells have therefore focused on optimizing the cytokine milieu as well as antigen presentation and load during T cell priming to generate effective, sustained memory responses. Another major question that still remains is precisely how naïve cell clones from a pool containing tremendous TCR diversity can be effectively selected for and primed for the most impactful and efficient immune response.

Unlike $CD4^+$ T cells, the diversity of $CD8^+$ T cell effector function beyond their cytotoxicity and production of inflammatory cytokines has only more recently gained appreciation[15], especially with the discovery of specialized subsets of memory T cells residing in various tissues of the body. As $CD4^+$ T cells were initially characterized for their "helper" roles in regulating antibody production, the literature, as a result, may have been better focused on defining subsets based on specific functionality (T_H1, T_H2, etc.). $CD8^+$ T cells were originally discovered and characterized based on their cytotoxic functions[16]. Therefore, our understanding and subsetting of $CD8^+$ T cells has relied on their compartmentalization[17], which encompasses their ability to transit to tissues to execute their cytotoxic program, as well as their ability to be recalled from circulation or secondary lymphoid organs in a memory response.

Compartmentalization of $CD8^+$ T cell memory

Nearly two decades ago, studies from Sallusto, Lanzavecchia and colleagues identified two overarching subsets of memory $CD8^+$ cells that could be distinguished on the basis of CCR7 expression and were described to have distinct effector functions, including the differential production of perforin, IFNγ and IL-2[18]. Notably, it was concluded that the $CCR7^-$ or "effector

memory" populations of $CD8^+$ T cells were more suited for homing to inflamed tissue sites, as compared to those "central memory" cells expressing CCR7, which had lymph node homing capacities by virtue of CD62L (L-selectin) expression. This study marked the first description of potentially compartmentalized memory subsets. Originally named as effector (T_{EM}) and central memory (T_{CM}) based on their expression patterns of CD62L and CCR7[18-20], the stability of these particular markers, functionality of these subsets and their ontogeny has come into further question[21,22]. Therefore, for the purposes of our studies, we will define them as circulating memory cells (T_{circ}).

One particular area of interest following the discovery of these subsets was the $CD44^{hi}$ "effector" memory P14 T cells found in non-lymphoid tissues such as the gut and lungs long after infection of mice with LCMV [23]. Memory-like cells in non-lymphoid tissues were in fact previously described in harvested sheep organs by Charles Mackay and colleagues[24]. Pivotal work that followed from the Masopust and Carbone groups[25-27] went on to demonstrate that a large portion of these cells were, in fact, in disequilibrium with the circulation, or "resident". In the memory phase after infection of mice with herpes simplex virus (HSV) by skin scarification, the latently infected dorsal root ganglia containing HSV-specific memory T cells was transplanted into congenically distinct naïve recipient mice, where, upon recall challenge at a different site, the memory T cells were seen to remain "resident" in the original infected transplanted ganglion and not migrate to the adjacent infected site[26]. Transplantation of a portion of the small intestine following systemic LCMV infection into naïve mice also revealed a resident memory population of $CD8^+$ T cells in the intestinal epithelium[27], showing that this phenomenon could manifest in diverse tissue sites. These findings, along with parabiosis experiments that were presented in subsequent studies[28,29] revealed a distinct population of memory $CD8^+$ T cells that were maintained long-term in non-lymphoid tissues, now referred to

broadly as tissue resident memory cells (T_{RM}), that expressed a distinguishing surface phenotype compared to the re-circulating memory $CD8^+$ T cells (T_{circ}). The study of tissue resident memory cells, as we describe in detail in the following section, has also led to the identification of distinct diverse functions of $CD8^+$ T cells residing in various lymphoid and nonlymphoid tissues.

II. TISSUE RESIDENT MEMORY CD8$^+$ T CELLS

Discovery and function of tissue resident memory T cells

The first study to specifically demonstrate functional relevance of T_{RM} was from Gebhardt, Carbone and colleagues, who showed that a non-recirculating population of HSV-specific memory CD8$^+$ T cells provided enhanced protection against rechallenge by skin scarification with HSV[26]. Here, it was also described that these skin-resident memory T cells expressed distinguishing surface markers VLA-1 and CD103 expression in comparison to T_{circ} found in the spleen. While this was the first study to definitively ascribe function to this non-recirculating population of cells, earlier studies from Kupper and colleagues investigating routes of vaccinia virus (VV) immunization demonstrated a dramatic increase in protection against VV rechallenge when utilizing a skin scarification based method for immunization[30,31], which was originally attributed to the more efficient imprinting of homing molecules during priming. Later studies also reaffirmed that protection against VV was enhanced by VV-specific skin T_{RM}[28], through elegant parabiosis and re-challenge experiments. These observations led to the conclusion that T_{RM} provide enhanced protection against barrier-invading pathogens. Not only were these cells observed in the skin, protecting against pathogens breaching the skin barrier, but were also, early-on, described in the brain[32], salivary glands[33], female reproductive tract[34], gut[35] and lungs[36]. Most notably, HSV-specific T_{RM} in the vagina were shown to provide protection against a lethal dose of intravaginal HSV-2, while HSV-specific T_{circ} alone failed to give protection in this setting[34]. More recent literature also describes resident memory T cells that persist and provide similar protection in the adipose tissue[37], thymus[38], and lymph nodes[39].

Sequencing-based investigations of T_{RM} in the skin, lungs, intestine and brain[40-42] revealed transcriptional differences between T_{RM} and T_{circ} extending beyond their expression of markers such as CD103. These profiles suggested that T_{RM} could have unique functionality as

compared to T_{circ}, given their increased transcription of effector cytokines such as IFNγ and Granzyme B. In recall responses of T_{RM} in the female reproductive tract[29] and skin[43], it was determined that part of the critical functionality of T_{RM} hinged on their rapid production of IFNγ that up-regulated VCAM-1 on endothelial cells and induced production of CXCL9 and CXCL10 in the tissue to recruit circulating memory and effector cells that could help clear the invading pathogen more rapidly. Not only did IFNγ induce the recruitment of memory $CD8^+$ T cells through this cascade, it was further noted that this initial response by T_{RM} was also critical for the activation and recruitment of other lymphoid and myeloid cells, including those involved in wound repair, barrier defense and antigen presentation[44,45]. With this "sensing and alarm" functionality, not only are T_{RM} able to protect against challenges with pathogens matching their antigen specificity, but they are also able to provide cross-protection against other non-specific pathogens upon their triggering with peptide:MHC[43,44]. In addition to more "traditional" anti-viral T_{RM} producing IFNγ, there have been further subsets of T_{RM} described in both humans and mice that are skewed, for example, towards IL-17 or IL-22 production, with distinct transcriptional signatures from one another[46-50]. IL-17 producing ("Tc17") T_{RM} induced by commensal microbial species such as *Staphylococcus epidermidis* not only provide enhanced protection against invasive species, but also enhance the wound healing capacity in the skin[47,48].

While the early literature describing T_{RM} and their functionality focused on anti-viral immunity, both $CD4^+$ and $CD8^+$ T_{RM} and "T_{RM}-like" cells have more recently emerged as critical players in parasitic infections[51-54], autoimmunity and anti-tumor immunity. In humans, auto-reactive T_{RM}-like cells have been described to mediate the pathogenesis of fixed drug eruptions[55], lupus nephritis[56], psoriasis[57], autoimmune diabetes[58], and vitiligo[59], among many others. In fact, the use of inhibitors against IL-15, a critical survival cytokine for skin T_{RM}, is seen to durably alleviate pathogenesis in vitiligo[59]. Although it is assumed that these cells are

autoreactive, how they are generated and maintained, if distinct from anti-viral T_{RM}, and subsequently activated in a pathogenic role remains to be understood. Furthermore, it is also likely that the cognate antigens for these pathogenic T_{RM} derive from microbial species, which then have the capacity to activate T_{RM} upon chronic breach of the epithelial barrier. While our studies mostly focus on the "classical" $CD8^+$ T_{RM} described in anti-viral immunity, it is important to note the diversity in functionality of these cells that persist in disequilibrium with the circulation.

The described transcriptional profile of T_{RM}[42] is also associated with improved prognosis in various cancers, with prominent case studies in lung cancer and melanoma. One of the first studies, describing a better prognosis in non small cell lung carcinoma (NSCLC)[60] in tumors with an increased $CD103^+CD69^+CD8^+$ population of tumor infiltrating lymphocytes, proposed that the increased cytotoxic phenotype could be due to stronger, more prolonged immune synapses between $CD8^+$ T cells expressing CD103 interacting with E-cadherin-expressing target cells, allowing for improved direct cytotoxicity[61]. While this particular mechanism has only been described *in vitro* by a single group and direct cytotoxicity in tumors cannot quantitatively explain the role that $CD8^+$ T cells play in tumor clearance[62], the improved prognosis of tumors containing T_{RM}-like cells has been reproduced in many studies of humans and mice[63-67], including breast cancer, melanoma, and NSCLC, to name a few. While the phenotype of these cells requires further exploration, the enhanced anti-tumor function of these T_{RM}-like cells in the tumor can even be ascribed to the rapid and enhanced production of IFNγ and other cytokines, known to be critical for anti-tumor activity[68,69].

T_{RM} positioning for recall responses within tissues

Part of the enhanced functionality of T_{RM} as compared to T_{circ} comes from their proper positioning within tissues that keeps them at the ready for when pathogens invade. In the liver,

for example, T_{RM} that have the capacity to protect against malaria don't reside in an epithelial layer, but actually persist within the sinusoid where they are able to better survey the blood and respond to malaria-infected cells[53]. While these cells do not express integrin CD103, they do express chemokine receptor CXCR6 and CD69 that may retain them and allow for proper localization to the sinusoids. In tissues with epithelial and subepithelial layers, $CD8^+$ T_{RM} are seen to persist in both layers and provide distinct protection against certain pathogens. For example, upon oral infection with *Yersinia pseudotuberculosis*, an additional population of $CXCR3^+CD8^+$ T_{RM} is seen to emerge in the lamina propria, lacking CD103 expression[70]. These cells use CXCR3 to associate near CXCL9 and CXCL10-producing macrophages during inflammation, which are in fact the site of replication for the infection. The subsets and localization of T_{RM} generated may depend heavily not only on the tissue site, but also on the pathogen at hand, and the location or load of infection.

Many pathogens invading epithelia also generate CD103-expressing populations of $CD8^+$ epithelial T_{RM}[71]. Epithelial T_{RM} (eT_{RM}) are also quite restricted in certain tissues to the areas of infection, such as in HSV-1 infection, where the $CD8^+$ T_{RM} are thought to displace the dendritic epidermal T cells (epidermal $\gamma\delta$T cells) that reside in the skin[72] epithelium, and display limited migration within the epidermis[73,74]. Upon pathogen recall, however, these eT_{RM} are not displaced, but actually proliferate and localize to the site of infection in an antigen-specific manner[75]. This property is also mirrored in the female reproductive tract[76]. Intriguingly, eT_{RM} also tend to have high levels of PD-1, TIM3, CD101, and other markers of chronic TCR stimulation in their profile at homeostasis. This could either be a reflection of sustained antigen recognition, for example of microbial or self-antigens in barrier sites, or an epigenetic or transcriptional adaptation that leaves them expressing these markers for enhanced self-regulation upon pathogen recall.

T_{RM} in humans

Although the bona-fide non-re-circulating properties of T_{RM} were established through experimentation in murine models, many early, pivotal findings in humans provided critical insights and therapeutic contexts that set the stage for the study of the basic mechanisms surrounding T_{RM}. Nestle and colleagues first observed "resident" skin cells from non-lesional skin of psoriatic patients that were able to mediate psoriasis-like inflammation upon transplantation onto mice[57]. This was among the first descriptions of supposedly non-circulating cells contributing to pathogenesis. Subsequent studies from Clark, Kupper and colleagues quantitated cutaneous leukocyte antigen (CLA) expressing cells in the skin, compared to the blood, and found that there were nearly double the number of T cells in the skin as found circulating through the entire body, suggesting that a majority of T cells in the skin were "resident"[77]. While these T cells were not all similar to the $CD8^+$ T_{RM} described in tissues, as significant proportion were also seen to be regulatory T cells[55,78,79], this strongly suggested that there were phenotypically distinct cells in human tissues that were in disequilibrium with the circulation. Later clinical studies by the same group using alemtuzumab (anti-CD52 antibody) to deplete circulating T cells revealed that in cutaneous T cell lymphoma, skin-resident cells were able to provide clinical protection[80] against the disease. In the skin, diseases that are T_{RM}-mediated as opposed to T_{circ}-mediated can also, in certain cases be distinguished by the pattern of inflammation or abnormality in the skin. For example, mycosis fungoides and fixed drug eruptions have well-demarcated skin lesions, as compared to CTCL, which presents with more diffuse skin erythema[55].

Notably, the human T_{RM} literature has provided more insights into the role of $CD4^+$ T_{RM} than those in mice, which have been largely focused on the various subsets of $CD8^+$ T_{RM}. Some pivotal studies in mice have demonstrated, in herpes simplex virus infection, that pathogen-

specific $CD4^+$ T_{RM} are mostly localized to sub-epithelial layers of the skin[73,81]. In some cases, such as $CD4^+$ T_{RM} in lymphoma, $CD4^+$ were observed in modest quantities in the epidermis[82]. This however does not seem to hold true for humans, as a significant portion of $CD4^+$ T_{RM} are seen to take up residence in the epithelium[46,79]. In certain mouse models, $CD4^+$ T_{RM}, predominantly shown in various mucosal tissues[45,83,84], are seen to play both pathogenic and protective roles, but have not been as extensively characterized as $CD8^+$ T_{RM}. $CD4^+$ T_{RM} in human tissues other than the skin also provide protection[85], such as in the bone marrow[86], lung[87,88], and skin[80]. Additionally, studies of human T_{RM} indicate that many have specificity for pathogens and commensal microbes, such as *S. Epidermidis* in the skin.

Elegant longitudinal investigations of healthy organ donors[88-90] by Farber and colleagues have pushed the needle in our understanding of circulating versus resident memory T cells in humans. In their survey of various lymphoid and mucosal organs as well as the blood, they find that virtually all $CD4^+$ and $CD8^+$ tissue resident cells express CD69, but that a greater proportion of $CD8^+$ T cells also express CD103 in tissues such as the intestine and salivary glands[91]. Several of the enriched transcripts in the $CD103^+$ cells show significant overlap with the described transcriptional programs in mouse[42,75] $CD8^+$ and $CD4^+$ T_{RM}, including enrichment of CD49a, CD103 and CD101 on $CD8^+$ T_{RM}. These studies suggest that many of the processes and signatures we find in mouse T_{RM} are also conserved in human T_{RM}, thereby solidifying the clinical relevance of mouse T_{RM} studies.

Priming of T_{RM}: does a specific precursor exist?

Initial observations based on the success of skin-scarification based smallpox vaccines suggested that this method of priming somehow provided enhanced protective immunity. One immunological consequence of skin scarification was later described to not only generate a robust circulating memory response but also the seeding of T_{RM} in peripheral tissues[28,31]. These

initial studies suggested that the site of priming may play a role in the effective generation of T_{RM}.

Initial studies in search of a precursor for T_{RM} have indicated that these cells derive[40] from memory precursor effector cells (MPEC)[7], which are KLRG1^{-} and CD127^{int} (IL-7 receptor). The addition of CX_3CR1 (fractalkine receptor) as another distinguishing marker of differentiation demonstrated that, in addition to lacking KLRG1, cells differentiating into T_{RM} in various tissues derived from precursors that also lacked CX_3CR1 expression[21,22]. However, these are also shared precursors with circulating memory cells, suggesting that there may not necessarily be a unique precursor for T_{RM} over T_{circ}. The generation of MPEC as compared to short-lived effector cells (SLEC) can be controlled by a number of different mechanisms, including the exploitation of gamma chain cytokine signaling[17], metabolic pathways[92-95] and asymmetric cell division[96], self-reactivity[97,98], and TGF-β signaling[99].

Very much like the circulating memory T cells that have the capacity to enter effector tissues, not only must T_{RM} originate from memory precursors, they must also have the license to traffic to and enter various tissues such as the skin and gut, endowed by induction of integrins and selectin ligands that allow for their rolling and extravasation[100]. This “imprinting” of homing capacity in T cells for their entry into non-lymphoid tissues like the gut and skin is thought to rely on priming by migratory dendritic cells from these respective tissues[101-103]. However, certain tissues are not as effective at priming memory responses and require a “prime and pull” to get T cells into the effector site, such as in the female reproductive tract for protective immunity against HSV-2[34], or even in the context of mucosal priming in the lung generating effective protection against vaginal challenge with *Chlamydia Trachomatis*[104]. The latter may also function by imprinting homing receptors that may be shared between the lung and other tissues. However, in studies of intravenous LCMV infection, where priming occurs in the spleen, there is

systemic distribution of T_{RM} across most tissues[27,105], suggesting that the site of priming may not be as critical for mucosal tissues, as long as the priming by the pathogen is robust enough to generate sufficient quantities of memory precursor cells with the ability to home to various tissues.

In fact, even *in vitro* activated cells (TCR transgenic splenocytes pulsed with peptide or CD3/28 activated cells) have the ability to differentiate into T_{RM}[35,106], but the timing of transfer after activation is critical, perhaps due to the fact that prolonged exposure to high-dose IL-2 in culture may drive the activated cells towards short-lived effector cells[107], or shift their metabolic state. While the days in culture prior to transfer may seem more tightly regulated for cells giving rise to T_{RM} as compared to circulating memory, this does not definitively suggest that there is a separate precursor or precursor state. It is important to note, that activated T cells can enter any mucosal tissue and take up residence even at steady state without inflammation. However, in the skin, this is more tightly regulated by local inflammation allowing for cells to seed the epithelia and may therefore have a narrower “window of opportunity”.

There are very limited pieces of evidence in favor of the argument that there may be different precursors for T_{RM} and T_{circ}. One study found that DNGR-1$^+$ DC were critical for T_{RM} but not T_{circ} generation[108]. Another study looking at TCR clones in the mouse skin[109] and draining lymph nodes following vaccination demonstrated that while there was some overlap in clones found in the skin and draining lymph nodes (used as surrogates for T_{RM} and T_{circ}, respectively), there were also many unique expanded clones found in each tissue suggesting that there could either be clonal selection or even differential precursors for either subset. However, neither of these observations definitively demonstrates that there is a unique T_{RM} precursor, and most studies have attributed the unique transcriptional signatures found in T_{RM} over T_{circ} to be driven by local cues in the tissue of their residence.

Differentiation and maintenance of T_{RM} in diverse tissues

As activated memory precursors enter the peripheral tissues, they do not yet express the markers that define them as tissue resident memory cells, namely their expression of signature markers such as CD69 and CD103. As activated T cells enter each tissue that they eventually take up residence in, they are exposed to a discrete set of cytokine signals that are thought to dictate their differential phenotype and terminal differentiation. After they enter inflamed tissues, T_{RM} "precursors" take a few days to a couple of weeks to up-regulate CD69 and then, in cases where pertinent, CD103[40,106].

Tissue resident lymphocytes, whether T_{RM} or innate lymphoid cells, have some shared transcriptional profiles across various tissues[42]. The major collective property is, of course, the program that drives their retention in the tissue. Mackay, Carbone and colleagues identified major transcription factors Hobit and Blimp, that are responsible for maintaining the residency program of various lymphocytes, such as NK, NKT and T_{RM} cells, in non-lymphoid tissues. Hobit and Blimp ChIP-seq revealed binding sites in Klf2, Ccr7, S1pr1, Tcf7 and other genes down-regulated in T_{RM}, suggesting that these transcription factors cooperate to repress these genes required for tissue egress[110-112]. While there is much debate about the role of signature integrin CD103 and its requirement in retention of lymphocytes in all tissues, one consensus feature of resident memory T cells is surface expression of CD69[113]. While CD69 is also a marker of recently activated T cells as a result of TCR triggering[114], the maintenance of CD69 expression in T_{RM} is proposed to be a result of the opposing axes of S1PR1 and CD69 expression [115,116]. During the differentiation of T_{RM}, it is thought that Klf2, the transcription factor controlling CCR7 and S1PR1 expression, must first be downregulated before CD69 is expressed [117]. While the precise mechanisms regulating CD69 induction on differentiating T_{RM} are not yet fully elucidated, it is thought that type I interferon or other inflammatory signals within the barrier sites

during differentiation of T_{RM} may play a role[85,117]. While CD69-dependent tissue retention has been shown to be an important property of T_{RM}, more recent literature has called into question the "permanent" residence of $CD69^{+}CD103^{-}$ T_{RM} in non-lymphoid barrier sites, as T_{RM} which eventually become resident in the lymph nodes are described to derive from this population of cells which were previously resident in the tissue[39]. The transcriptional profiles of these lymph node T_{RM} reflect their continued maintenance in the lymphoid tissue, which includes contrasting programs to those observed in non-lymphoid tissue-resident T_{RM}, and increased overlap with T_{circ}. While these lymph node resident T_{RM} are also critical for protection, our further discussions of T_{RM} will include mostly those found in the non-lymphoid tissues.

Despite certain shared programs, the T_{RM} occupying various non-lymphoid tissue niches have distinct survival requirements and phenotypes. As many barrier interfaces have high microbial loads, T_{RM} are more "exposed" to potentially pathogenic or inflammatory processes as compared to T_{circ}. To bolster themselves in the barrier, T_{RM} have been described to have enhanced protection against getting infected themselves, as demonstrated by their expression of IFITM3[36]. Their sensing of the environment may be mediated by their expression of aryl hydrocarbon receptor (AHR)[118]. Long-term survival of T_{RM} also seems to hinge upon enhanced fatty acid metabolic capacity[119] that maintains their profile in barrier sites such as the skin. Interestingly, the expression of these factors is thought to vary across each distinct tissue site that T_{RM} take up residence in, suggesting that their terminal differentiation is molded by these microenvironments. In particular, epithelial T_{RM} (eT_{RM}), which reside specifically in the epithelial layer of tissues, for the most part expressing integrin CD103, are seen to rely most heavily on these factors.

Another critical, perhaps shared, survival cytokine for T_{RM} is IL-15. Loss of IL-15 signaling results in decreased differentiation and impaired survival of T_{RM} in several non-

lymphoid tissues[40,53,59,120,121]. An interesting feature of IL-15 is its necessity to be trans-presented in certain contexts for productive STAT5 signaling[122], suggesting that continual cellular interactions, or high densities of cells are required in areas where T_{RM} are maintained. Intravital microscopy studies of eT_{RM} in fact demonstrate that these cells adopt a dendritic morphology and confined migration, which may allow them to more continuously receive critical survival signals[73,74]. The induction of IL-15 receptor (CD122) relies on the delicate balance of T-box transcription factors T-bet and Eomesodermin[121,123]. While these transcription factors need to be expressed early on during the differentiation of memory precursors, the terminal differentiation of eT_{RM} requires their ultimate down-modulation[121,124]. Overexpression of T-bet in activated T cells decreases the lodgment of T cells in non-lymphoid tissues long-term[124].

Yet another shared transcription factor in $CD8^+$ T_{RM} differentiation across tissues is Runx3[125]. Milner, Goldrath and colleagues demonstrate that loss of Runx3 in activated $CD8^+$ T cells prevents the efficient formation of T_{RM} in a number of tissues, but does not impact T_{circ}. Runx3 is quite non-specific to T_{RM}, as it is also known to be critical for the thymic development of $CD8^+$ T cells as well as the differentiation of effector as well as circulating memory cells[126]. However, Runx3 is also known to enable epigenetic and transcriptional programs in T cells through cooperation with other transcription factors, such as T-bet for effector cell differentiation[127], suggesting a wide spectrum of its involvement in maintaining $CD8^+$ T cell identity. An interesting aspect of Runx3 is its critical relationship to TGF-β signaling[128], including cooperation with Smad transcription factors to maintain programs of $CD8^+$ T cells[129].

TGF-β signaling and T_{RM}

In addition to Runx family transcription factors, T-box transcription factors can also be modulated via TGF-β, which is required for the down-regulation of Eomes in terminal eT_{RM} differentiation[121]. TGF-β, however is a pleiotropic cytokine that can regulate many critical fate

determinants in T cells. Most studies of TGF-β signaling in the differentiation of T_{RM} have relied on the use of TCR transgenic T cells where TGF-β signaling is impaired from the naïve $CD8^+$ T cell stage (dLck-Cre). This massive abrogation of signaling yields dramatic phenotypes of T_{RM} loss in most tissues[40,130-132], except for the liver, which does not rely on TGF-β signaling for terminal T_{RM} differentiation. This was initially investigated as a result of and attributed to the role that TGF-β plays in CD103 induction[133]. However, these mice also notably exhibit a higher frequency of $KLRG1^{hi}$ cells, that more resemble short lived effector cells rather than memory precursors[131], suggesting an impairment even in the generation of T_{RM} precursors. Moreover, TGF-β in naïve cells is also critical to restrain their proliferation by modulating IL-15 signaling[134]. Given that TGF-β can impact all of these potential stages of T_{RM} differentiation, the study and understanding of TGF-β signaling at each of these critical milestones in T_{RM} development, while important, remains incomplete. We further detail the intricacies of the TGF-β signaling pathway in $CD8^+$ T cells in the next section.

III. TGF-β SIGNALING IN IMMUNITY

TGF-β production, activation and signal transduction

One of the unique features of TGF-β as a cytokine is that it is produced and secreted in a latent form requiring activation. The TGF-β family consists of 3 members, TGF-β1,2,3, while the superfamily includes growth factors such as Activin and GDF family members. The predominant isoform of TGF-β involved in immune activity is thought to be TGF-β1, however growing literature now suggests that immune cells in certain non-lymphoid tissues may be regulated by TGF-β2 and TGF-β3[135] that are preferentially produced in their microenvironments. TGF-β is synthesized as an N-terminal latency associated peptide (LAP) connected to a C-terminal fragment containing the mature cytokine[136]. Upon translation, TGF-β-LAP assembles into a dimer and is cleaved by the endoprotease furin[137], which leaves a homodimer of the C-terminal mature cytokines non-covalently linked and structurally protected by LAP. This structure, referred to as the small latent complex, is then secreted and can be found bound to the surface of various cells in the body, or to the extracellular matrix, usually associated with latent TGF-β binding protein to form a large latent complex. The small latent complex contains an RGD motif that may facilitate the release of the mature cytokine into an active form[138].

The activation of TGF-β has been described to occur by many mechanisms *in vitro*, including through shear force induced by pipetting of the latent TGF-β complex, extreme heat, low pH and ionizing radiation. However, none of these mechanisms have been critically shown to play a role *in vivo* in the activation of TGF-β. Rather, *in vivo*, the activation of TGF-β has been shown to potentially occur either through enzymatic cleavage, by the recruitment of matrix metalloproteinases, or more robustly, through the activity of αV integrins that associate with β3, β5, β6, and β8 integrin chains. The αV integrins are expressed on a number of different types of cells and are thought to facilitate the activation of TGF-β by binding to the RGD motif in the

small latent complex. Once bound, it is hypothesized that activation occurs by way of a "straight-jacket" force, where αV-expressing cells anchor to latent TGF-β on the ECM or surface of cells, and with enough cytoskeletal force, release the active TGF-β at this cellular synapse[138-140]. This favorable model proposed by Tim Springer and colleagues is based on the structure of integrin αVβ6[141], but whether this facilitates the release of cytokines *in vivo* in a spatially confined area, and if this would apply to other αV integrins in the family such as αVβ8[142], is still unknown. However, activation by αV integrins is now acknowledged to be the predominant mechanism by which TGF-β is distributed for signaling across the immune system. Elegant studies mutating the RGD motif to an RGE motif in LAP yields a multi-organ inflammatory phenotype in mice akin to complete deficiency in TGF-β1. While not yet explored in depth, the manner in which TGF-β is activated may play a large role in determining the diverse functional outcomes resulting from TGF-β signaling.

Once TGF-β is activated, it can bind to a homodimer of TGF-βRII, which then complexes with TGF-βRI to form a tetrameric complex[143]. TGF-βRII phosphorylates TGF-βRI, which recruits and phosphorylates the receptor Smads (R-Smads) Smad2 and Smad3 which form a homo- or heterodimer that complexes with Smad4 to translocate into the nucleus for transcriptional activity. TGF-β can also non-canonically facilitate the activation, in certain cases, of R-Smads Smad1 and Smad5[144] and also engage non-Smad pathways such as MAP Kinase, Rho GTPase, PI3K and RAGE signaling[145]. Once Smads are activated, they trigger a negative feedback loop that induces the inhibitory Smad (I-Smad) Smad7, and in many cases also facilitates the down-modulation of TGF-βRII activity[146]. Smads themselves bind to DNA with relatively low affinity[147], and often times require association with other transcription factors to carry out their transcriptional activity, such as FoxO1 and FoxH1[148] transcription factors. Additionally, Smad transcriptional outcomes are heavily dictated by the other existing "master"

transcription factors that are active within a given cell[149], offering further reasoning behind the pleiotropism and context-dependency of TGF-β signaling.

TGF-β in adaptive immunity

The prominent characterization of TGF-β as an immunosuppressive cytokine stems from the observations that mice deficient in TGF-β1 die very early on due to multi-organ inflammation[150,151]. This phenotype can be rescued in β2 microglobulin or MHC Class II deficient mice[152,153], suggesting that many of these effects are from uncontrolled cascades in the adaptive immune system, with T cells potentially being an important TGF-β-regulated target. Initial studies that took a dive into investigating the effects of TGF-β signaling on T cells utilized transgenic mice with a dominant negative TGF-βRII driven by a CD4[154] or CD2 promoter[155]. Curiously, both mice yielded distinct phenotypes. When the dominant negative TGF-βRII was driven by the CD4 promoter, the resultant multi-organ inflammation mirrored that of the TGF-β1-deficient mice, with just a later onset. However, when driven by the CD2 promoter, overt inflammation was not apparent- only an expansion of $CD8^+$ T cells. These conflicting observations may have been due to opposing effects of TGF-β on cells other than T cells in the CD2 mice, the construction of transgenic mice, differences between animal facilities or, most likely, due to the incomplete abrogation of TGF-β signaling through the use of dominant negative TGF-βRII. More recent studies utilizing TGF-βRII floxed mice crossed to a CD4-Cre confirm inflammatory disease in the absence of TGF-β signaling in T cells within one month of age[133]. However, using a distal Lck-Cre promoter to delete TGF-βRII in T cells once they have undergone thymic development did not yield any such inflammatory disease[133], though the mice do display some low level of activation at baseline.

Further studies have explored the regulation of T cell subset differentiation by TGF-β. TGF-β is known to inhibit the differentiation of T_H1 cells by inhibiting T-bet[156] and also

attenuating IL-12 responsiveness[157] through down-regulation of the receptor in $CD4^+$ T cells. TGF-β also inhibits T_H2 differentiation by though both direct and indirect modulation of GATA3[158,159]. However, ectopic expression of GATA3 seems to be able to overcome TGF-β signaling to induce T_H2 generation, reaffirming that TGF-β's activity is highly dependent on the signaling context. These phenotypes are also confirmed in models of over-expression of inhibitory Smad7 that lead to an enhancement of T_H1 and T_H2 differentiation[160].

In addition to suppressing the differentiation of T_H1 and T_H2, TGF-β also promotes the differentiation of T_{reg}, T_H17 and T_H9 $CD4^+$ cells. While TGF-β may be more critical for induced T_{reg} differentiation, working with IL-2 to induce FoxP3[161], it is also important for the maintenance of nT_{reg}, preventing their apoptosis by up-regulation of Bcl-2. Additionally, TGF-β is critical for the induction of T_H17 cells, in cooperation with IL-6. While TGF-β1 is the predominant isoform known to be utilized by T lymphocytes, there is also evidence that exposure to TGF-β1 as compared to TGF-β3 yields different subsets of T_H17 cells[162]. This may occur by activation of canonical versus non-canonical TGF-β signaling pathways. TGF-β also promotes the homeostasis and development of T cells by modulating IL-7R expression during thymic development [163]. Taken together, these studies show that TGF-β has a context dependent effect on T cell differentiation.

The role of TGF-β in the regulation of $CD8^+$ T cells

When it comes to the regulation of $CD8^+$ T cells, the role of TGF-β becomes less clear as exclusively an immunosuppressive factor. As Smads are known to act through association with other transcription factors to exert their transcriptional activity[149], given the different master transcription factors at play in the development and differentiation of $CD8^+$ as compared to $CD4^+$ T cells, it is no surprise that TGF-β signaling may have varying effects on both cell types. TGF-β is known to act on $CD8^+$ T cells at nearly all stages of their life cycle. Based on receptor

expression patterns derived from ImmGen (**Figure 1.1**), $CD8^+$ T lymphocytes may not become maximally responsive to TGF-β until they have fully developed in the thymus and exited to the secondary lymphoid organs (SLO). This also offers an explanation for the lack of an abnormal phenotype observed in dLck-Cre mice crossed to TGF-β$RII^{fl/fl}$, where at homeostasis, there appears to be no overt inflammation.

In the thymus, the TGF-β target gene CD103 is induced on $CD8^+$ T cells in a Runx-dependent manner[129], suggesting that there may be some level of TGF-β or alternative Smad signaling at this stage. Additionally, TGF-β signaling is thought to promote the development of $CD8^+$ T cells in the thymus[163] and induce the expression of IL-7R indirectly through down-modulation of Gfi-1. Once $CD8^+$ T cells exit to the SLO, TGF-β is described to regulate the homeostatic proliferation of naïve $CD8^+$ T cells through modulation of sensitivity to IL-15 (CD122) [134]. As $CD8^+$ T cells are activated, the opposing roles of TGF-β on sub-populations of cells becomes more apparent. Upon LCMV or *Listeria Monocytogenes* infection, antigen-specific $CD8^+$ T cells differentiate, broadly, into short lived effector cells (SLEC) and memory precursor effector cells (MPEC). TGF-β actually promotes the contraction of SLEC through up-regulation of the pro-apoptotic protein Bim and down-regulation of anti-apoptotic protein Bcl-2[164], which is counteracted by IL-15/STAT5 signaling in MPEC to promote their survival [99]. Not only does this demonstrate context-dependent signaling of TGF-β, but may also indicate a signaling "hierarchy". Additionally, on effector cells, TGF-β signaling is known to dampen the polyfunctionality of $CD8^+$ T cells[165], reducing their production of IFNγ and Granzyme B. As we have already discussed, TGF-β can also, on MPEC, promote the up-regulation of CD103 for the terminal differentiation into eT_{RM}. Not only does TGF-β regulate CD103 expression in this process, it also regulates the transcription of other key target genes such as VLA-1[132] and PD-1[166] [143], and the balance of T-box transcription factors. These targets are also mirrored in tissue

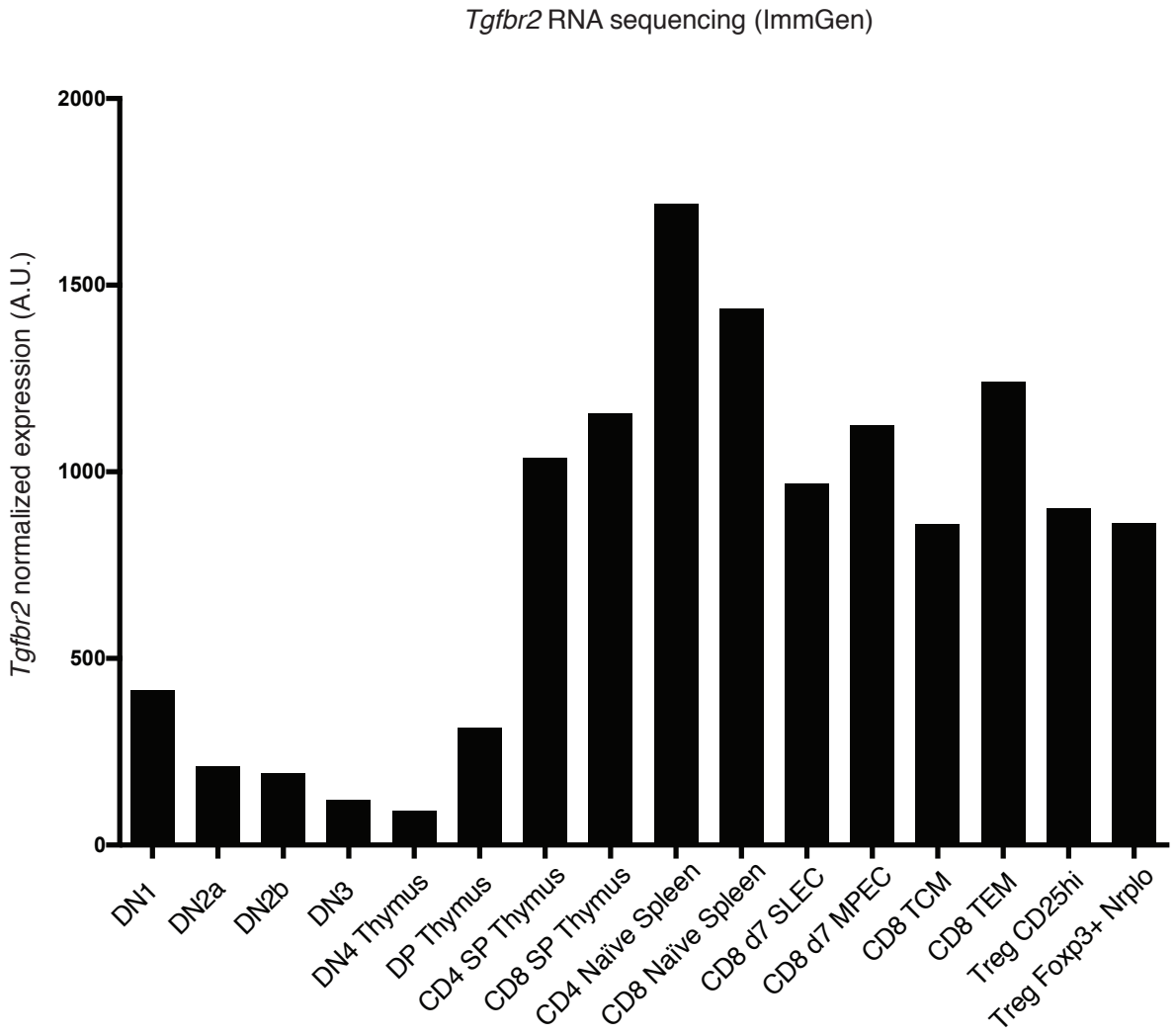

Figure 1.1 ImmGen Ultra-low input RNA sequencing of *Tgfbr2* transcript levels in T cells.

resident innate lymphoid cells and natural killer cells, through both canonical and non-canonical TGF-β signal transduction[135,167-169]. Interestingly, the differentiation of lung T_{RM} is dependent on TGF-β, but independent of Smad4[170], suggesting that there may either be compensatory mechanisms at play in the TGF-β signaling pathway or that in the lung there is an alternative pathway of activation that leads to CD103 up-regulation. The context of TGF-β signaling for T_{RM} differentiation may vary between non-lymphoid tissues based on the isoform of TGF-β within the tissue, its abundance and possibly mechanism of activation.

Sources of TGF-β and its activation in adaptive immunity

TGF-β requires activation from its latent form, and many studies have looked into the role of αV integrins, namely αVβ6 and αVβ8, which are known to activate the cytokine from its latent form. Loss of both of these integrins mimics the immune phenotype seen in TGF-β1 deficient animals, with multi-organ inflammation[171], suggesting that these two integrins control the activation of TGF-β as it pertains to the immune system. The expression patterns of αVβ6 and αVβ8 is quite distinct, with αVβ6 seen to be expressed pretty much exclusively in epithelial cells, while αVβ8 has a broader expression pattern that spans $CD4^+$ T cells, T_{reg}, dendritic cells (DC), and even some epithelial cells in the skin[171,172]. For the differentiation of peripheral T_{reg} and T_H17, it is abundantly clear – reproduced by both the Sheppard and Lacy-Hulbert groups in separate knockout models-- that the activation of TGF-β is dependent on DC expressing integrin αVβ8[173-177]. A most intriguing aspect of these findings is that, at least *in vitro*, the successful receipt of TGF-β signals by $CD4^+$ T cells for their differentiation is contingent on being primed by DC which, in *cis*, can both present the cognate antigen and also activate TGF-β[174]. This suggests that cellular interactions may be involved in the activation and presentation of TGF-β to T cells for the productive receipt of active cytokine during their differentiation.

This may be regulated differently in the maintenance of T_{reg}, which express their own $\alpha V\beta 8$ and, under inflammatory conditions, provide themselves with active TGF-β that maintains their function[178]. Curiously, however, mouse models of selective TGF-$\beta 1$ deficiency in T_{reg} suggest that TGF-$\beta 1$ production by T_{reg} alone is dispensable for their immunosuppressive capacity, but that TGF-$\beta 1$ production from activated $CD4^+$ T cells may provide the latent TGF-β substrate for activation and differentiation of T_H17 cells[179,180].This suggests that activation and production of TGF-β may be distributed among different cells even in a single differentiation process. This could also be facilitated by the expression of glycoproteins such as GARP, highly expressed on T_{reg}, or LRRC33 that sequester latent TGF-β on the surface of cells[181-187].

For $CD8^+$ T cells, the mechanisms of TGF-β activation operative at each stage of differentiation are largely unknown. It is now known that $\alpha V\beta 6$ and $\alpha V\beta 8$ expressed on epithelial cells in the skin and gut are critical for the homeostatic maintenance of eT_{RM} within these tissues[172]. However, the contexts of TGF-β activation and signaling for effects during T cell priming, expansion, contraction and other differentiation processes within tissues remains unexplored.

IV. DISSERTATION OBJECTIVES

Since TGF-β can act at many stages in the life cycle and differentiation of $CD8^+$ T cells, we sought to map the contexts and consequences of TGF-β signaling at various stages through the lens of the eT_{RM} differentiation process, starting with the role of DC-mediated TGF-β activation. We hypothesized that DC may facilitate the activation of TGF-β in tissues such as the skin, resulting in the induction of an eT_{RM} program, including down-regulation of Eomesodermin and induction of CD103 and other critical signature transcripts. Therefore, we generated mice that would allow us to test this hypothesis, by conditional deletion of αV integrin on dendritic cells. Second, we sought to better understand the spatiotemporal context of TGF-β signaling in the skin during eT_{RM} terminal differentiation. To this end, we used an intravital-microscopy based approach to monitor $CD8^+$ T cells during their terminal differentiation in the skin. Here, we present the surprising results of our studies of TGF-β's role in the differentiation of eT_{RM}.

CHAPTER 2: TGF-β conditions naïve CD8$^+$ T cells for tissue-resident memory formation

Parts of this chapter are currently in revision as:

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ABSTRACT

Epithelial resident memory T cells (eT_{RM}) serve as sentinels in barrier tissues to guard against previously encountered pathogens. How eT_{RM} are generated has significant implications for efforts to elicit their formation by vaccination or even prevent their long-term persistence in autoimmune diseases where they may mediate pathology. One critical cytokine required for the formation of eT_{RM} is TGF-β, a pleiotropic immunoregulatory cytokine that requires activation from a latent form. While the importance of TGF-β in eT_{RM} formation is established, the context of its activation during differentiation has yet to be determined. Here, we investigated the role of dendritic cell (DC)-mediated TGF-β activation on the formation of eT_{RM}. The conditional deletion of the TGF-β activating integrin αV from DC resulted in a loss of eT_{RM} in the skin which could not be attributed to the role of DC-mediated TGF-β activation in the skin during terminal differentiation in the skin or during priming in the lymph node (LN). Instead, we show that during immune homeostasis, TGF-β epigenetically conditions resting naïve CD8$^+$ T cells and prepares them for eT_{RM} formation. Naïve T cell conditioning occurs in LNs but not the spleen, through MHC-I-dependent interactions of naïve T cells with peripheral tissue-derived migratory DCs that activate latent TGF-β. We also find that this homeostatic interaction facilitates the preferential conditioning of naïve CD8$^+$ T cell clones that exhibit lower self-reactivity. We therefore describe

a novel mechanism by which the pre-immune repertoire is actively conditioned and selected for a specialized memory differentiation fate by TGF-β.

INTRODUCTION

Tissue resident memory T cells (T_{RM}) have emerged as critical players in adaptive immunity, providing long-lived, enhanced protection against pathogens in barrier sites[26] by enabling rapid innate and adaptive functions against barrier-invading species[29,43]. While their importance in barrier immunity is well established, and their role in anti-tumor immunity, as well as function in driving certain autoimmune pathologies has gained appreciation over the past decade, the mechanisms by which T_{RM} are generated are still incompletely understood. Notably, T_{RM} are quite transcriptionally[40] and functionally[75] distinct from circulating memory cells, relying on different survival factors[121] and acquiring adaptations that allow them to persist and function optimally in non-lymphoid tissues. Whether a distinct precursor exists for T_{RM} as compared to circulating memory T cells is still yet to be uncovered.

While there are shared programs across the spectrum of described T_{RM} subtypes [42], T_{RM} residing in distinct tissues possess unique transcriptional profiles[42,53,132], thought to be molded by the cytokine milieu within the tissue of residence. One of the critical cytokines in the differentiation and maintenance of T_{RM} is TGF-β. Initially, TGF-β was thought to function in T_{RM} formation predominantly through the regulation of signature integrin CD103[40,130], a marker of epithelial residence. However, more recent studies have uncovered additional roles for TGF-β[131,132], including impacting T-bet and Eomes[121], which, in turn, regulate expression of the receptor for IL-15[123], a critical eT_{RM} survival factor. TGF-β is thought to work in concert with several other transcription factors that are known to drive differentiation of $CD8^+$ T cells. In fact, Runx3, which requires TGF-β signaling for its activity and functions in a critical regulatory loop with the signaling pathway, has been identified as a master regulator of the T_{RM} program[125]. However, T_{RM} in each tissue depend on TGF-β signaling to varying extents for their program,

with the skin being one of the most reliant, and the liver being nearly independent of TGF-β signaling in the tissue[53].

TGF-β is a pleiotropic immunoregulatory cytokine that plays a significant role in homeostasis and differentiation in the adaptive immune system. While many cells, both hematopoietic and non-hematopoietic, have the capacity to produce TGF-β at steady state or upon inflammation, the effects of the cytokine on downstream signaling are tightly regulated through a variety of mechanisms, namely the production of TGF-β in a latent complex requiring activation. Latent TGF-β can be found abundantly in the extracellular matrix (ECM) within tissues or bound to the surface of cells, for instance through proteins such as GARP that can sequester the latent cytokine on the surface of regulatory T cells. In the immune system, much of the activity of TGF-β is thought to be regulated *in vivo* through the activity of αV integrins, pairing with a multitude of beta chains, that can be expressed on many cells, including epithelial cells, T_{reg}, fibroblasts and dendritic cells. αV integrins bind to an RGD motif on the latent TGF-β protein and are hypothesized to work either in concert with matrix metalloproteinases that cleave the latent protein, or through a mechanical “straight-jacket”-like force that may depend on cellular interactions with ECM or other TGF-β-sequestering cells, thereby presenting the cytokine to cells for downstream signaling via an established synapse.

Dendritic cell-expressed αVβ8 integrin, in particular, has been shown to play a critical role in the differentiation of $CD4^+$ T cells into T_H17[174,177] and peripheral T_{reg}[173]. The productive receipt of TGF-β signals in the differentiation of these $CD4^+$ subsets is thought to occur during cognate interactions of T cells with dendritic cells during activation. Intriguingly, *in vitro* studies indicate that αV integrin and peptide:MHC-II need to be expressed on the same dendritic cell for T_H17 and peripheral T_{reg} differentiation, suggesting that the cytokine may only be activated and presented productively to T cells in *cis* with an established immunological synapse. While this

has been explored during $CD4^+$ T cell differentiation, studies of TGF-β activation *in vivo* in the context of $CD8^+$ T cell differentiation are limited. Elegant studies have shown that epithelial-expressed αVβ6 and αVβ8 have been shown to be critical for the TGF-β activation that maintains $CD8^+$ epithelial T_{RM} (eT_{RM})[172]. However, existing literature suggests that TGF-β may play a significant role in the establishment of a T_{RM} program prior to their lodgment in the epithelium, such as by influencing the balance of T-box transcription factors[121,124]. The spatiotemporal context of TGF-β signaling during this differentiation process remains unknown.

Here, we sought to investigate the contribution of TGF-β activation by dendritic cells (DC) to the differentiation of tissue resident memory T cells by generating mice that conditionally lack αV integrin on DC. Upon examination of these animals, we observed a pronounced deficiency in the $CD8^+$ epidermal T_{RM} (eT_{RM}) compartment. While we initially hypothesized that this may be due to the contributions of DC to local TGF-β signaling in the skin during terminal eT_{RM} differentiation, we found that skin DC are dispensable in this stage. Instead, we uncovered a surprising homeostatic role for αV-expressing DC in providing TGF-β signals to naïve $CD8^+$ T cells. This homeostatic TGF-β signaling epigenetically conditions naïve $CD8^+$ T cells for the capacity to differentiate into eT_{RM} upon activation. Epigenetic conditioning occurs in lymph nodes through non-cognate interactions of low self-reactive naïve $CD8^+$ T cells with migratory DC that traffic from peripheral tissues to draining lymph nodes at steady state. Our findings present a novel mechanism by which the pre-immune repertoire is actively conditioned for the capacity to give rise to a specialized immune cell subset.

RESULTS

Integrin αV on dendritic cells is necessary for epidermal T_{RM} formation

In order to investigate the role of TGF-β activation by DC on tissue resident memory cell formation, we first crossed $CD11c^{Cre}$ BAC transgenic mice[188] to mice with *lox*P-flanked integrin

alpha V *Itgav* alleles ($\alpha V^{fl/fl}$), the shared alpha chain for all TGF-β-activating integrins. Cre recombinase in $CD11c^{Cre}$ BAC transgenic mice is reported to be active in greater than 95% of conventional dendritic cells (CD11c and MHC Class II-expressing), with very limited activity in other myeloid subsets and lymphocytes. Accordingly, in our $CD11c^{Cre}$ x $\alpha V^{fl/fl}$ (hereafter referred to as "αV-ΔDC" mice), αV protein was absent from the surface of the majority of conventional DC (**Figure 2.1A**), and the floxed allele was deleted in DC but not other cell types (**Figure 2.1A**). Deletion of the integrin on DC did not disrupt DC homeostasis in the secondary lymphoid organs or non-lymphoid tissues such as the skin, as we found the proportion and number of distinct DC populations to be consistent between αV-ΔDC and WT littermate control mice ($CD11c^{Cre}$ x $\alpha V^{fl/+ \text{ or } +/+}$) (**Figure 2.1B-C**). We observed a slight expansion of $CD8^{+}CD44^{hi}CD62L^{low}$ ("T_{EM}") population in the spleen (**Figure 2.1D**) beginning around 3 weeks of age, but not in the lymph nodes, presumably due to the inability of cells lacking CD62L to enter the lymph nodes. Despite this expansion of "effector memory-like" cells in the spleen, the mice showed no signs of pathology until at least 6 months of age. This was similar to mice which lack integrin β8 (the beta chain preferentially expressed with αV on DC) on DC that develop colitis-like inflammation in the gut, which is attributed to the lack of efficient peripheral T_{reg} and T_H17 generation[173,174]. All experiments were conducted when mice were between 8 and 16 weeks of age to avoid confounding factors due to overt inflammation.

As skin eT_{RM} are thought to be the most critically dependent on TGF-β signals for their program[121], we performed immunofluorescence staining of the skin to examine the compartmentalization of $CD8^{+}$ T cells. Curiously, we observed a pronounced defect in $CD8^{+}$ T cells in the skin epidermis of αV-ΔDC mice compared to WT mice (**Figure 2.2A-B**). Upon quantification of the histology, we found no evident numerical defect in the $CD8^{+}$ T cells in the dermis of these mice, or even a deficiency in $CD3^{bright}CD8^{+-}$ dendritic epidermal T cells (DETC)

Figure 2.1 Characterization of αV-ΔDC mice. (A) αV expression in splenic CD11c^{+} MHC II^{+} DC from αV-ΔDC or littermate control WT mice by flow cytometry (left) and genomic PCR of αV locus in αV$^{fl/+}$ mice, from sorted subsets in CD11c-Cre^{-} (first column) or CD11c-Cre^{+} mice. **(B)** Frequency of CD11c^{hi} MHC II^{int} resident DC (rDC), CD11c^{int} MHC II^{hi} migratory DC (mDC) subsets CD11b^{+} and CD8^{+} or CD103^{+} in LNs of αV-ΔDC or WT mice. **(C)** Frequency of CD11c^{+} MHC II^{+}, CD11b^{+} CD207^{+} Langerhans cells, dermal CD11b$^{–}$ CD207^{+} cDC1, CD11b^{+} CD207$^{–}$ cDC2, and CD11b$^{–}$ CD207$^{–}$ DN dDC in skin of αV-ΔDC or WT mice. **(D)** Frequencies of CD44$^{lo/int}$ naive, CD44^{hi} CD62L^{hi} central memory, and CD44^{hi} CD62L^{lo} effector/effector memory phenotype CD8^{+} T cells in spleens and LNs of 10 week-old αV-ΔDC or WT mice. Each experiment was performed at least twice with similar results. ****: $p<0.0001$, n.s.: not significant.

Figure 2.1 (Continued)

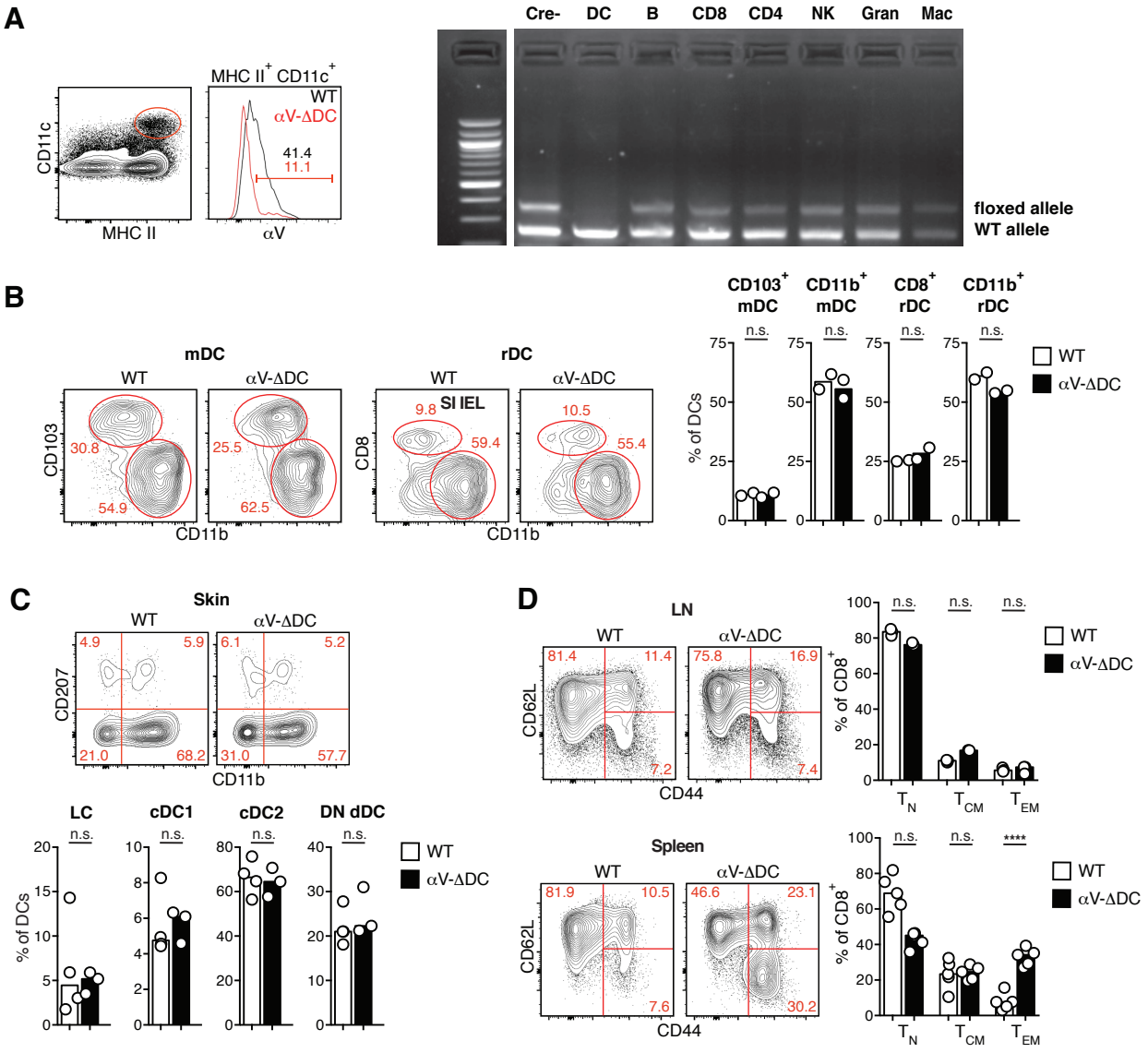

Figure 2.2 Defective epidermal T_{RM} formation in absence of αV integrins on DCs. (A) Histological cross-sections from ear skin of $CD11c^{Cre}$ x $\alpha V^{fl/fl}$ ('αV-ΔDC') and $CD11c^{Cre}$ x $\alpha V^{+/+}$ ('WT') littermate control mice. Arrowheads indicate $CD8^+$ $CD3^{dim}$ T_{RM} in the epidermis of WT, but not αV-ΔDC mice, while arrows indicate $CD3^{bright}$ DETC present in both strains. Dashed lines indicate dermal-epidermal border. Scale bar = 50 μm. Epi: epithelium, Derm: dermis. **(B)** Density of $CD8^+$ and $CD3^{bright}$ $CD8^-$ T cells in epidermis (left) and of $CD8^+$ $CD3^+$ cells in the dermis (right). Data are from 4 or 3 sections from 2 animals for each group. (**C,D**) Expression of the tissue residence marker CD103 (as well as CD69 on T cells) on indicated immune cell subsets ($Thy1^+$ $CD8\beta^+$ T cells, $Thy1^+$ $CD4^+$ T cells, $Thy1^+$ $TCR\delta^+$ γδT cells, $CD11c^+$ MHC II^+ $CD11b^-$ DCs) from skin of αV-ΔDC and WT littermate control mice. (**E**) Expression of CD103 and CD69 on $CD8^+$ T cells in small intestine epithelium (SI IEL), lamina propria (SI LP), salivary gland (SG), and lung of αV-ΔDC and WT littermate control mice. Scale bar = 50 μm. Each experiment shown was performed at least twice with similar results. **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$, n.s.: not significant.

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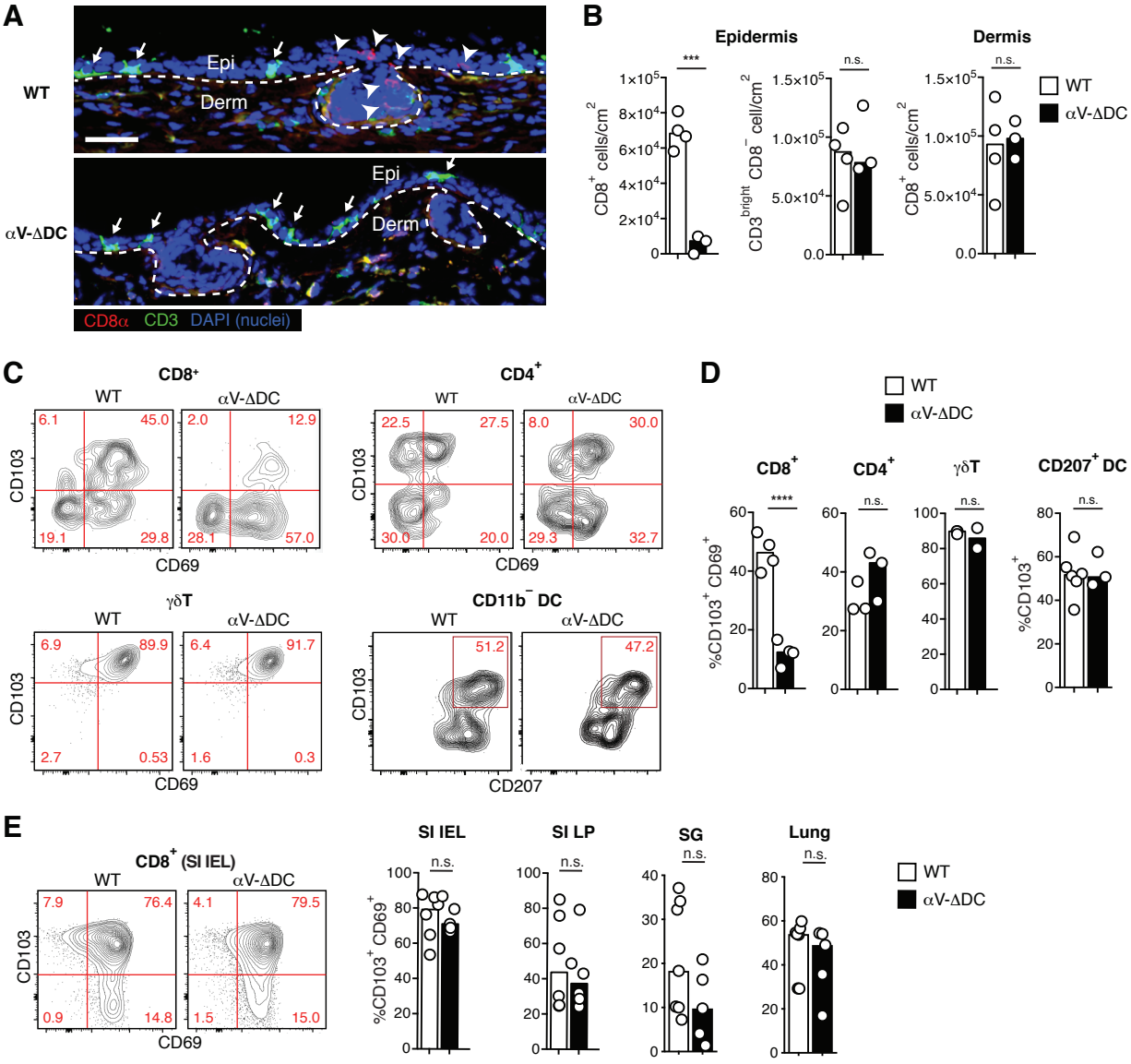

(**Figure 2.2B**). Accordingly, flow cytometry based characterization of the skin at steady state revealed a selective decrease in both the frequency and absolute numbers of $CD8^+$ T cells in the skin co-expressing eT_{RM} signature surface markers CD69 and CD103 in αV-ΔDC mice (**Figure 2.2C-D**). This deficiency in CD103 (αE integrin), a TGF-β-dependent marker, was exclusive to $CD8^+$ T cells in the skin, as examination of $CD4^+$ T cells, $\gamma\delta$T cells and dendritic cell populations known to express CD103 in the skin revealed no defect in their expression of CD103, or in their overall representation in the tissue (**Figure 2.2C-D**). Upon examination of other tissues such as the lungs, gut and salivary glands, however, we did not see a reduction in the $CD103^+CD69^+$ population of $CD8^+$ T cells (**Figure 2.2E**).

Unlike mucosal tissues which are more amenable to seeding of T_{RM} at steady state[23,35], the skin requires inflammation for efficient accumulation and generation of eT_{RM}[26,106]. We wondered whether induction of inflammation would rescue the defect seen in the skin eT_{RM} compartment. To this end, we inflamed the ear skin of αV-ΔDC or WT littermate control mice either through mechanical irritation using a tattooing device[74], or by treatment with the contact sensitizer 2,4-dinitrofluorobenzene (DNFB)[106](**Figure 2.3**). Four weeks after induction of inflammation through either means, we observed an even more pronounced defect in the eT_{RM} compartment in the skin of mice, reflected by both a reduction in $CD103^+CD69^+$ cells by flow cytometry (**Figure 2.3A-C**) as well as an absence of $CD8^+$ T cells in the epidermis upon histological analysis (**Figure 2.3D**), despite an intact $CD4^+$ T cell and DETC compartment. Therefore, our initial characterization revealed that αV expression on dendritic cells is selectively critical for the efficient generation of $CD8^+$ eT_{RM} in the stratified skin epithelium, but not other local $CD103^+$ immune cells in the skin.

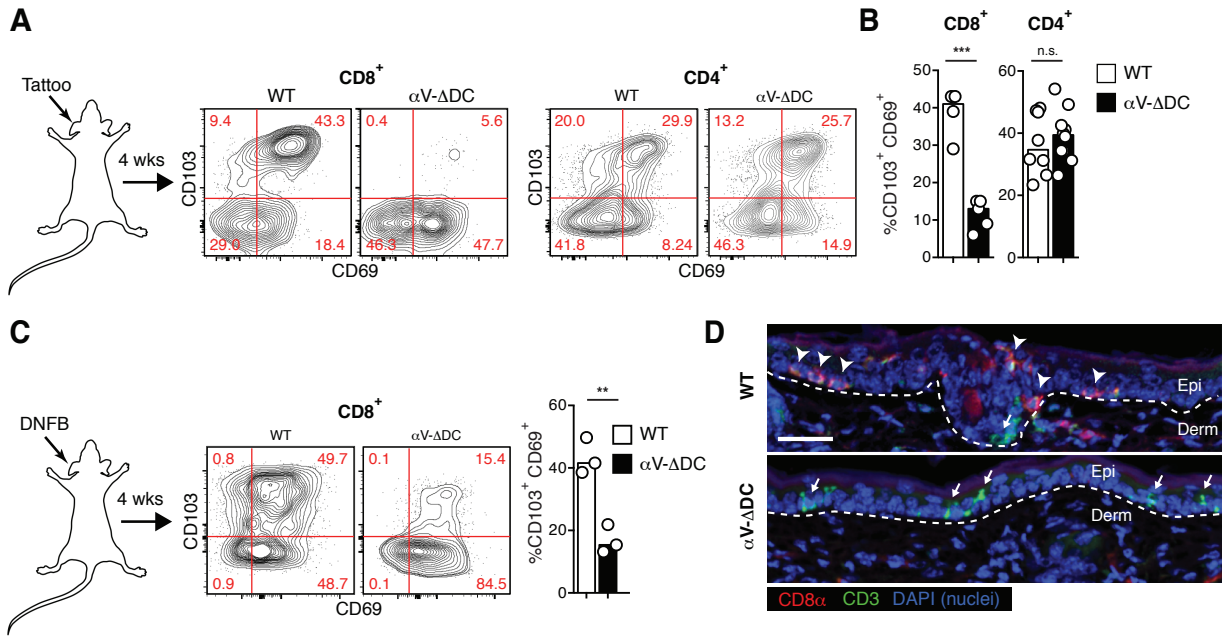

Figure 2.3 αV-ΔDC mice are impaired in epidermal T_{RM} formation upon inflammation. (A-C) Expression of CD69 and CD103 on skin $CD8^+$ or $CD4^+$ T cells 4 weeks after sterile inflammation induced by mechanical irritation with a tattooing device (A, B) or topical DNFB treatment (C). **(D)** Ear skin 4 weeks after DNFB treatment. Note the enrichment of $CD8\beta^+$ $CD3^{dim}$ eT_{RM} (arrow-heads) in WT mice, but their absence in the epithelium of αV-ΔDC, which is instead still populated by $CD3^{bright}$ DETC (arrows). Scale bar = 50 μm. Each experiment shown was performed at least twice with similar results. **: p<0.01, ***: p<0.001, n.s.: not significant.

Local TGF-β activation by αV- expressing dendritic cells in the skin is not critical for eT_{RM} differentiation

As terminal differentiation of eT_{RM}, as determined by CD103 induction, is thought to be initiated locally in the skin[40], we hypothesized that dendritic cells in the skin may activate a pool of TGF-β during inflammation, perhaps through interactions with skin-infiltrating T cells, that would facilitate the efficient terminal differentiation of eT_{RM}. Shortly upon induction inflammation, we observed an increase in αV integrin on the surface of dendritic cells in the skin, but not in the lymph node (**Figure 2.4A**). To test whether this contributed to local eT_{RM} differentiation in the skin, we adoptively transferred *ex vivo* activated TCR transgenic OT-I T cells (specific for the model antigen chicken ovalbumin) intravenously and inflamed the skin by mechanical irritation with a tattoo gun. Upon assessment of the skin for eT_{RM} formation 4 weeks later, we observed that OT-I in both αV-ΔDC and WT mice were able to give rise to eT_{RM} with similar efficiency (**Figure 2.4B**), unlike the endogenous pool in αV-ΔDC mice. We also observed the same result upon intradermal injection of cells into mice, as well as injection of polyclonal effector T cells. We also considered whether dendritic cells were at all involved in this terminal differentiation process. To this end, we generated bone marrow chimeras with Zbtb46-DTR (zDC) bone marrow or CD11c-DTR bone marrow, to probe the role of either cross-presenting or all classical dendritic cells, respectively, in eT_{RM} terminal differentiation. 8 weeks after irradiation and reconstitution (**Figure 2.4C**), we inflamed the ear skin of mice, adoptively transferred *ex vivo* activated OT-I T cells, and injected diphtheria toxin over the course of 4 weeks to deplete the respective subsets of DC. Interestingly, even the depletion of these DC did not impact terminal differentiation of OT-I into eT_{RM} in the skin (**Figure 2.4C**), despite a notable quantitative decrease in these DC populations (**Figure 2.4E**). We therefore concluded that local activation of TGF-β by αV expressing DC in the skin was not critical for eT_{RM} formation.

Figure 2.4 Local TGF-β activation by DC is dispensable for terminal eT_{RM} differentiation. (**A**) Ear skin of WT mice was inflamed by tattoo on one side and after 72 hours, ear skin and draining lymph nodes were harvested. Gated on $CD11c^+$ cells, αV integrin expression was measured using internal control uninflamed ears and lymph nodes from the same mouse. (**B**) 10^6 Thy1.1/2 congenic *ex vivo* activated OT-I effector cells were intravenously injected into Thy1.2 αV-ΔDC or WT recipients, whose ears were simultaneously inflamed through tattoo injury. After 4 weeks, CD69 and CD103 expression was assessed on transferred OT-I and host $CD8^+$ T cells in skin. (**C**) Mice were lethally irradiated and 10^6 bone marrow cells were transferred from Zbtb46 (zDC) DTR, CD11c DTR or WT mice. 8 weeks after transfer and reconstitution, the experiment in (B) was conducted, along with sustained injections of diphtheria toxin (DTx) every 3 days. Each experiment shown was performed at least twice with similar results. **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$, n.s.: not significant.

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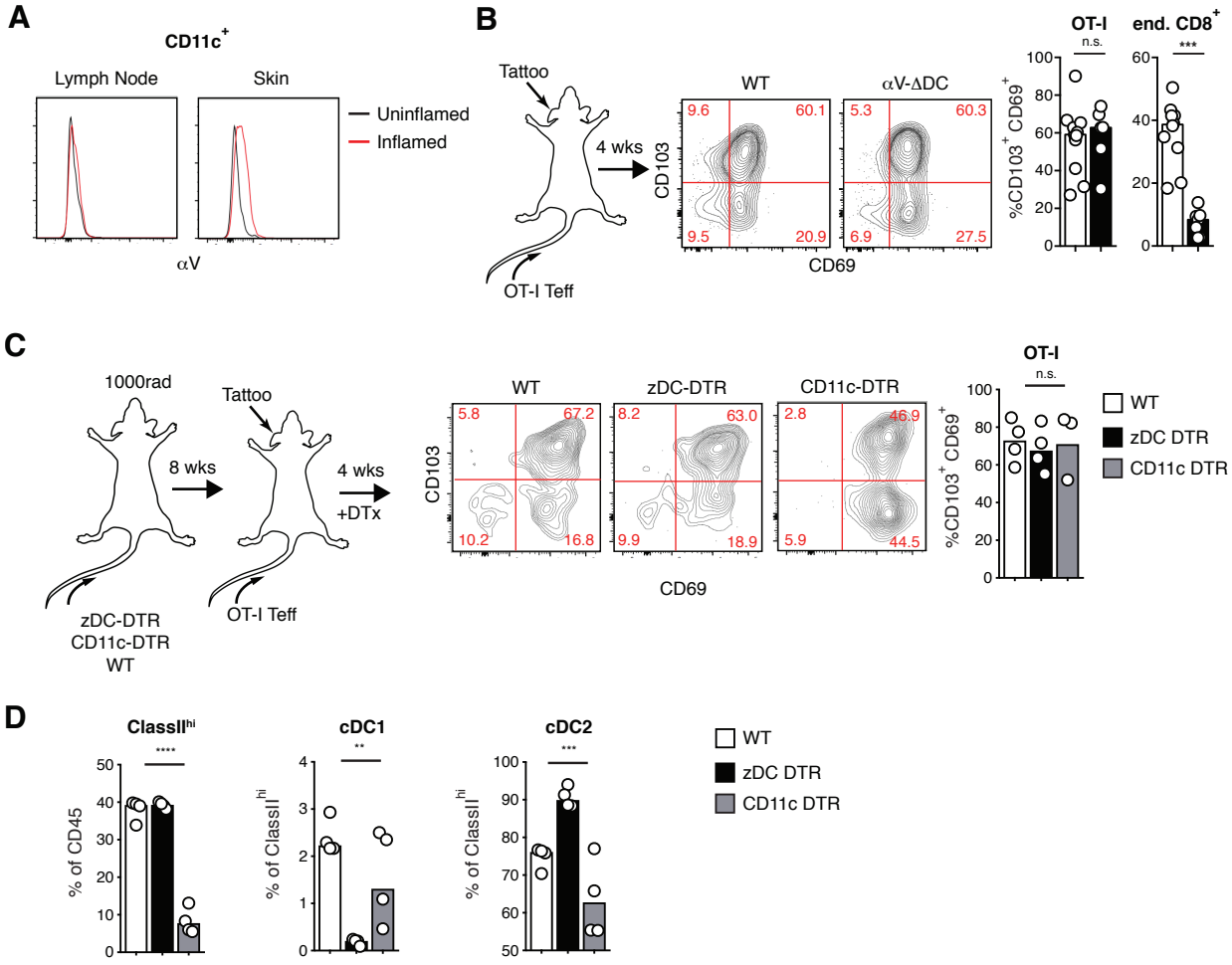

Effect of dendritic cell expressed αV on CD8⁺ T_{RM} formation occurs prior to priming

Alternatively, we considered that CD8$^+$ T cells may be exposed to TGF-β by DC upon activation, through stabilization of cognate interactions via the immunological synapse. We hypothesized that such a context of signaling could confer upon activated T cells the capacity for enhanced eT_{RM} generation, for instance by efficient precursor generation by increasing metabolic fitness[96], or even by imprinting of skin homing capacity by induction of selectin ligands[189] and skin-tropic chemokine receptors[101,103]. We therefore adoptively transferred naïve OT-I T cells into αV-ΔDC or WT littermate control mice and vaccinated mice for T_{RM} induction *in vivo* by tattoo with OVA-encoding plasmid DNA on the ear skin (**Figure 2.5A**). To our surprise, even *in vivo* primed OT-I in αV-ΔDC mice were able to generate similar frequencies and numbers of eT_{RM} in the skin as compared to WT hosts, much unlike the endogenous CD8$^+$ compartment (**Figure 2.5A**).

Since TGF-β activation by αV-expressing DC seemed to be important for eT_{RM} formation, but not during priming or local differentiation in the skin, we considered other contexts during which CD8$^+$ T cells may interact with DC and thus receive TGF-β signals to impact their differentiation. An often overlooked stage of CD8$^+$ T cell development or homeostasis is the period of time after thymic egress where naïve T cells circulate through secondary lymphoid organs for anywhere from days to years before they are even activated[190]. It is known that resting T cells depend on "tonic" interactions with self-peptide:MHC-I for their maintenance during this period prior to activation[191]. Therefore, we hypothesized that at the same time, during immune homeostasis, naïve CD8$^+$ T cells may be exposed in secondary lymphoid organs to active TGF-β that would thereby pre-condition them for enhanced eT_{RM} formation upon eventual foreign antigen encounter at activation. To investigate this, we adoptively transferred naïve OT-I cells into αV-ΔDC mice, where this conditioning may be lost, and WT mice, where the

Figure 2.5 Effect of DC-expressed αV on $CD8^+$ eT_{RM} formation occurs prior to priming.

(**A**) 10^5 $CD44^{lo}$ $CD62L^{hi}$ naïve OT-I cells were adoptively transferred into αV-ΔDC or WT recipients, whose ears were simultaneously tattooed with OVA-encoding plasmid DNA to prime OT-I in skin-draining LNs. After 4 weeks, CD69 and CD103 expression was assessed on transferred OT-I and host $CD8^+$ T cells in skin. (**B, C**) 10^6 $CD44^{lo}$ $CD62L^{hi}$ naïve OT-I cells were adoptively transferred into αV-ΔDC or WT recipients. 3 weeks later, OT-I cell frequency in pooled LNs and spleen (SLO) was determined in some animals (B), while the remaining animals were vaccinated by ear tattoo with OVA-encoding plasmid DNA (C). After 4 weeks, CD69 and CD103 expression was assessed on transferred OT-I and host $CD8^+$ T cells in skin. (**D**) 10^6 Thy1.1/2 congenic $CD44^{lo}$ $CD62L^{hi}$ naïve OT-I cells (1° OT-I) were adoptively transferred into αV-ΔDC or WT recipients. 3 weeks later, a second batch of 10^5 CD45.1 congenic OT-I cells (2° OT-I) was transferred into the same recipients and mice were vaccinated by ear tattoo with OVA-encoding plasmid DNA. After 4 weeks, the ratios of OT-I derived from the second and the first injected batch was assessed in the pools of $CD69^+$ $CD103^+$ T_{RM} in skin and of $CD44^+$ $CD8^+$ T cells in spleen. Each experiment shown was performed at least twice with similar results. *: $p<0.05$, ***: $p<0.001$, n.s.: not significant.

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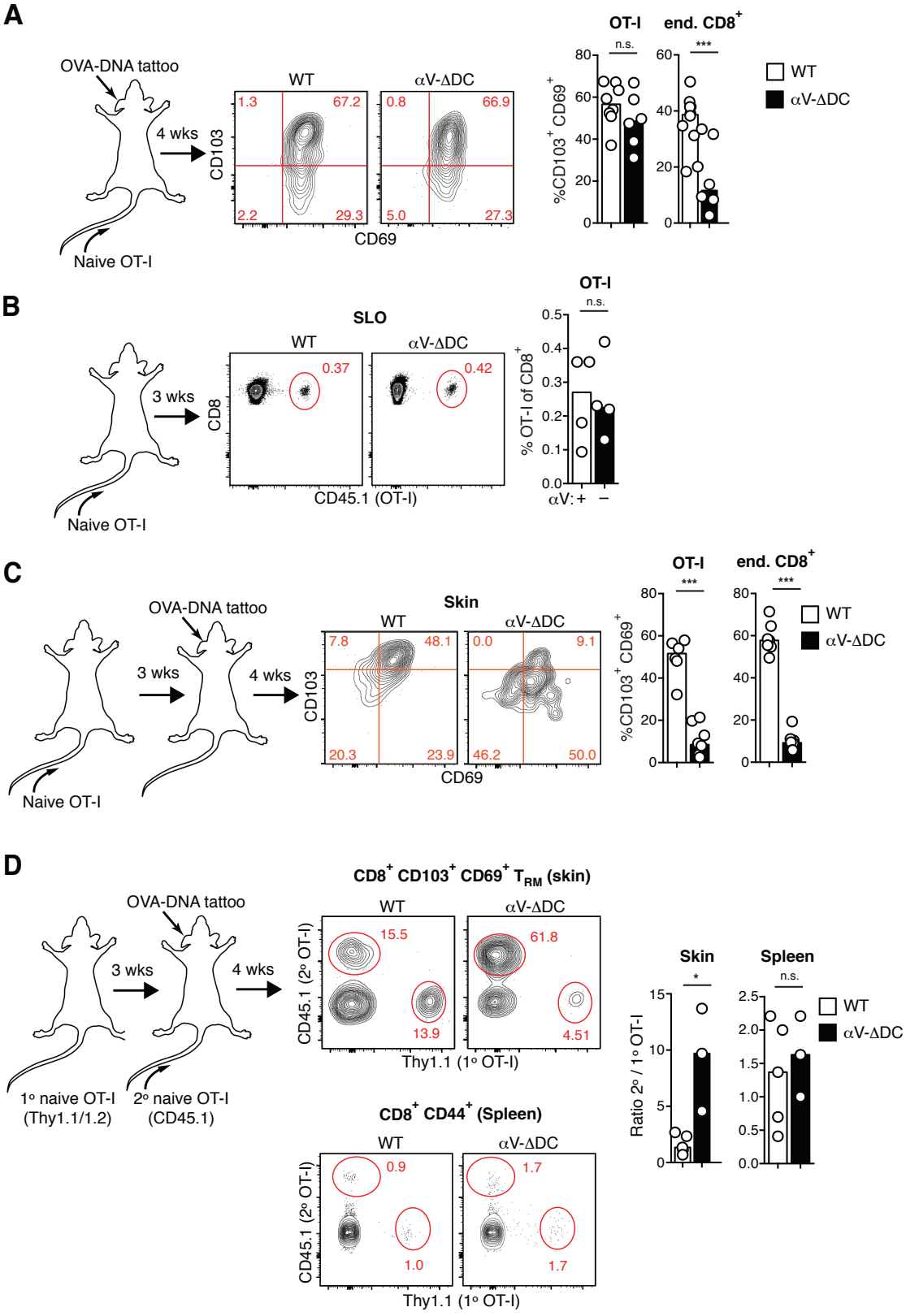

conditioning might be sustained, and allowed them to equilibrate in these microenvironments for 3 weeks. After this equilibration period, we observed that the frequency of naïve OT-I in the secondary lymphoid organs was comparable between those incubated in αV-ΔDC and WT (**Figure 2.5B**). We then vaccinated mice by OVA-DNA tattoo of the ear skin. Four weeks following vaccination, we observed a reduction in the frequency of $CD103^+CD69^+$ OT-I eT_{RM} in the vaccinated skin of αV-ΔDC mice, but not in WT mice, matching the reduction we observed in the eT_{RM} derived from the endogenous polyclonal $CD8^+$ T cell compartment in αV-ΔDC mice(**Figure 2.5C**). To control for any potential added variability in the T cell priming efficiency in each host, we repeated the experiment with an internal control, transferring in a first batch of naïve OT-I cells, waiting for 3 weeks, then transferring a second batch of congenically distinct naïve OT-I at the time of OVA-DNA tattoo vaccination (**Figure 2.5D**). Cells from both batches contributed equally to the pool of eT_{RM} in the skin of WT mice, suggesting that their capacity to form eT_{RM} had not been significantly altered over the course of the 3 week incubation period (**Figure 2.5D**). In contrast, in αV-ΔDC mice cells of the first batch were 10-fold less efficient at becoming eT_{RM} than those of the second batch (**Figure 2.5D**), demonstrating that the capacity of naïve $CD8^+$ T cells to form eT_{RM} declines over time in the absence of DC expressing αV. Interestingly, we did not observe such a bias in circulating memory populations ($CD44^{hi}$) of OT-I in the spleen (**Figure 2.5D**). Therefore, we concluded that continual exposure to αV expressing DC sustains the capacity of naïve $CD8^+$ T cells to efficiently form skin eT_{RM}, without necessarily impacting their ability to give rise to circulating memory populations.

Naïve $CD8^+$ T cell exposure to TGF-β at homeostasis shapes their epigenetic potential

Given that the differentiation of circulating memory cells was not as sensitive as eT_{RM} to pre-conditioning in an environment with αV-expressing DC, we considered properties that distinguish T_{RM} from circulating memory $CD8^+$ T (T_{circ}) cells. While T_{RM} have distinguishing

transcripts from T_{circ}[40,42], many of these T_{RM} signature genes were not yet expressed in naïve $CD8^+$ T cells, as shown by ultra-low input RNA sequencing that we compiled from the ImmGen database (**Figure 2.6**).

We therefore hypothesized that homeostatic exposure to αV-expressing DC may induce a permissive epigenetic state in naïve $CD8^+$ T cells that enhances their capacity to form eT_{RM} upon activation by poising these genes relevant to eT_{RM} formation. To assess alterations in genome-wide chromatin accessibility, we purified $CD44^{lo}$ naïve $CD8^+$ T cells from both αV-ΔDC and WT mice and performed the assay for transposase accessible chromatin using high throughput sequencing (ATACseq). We found that 13Mb of chromatin was accessible in naïve $CD8^+$ T cells, accounting for less than 0.04% of the mouse genome, with 496kb (3.8%) being more accessible in naïve $CD8^+$ T cells from WT mice and 391kb (3%) being more accessible in naïve cells from αV-ΔDC mice. Peak calling and merging yielded fairly low numbers of differentially accessible regions (DARs) in cells from both WT and αV-ΔDC mice (**Figure 2.7A**). We observed that the DARs from WT cells were preferentially clustered around transcription start sites (TSS), suggesting that they may already be expressed or poised for expression, whereas DARs in αV-ΔDC cells were spread across even sites quite distal to the TSS of the most proximal gene (**Figure 2.7B**). Upon further analysis of these DARs, we found that those in naïve cells from αV-ΔDC mice were enriched for binding motifs of interferon regulatory factors (IRF) and T-box (e.g. T-bet and Eomesodermin) transcription factor motifs (**Figure 2.7C**), while DARs in naïve cells from WT mice were most enriched for Krüppel-life factor (KLF) and other zinc finger DNA-binding proteins, as well as Runx family members, such as Runx3 (**Figure 2.7C**). The increased accessibility of these motifs in naïve cells suggests that some epigenetic changes driving eT_{RM} programs may already be active prior to activation. Indeed, among a set of 21 signature skin eT_{RM} up-regulated transcripts[40], we found 5 of these genes linked to DARs

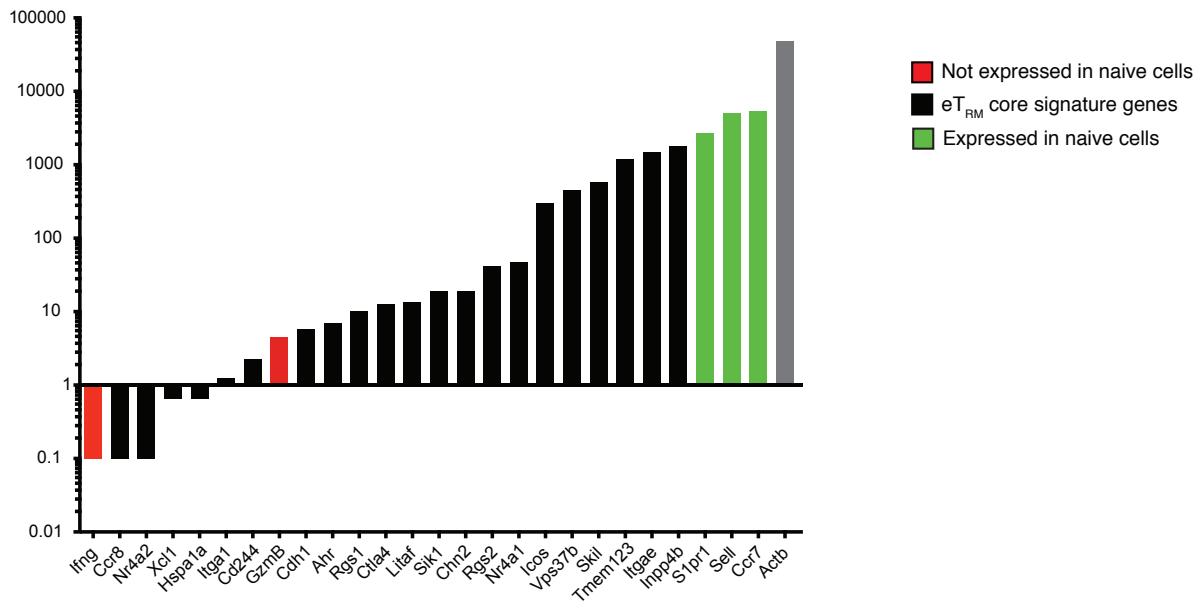

Figure 2.6 eT$_{RM}$ core signature genes are not highly transcribed in naïve CD8^{+} T cells. ImmGen database RNA-seq gene expression analysis from naïve CD8 T cells reveals variable expression of eT$_{RM}$ genes in naïve CD8^{+} T cells. Green bars denote selected genes known to be expressed at the protein level in naïve T cells, red bars denote selected genes known to be expressed at very low levels or not to be expressed in naïve T cells. Black bars denote skin eT$_{RM}$ genes.

Figure 2.7 Naïve CD8$^+$ T cell exposure to TGF-β at homeostasis shapes their epigenetic potential to form eT$_{RM}$. (**A**) Volcano plot of chromatin accessibility changes between CD62L^{hi}CD44^{low} naïve CD8$^+$ T cells from WT and αV-ΔDC mice, considering merged DARs. (**B**) Cumulative distributions of distances from DARs in cells from WT (green) and αV-ΔDC (blue) animals to the closest transcription start site. The two-sample Kolmogorov-Smirnov was used to compare cumulative distributions. (**C**) Enrichment of indicated transcription factor binding motif families in DARs. (**D**) Normalized chromatin accessibility near the *Itgae* (top), *Ccr8* (middle), and *S1pr5* (bottom) loci. The rectangles mark detected DARs.

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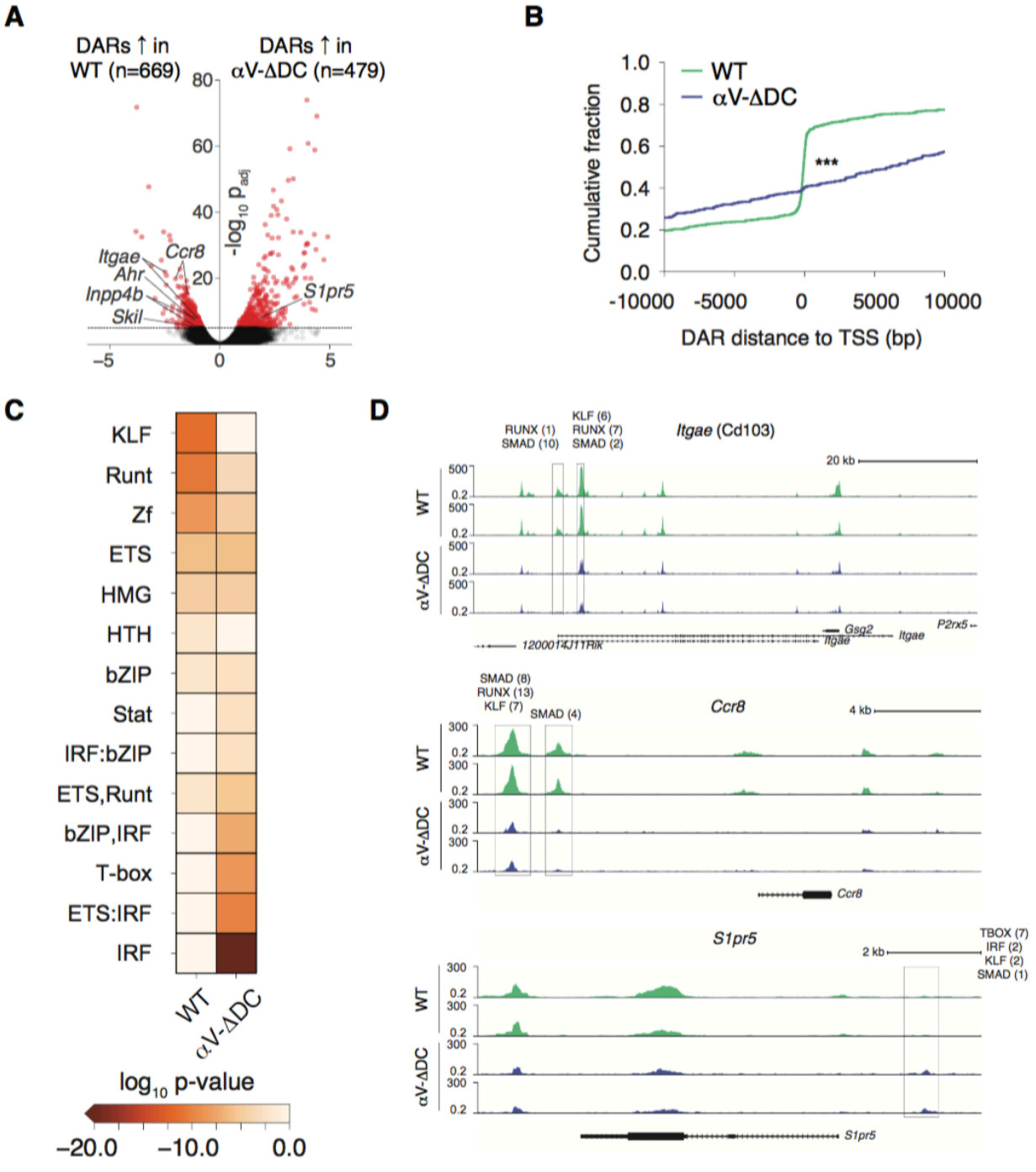


in our WT cells, including *Itgae* (coding for CD103), *Ahr*, and *Ccr8* (**Figure 2.7A**). The DARs proximal to these genes contained clustered Runx, KLF, and Smad motifs (**Figure 2.7D**), suggesting involvement of TGF-β with these associated changes. Additionally, one down-regulated gene in the eT_{RM} signature, *S1pr5*, was accordingly linked to a DAR upstream of its TSS in cells from αV-ΔDC mice, with T-box and IRF motifs (**Figure 2.7D**).

Notably, Runx3 has recently emerged as a master transcriptional regulator of eT_{RM} differentiation[125], which is supported by the trend in our ATACseq data.. This is interesting in the context of TGF-β signaling as well, Runx family transcription factors (TFs) are not only induced by TGF-β, but also interact with and enable functions of the TGF-β regulated Smad TFs[129,192]. Moreover, when we conducted gene set enrichment analyses of the DARs in WT cells, we found that many of the up-regulated biological pathways converged upon TGF-β and Smad signaling-associated processes (**Figure 2.8A**). Taken together with the Runx3 studies, our data suggests that the role of Runx3 during eT_{RM} differentiation may be, at least in part, based on its requirement for TGF-β mediated gene regulation.

Furthermore, gene ontology enrichment revealed that the DARs in WT cells were linked to cell cycle arrest, quiescence, and memory over effector programs, while the αV-ΔDC DARs were more closely linked to cell proliferation, activation and effector over memory programs (**Figure 2.8A-B**). These observations therefore show that DC-dependent exposure to active TGF-β in secondary lymphoid organs (SLO) renders multiple critical eT_{RM} genes more accessible in naïve $CD8^+$ T cells, potentially facilitating their accelerated transcriptional activation and more efficient downstream eT_{RM} formation.

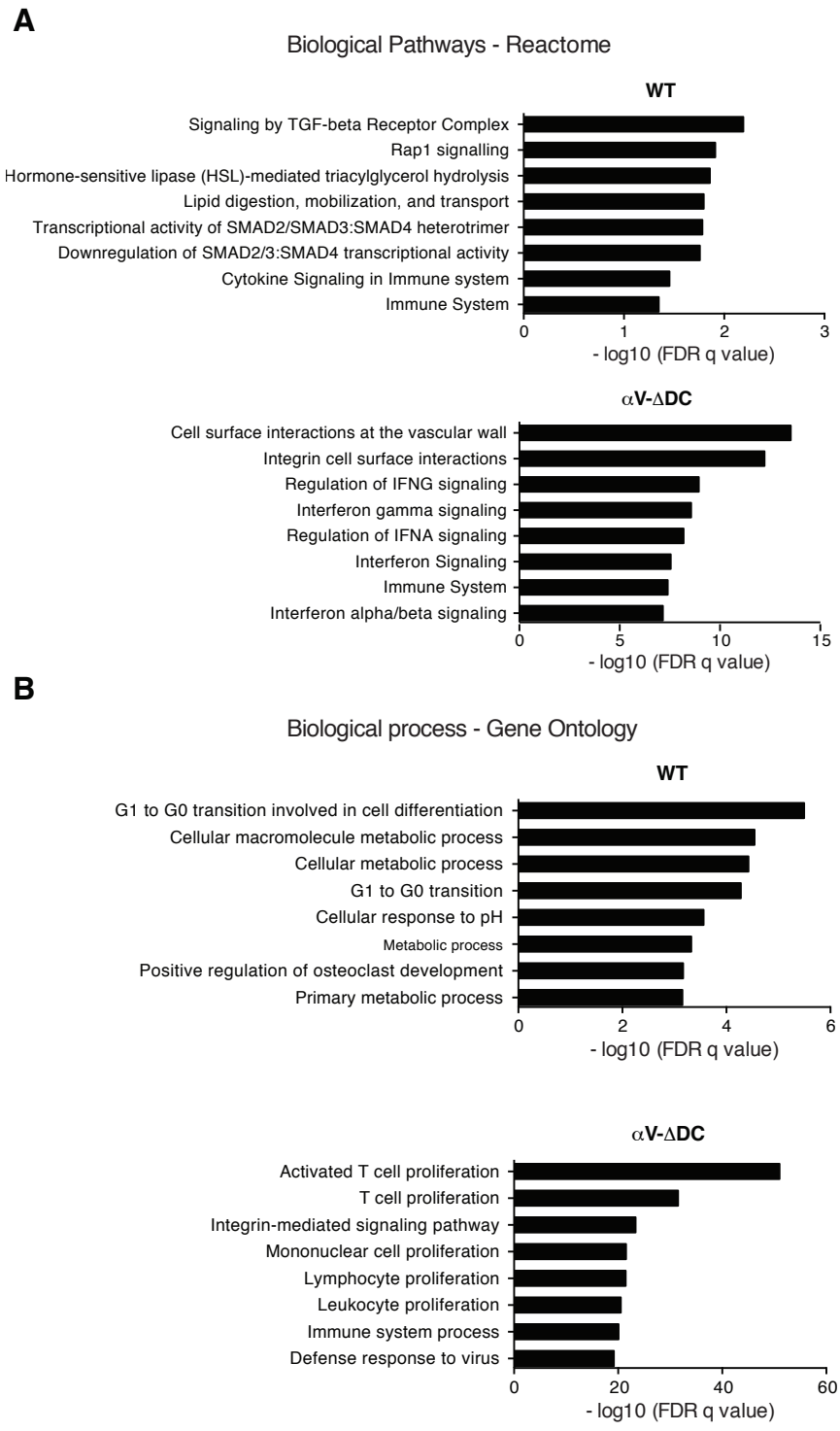


Figure 2.8 GREAT analysis of DARs in cells from WT and αV-ΔDC mice. Top 8 enrichment hits each from the MSigDB Reactome database (A) and the Gene Ontology (GO) Biological Process database (B), ranked by FDR q-value and plotted as $-\log_{10}$(FDRq).

CD103 is a surrogate marker for TGF-β dependent conditioning in naïve CD8$^+$ T cells

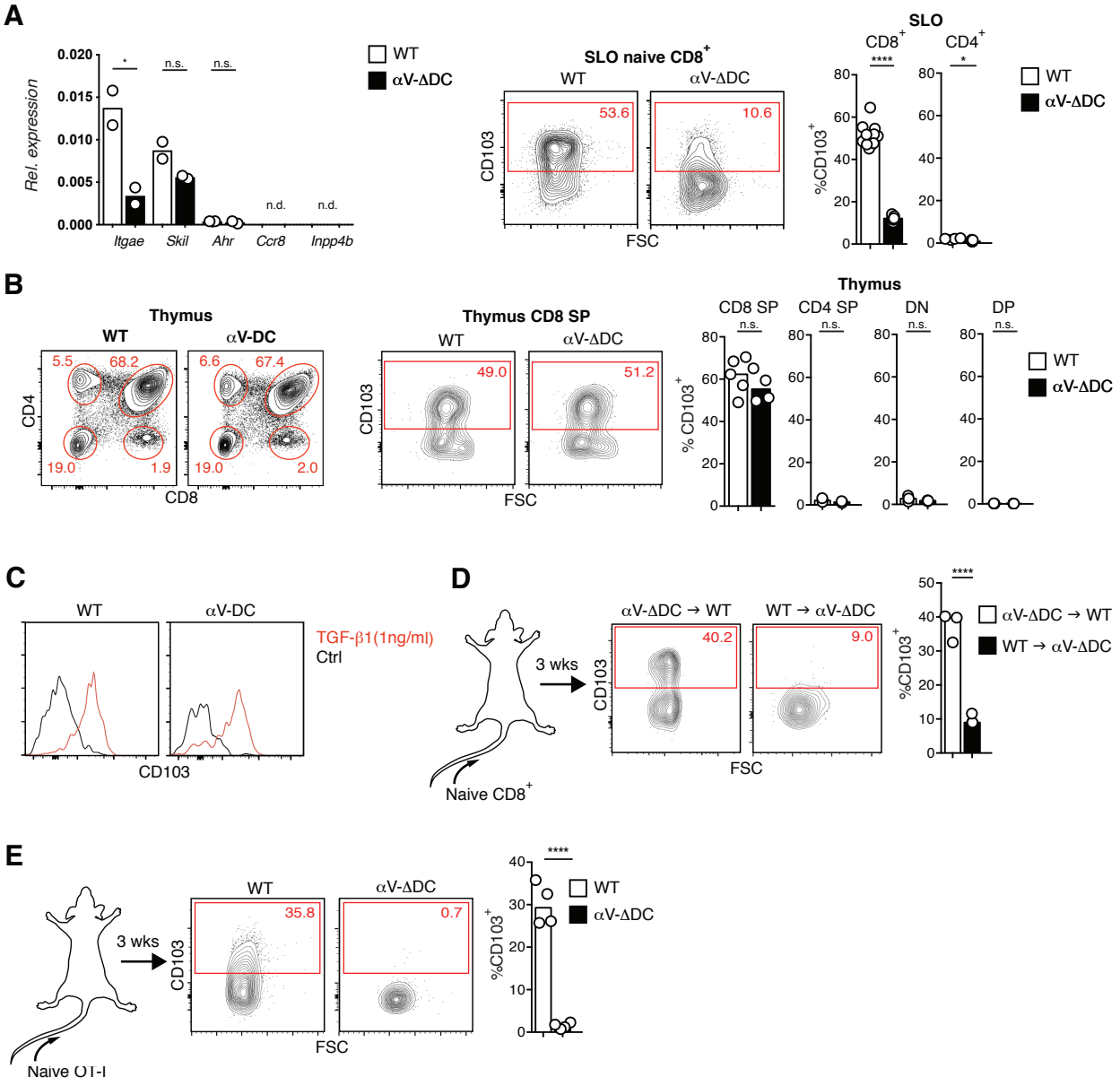
While many of the eT$_{RM}$-associated DARs in WT are not highly transcribed in naïve cells (**Figure 2.9A**), we observed a DAR in the WT cells containing Runx and Smad motifs near the TSS of *Itgae*, suggesting that this may be a gene readily expressed by naïve T cells[129]. Indeed, we found both at the mRNA and protein level, CD103 was expressed by naïve CD8$^+$ T cells in the secondary lymphoid organs of WT but not in αV-ΔDC mice (**Figure 2.9A**). As CD103 is expressed on conditioned naïve T cells and is known to be a TGF-β dependent gene[129,133], we thought to use it as a surrogate marker for conditioning.

During CD8$^+$ thymic development, CD103 is up-regulated on CD8$^+$ single positive cells in a TGF-β and Runx-dependent manner before their egress from the thymus into the secondary lymphoid tissues[129]. As naïve CD8$^+$ T cells from secondary lymphoid organs in αV-ΔDC mice lack CD103 on their surface, we wondered whether this defect came from their thymic development or if this was instructed in the periphery. Upon examination of CD8$^+$SP thymocytes in αV-ΔDC mice, we found no deficiency in CD103 expression (**Figure 2.9B**) unlike our observations in the SLO, suggesting that their lack of CD103 expression in the periphery was not due to an intrinsic inability of these cells to up-regulate the integrin. We further confirmed this by isolating and treating naïve CD8$^+$ T cells from WT or αV-ΔDC mice with TGF-β-1 *in vitro*, which was able to induce their expression of the CD103 (**Figure 2.9C**). Upon transfer of naïve CD8$^+$ T cells from αV-ΔDC into WT and equilibration for 3 weeks, we found that they were also able to regain expression *in vivo* (**Figure 2.9D-E**). Conversely, both naïve polyclonal WT as well as OT-I T cells lost their CD103 expression *in vivo* upon transfer into αV-ΔDC hosts (**Figure 2.9D-E**). Together, our data suggest that CD103 expression must be actively maintained after its original induction in the thymus, thereby making it a sensitive marker of conditioned naïve CD8$^+$ T cells.

Figure 2.9 CD103 is a TGF-β dependent marker of naïve CD8$^+$ T cell conditioning.

(**A**) CD62L^{hi}CD44^{low} naïve CD8$^+$ T cells from WT and αV-ΔDC mice were enriched by FACS sorting, RNA was isolated and RT-PCR was performed. Relative expression was determined using beta actin as a housekeeping gene (left). Expression of CD103 on CD62L^{hi}CD44^{low} naïve CD4$^+$ and CD8$^+$ T cells (pooled from LNs and spleen) (right) and on (**B**) thymocyte subsets from αV-ΔDC and WT littermate control mice. (**C**) CD103 expression of naive CD8$^+$ T cells from αV-ΔDC or WT mice following 3 days of culture in 5 ng/ml of IL-7, 100ng/mL IL-15 in the presence or absence of 1 ng/ml of activated murine recombinant TGF-β1. (**D**) 10^6 naïve polyclonal CD8$^+$ T cells from αV-ΔDC were adoptively transferred into CD45.1 congenic C57BL/6 mice or vice versa and re-isolated 3 weeks later from pooled LNs and spleens for analysis of CD103 expression. (**E**) 10^6 naïve OT-I T cells were adoptively transferred into αV-ΔDC or WT hosts and isolated from pooled LNs and spleens 3 weeks later for analysis of CD103 expression. Each experiment was performed at least twice with similar results. *: $p<0.05$, ****: $p<0.0001$, n.s.: not significant.

Figure 2.9 (Continued)



We then sought to map the kinetics of CD103 expression as $CD8^+$ T cells transitioned from a naïve state to eT_{RM}. While naïve cells express CD103 in a bimodal fashion (**Figure 2.9A**), they lose this expression upon activation (**Figure 2.10A**), likely due to the down-regulation of TGF-βRII immediately following T cell activation[99]. After differentiation, we find that memory cells in the non-lymphoid selectively up-regulate CD103 (**Figure 2.10A**, bottom) upon exposure to tissue cues during terminal eT_{RM} differentiation. In fact, even $CD44^{hi}$ cells in the secondary lymphoid organs do not regain CD103 expression (**Figure 2.10A**, top), despite residing in the same milieu as naïve $CD8^+$ T cells. Therefore, CD103 expression on naïve $CD8^+$ T cells is distinct from that expressed on eT_{RM}, and suggests a unique meaning for CD103 expression on naïve cells.

Naïve T cells are selectively conditioned in the lymph node

Since the majority of $CD8^+$SP thymocytes express CD103, it is possible that the $CD103^+$ naïve $CD8^+$ T cells we observed in the SLO of WT mice were enriched in recent thymic emigrants (RTE). To assess the magnitude of the RTE contribution to the CD103+ population of naïve $CD8^+$ T cells, we treated WT mice with the functional sphingosine-1-phosphate receptor antagonist FTY720 (fingolimod) in order to block lymphocyte egress from the thymus, as well as in-between the SLO. Presumably, if CD103+ naïve $CD8^+$ T cells were predominantly RTE, this treatment would be predicted to yield a decrease in CD103 expression in the SLOs. Interestingly, however, we found that the frequency of CD103 expressing cells, as well as their overall MFI, did not decrease, but rather increased on cells “trapped” in the lymph node (LN), while decreasing in the spleen (**Figure 2.11A**). This not only suggested that RTEs do not necessarily make a significant contribution to our pool of $CD103^+$ naïve cells in the SLO, as corroborated by studies of $CD8^+$SP thymic output[190], but also that CD103 is preferentially induced and sustained in the LNs, but not in the spleen.

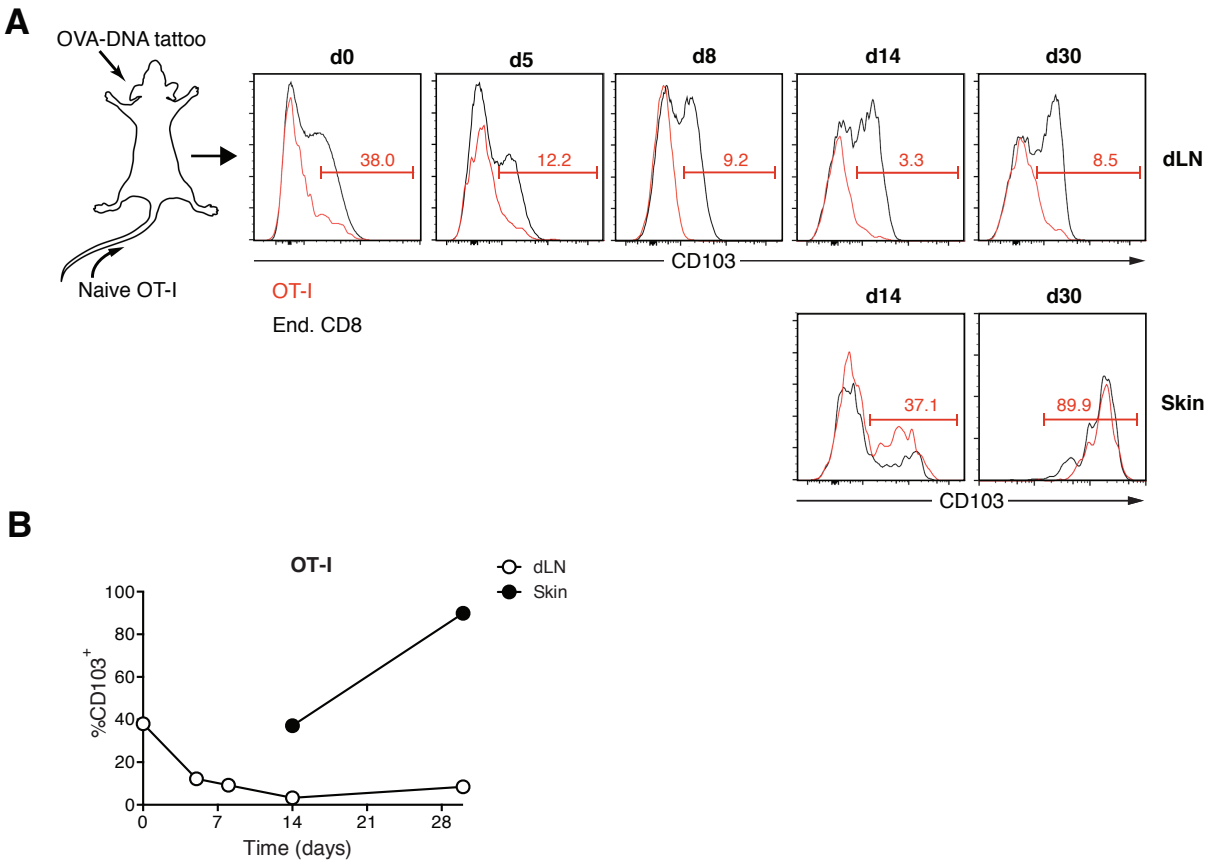
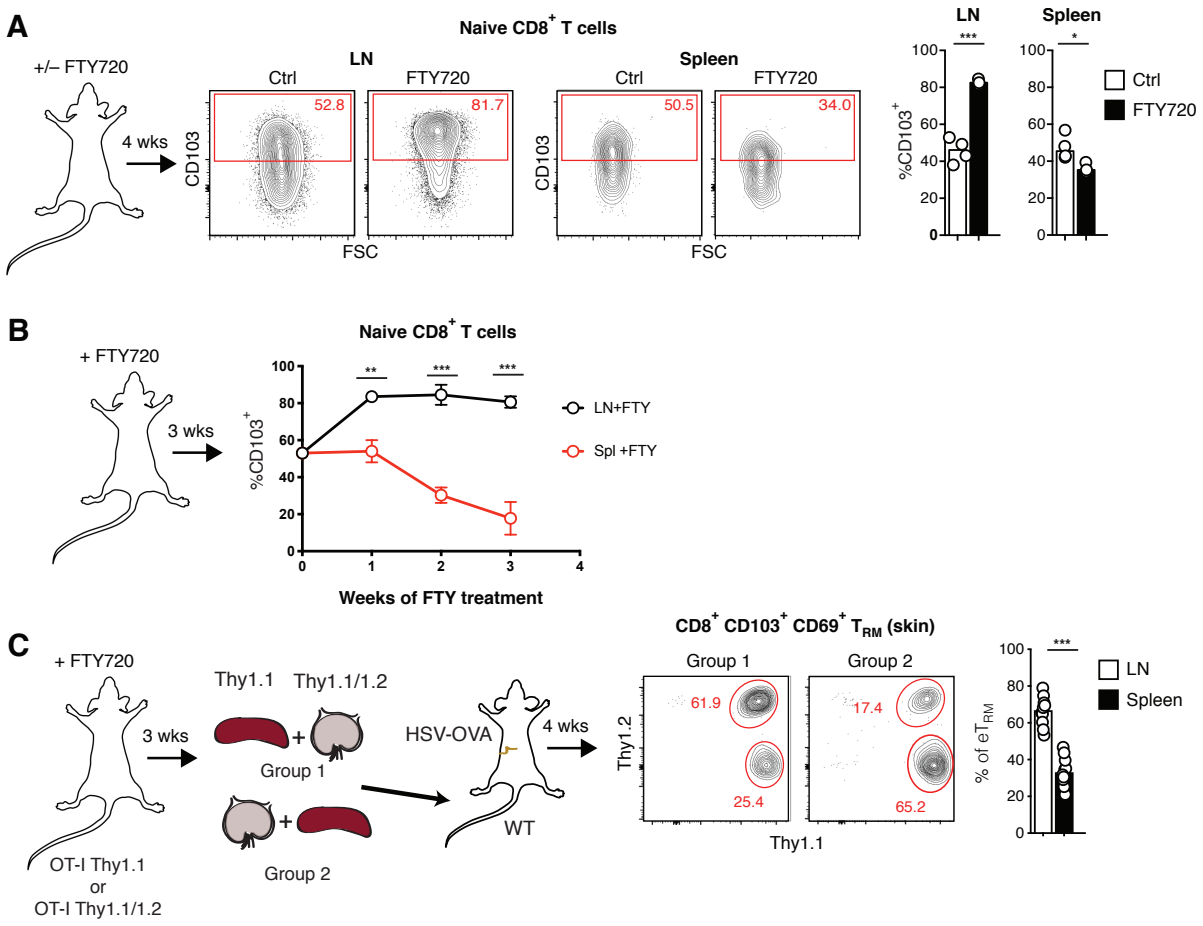


Figure 2.10 CD103 expression on naïve CD8$^+$ T cells is distinct from that of eT$_{RM}$. (**A**) Naïve OT-I were adoptively transferred into WT mice, which were vaccinated with OVA-plasmid DNA delivered through tattoo of the ear skin. CD103 expression on OT-I cells was assayed at indicated time-points after vaccination in the draining cervical LN (top row) and ear skin bottom row). Endogenous CD8$^+$ T cell at each site are shown for reference. (**B**) Plot of (A).

Figure 2.11 Naïve $CD8^+$ T cells are conditioned for eT_{RM} formation in the lymph nodes.

(**A**) C57BL/6 mice were treated with FTY720 to block lymphocyte egress from all lymphoid tissues. After 4 weeks of continued treatment, naïve $CD8^+$ T cells in LNs and spleen were analyzed for CD103 expression. (**B**) C57BL/6 mice were treated with FTY720 and harvested at weekly time points for analysis of CD103 expression on naïve $CD8^+$ T cells in LNs and spleen. (**C**) OT-I mice of two different congenic markers (Thy1.1 and Thy 1.1/1.2) were treated with FTY720 for 3 weeks, at which time the LNs and spleens were harvested separately and naïve $CD8^+$ T cells were enriched. 10^5 naïve $CD8^+$ T cells each from LNs and spleens (of distinguishing congenic markers) were then competitively transferred into C57BL/6 mice as indicated and infected with 10^6 p.f.u. HSV-OVA by scarification of the flank skin. 4 weeks later, skin was analyzed and contributions from the LN and spleen pool to the eT_{RM} in the flank skin was determined in both groups of mice. Each experiment was performed at least twice with similar results. **: $p<0.01$, ***: $p<0.001$, n.s.: not significant.

Figure 2.11 (Continued)



In order to understand the kinetics by which CD103 is induced or lost as a conditioning marker on naïve $CD8^+$ T cells, we conducted a time course experiment. To this end, we treated WT mice with FTY720 for up to 4 weeks and harvested spleen and LN for analysis of CD103 expression at one week intervals (**Figure 2.11B**). Based on the observation that conditioning may occur in the lymph node, using CD103 as a surrogate marker of conditioning, we found that the maximum induction of CD103 on naïve cells occurs within one week of being "locked" in the LN (**Figure 2.11B**). However, in the spleen, where cells presumably lose their conditioning, the decrease in CD103 expression takes up to 3 weeks to reach its lowest plateau (**Figure 2.11B**), suggesting that the loss of conditioning occurs much more slowly than the acquisition of CD103 in the LN.

To test whether locking cells in the LN as compared to the spleen would have any impact on eT_{RM} differentiation, we treated OT-I mice of two different congenic markers with FTY720 for 3 weeks, matching the kinetics of the conditioning time course we had previously conducted. After 3 weeks, we isolated naïve $CD8^+$ T cells from SLO, keeping cells from the LN and spleen separate. We then competitively transferred, in equal ratios, 10^5 naïve cells from each donor from the LN or spleen. The transferred cells were distinguished on the basis of congenic markers (**Figure 2.11C**) and recipient mice were challenged with HSV-OVA on the flank skin. Upon harvest of the challenged skin site 4 weeks later, we found that, regardless of the donor mouse, cells originally isolated from the LN were enriched in the eT_{RM} pool over those from the spleen (**Figure 2.11C**). Therefore, we concluded that naïve $CD8^+$ T cells receive essential homeostatic signals in the LN, and not the spleen, that confers $CD8^+$ T cells with enhanced capacity to become eT_{RM}.

To test this hypothesis in an independent mouse model, we analyzed lymphotoxin alpha-deficient ($LT\alpha^{-/-}$) mice, which lack LNs, but still have spleens[193]. While CD103 expression was

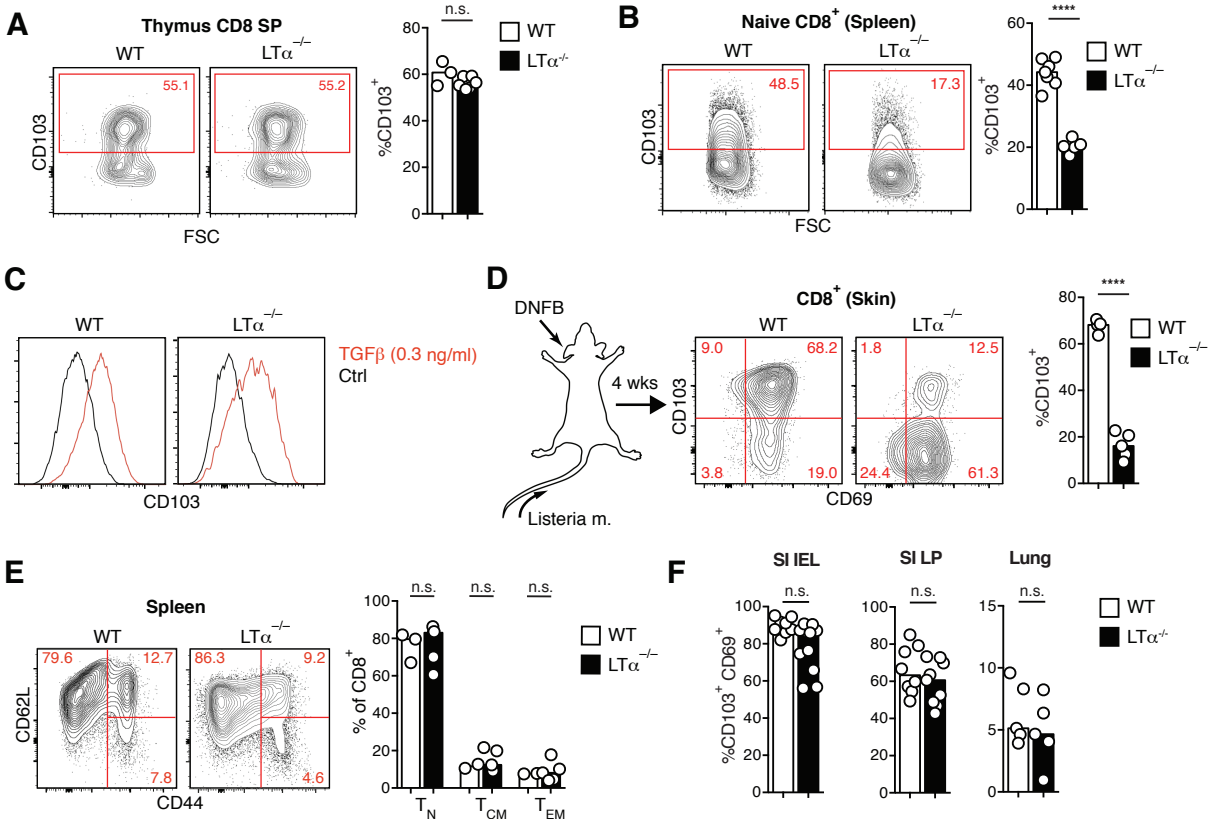
normal in thymocytes of these mice (**Figure 2.12A**), upon examination of naïve $CD8^+$ T cells in the spleen, we found a loss of CD103 expression in $LT\alpha^{-/-}$ mice (**Figure 2.12B**), similar to αV-ΔDC mice. We were also able to rescue this deficiency in CD103 maintenance upon treatment of naïve $CD8^+$ T cells isolated from the spleens of $LT\alpha^{-/-}$ mice with TGF-β1 *in vitro* (**Figure 2.12C**), reaffirming the importance of TGF-β signaling for the maintenance of CD103 in naïve $CD8^+$ T cells. To test whether this mimicked the eT_{RM} phenotype we observed in αV-ΔDC mice, we performed a "prime and pull" challenge[34] to account for the impairment in immune response to local skin challenge in the absence of draining LNs. Indeed, 4 weeks after intravenous Listeria infection and DNFB-based "pull" of activated T cells into the skin, we observed a profound reduction in the frequency of $CD103^+$ eT_{RM} in the skin of $LT\alpha^{-/-}$ mice (**Figure 2.12D**). As we found in the mucosal tissues of αV-ΔDC mice, there was no evident impairment in the $CD103^+CD69^+$ population of $CD8^+$ T_{RM} in the gut or lungs, or in circulating memory pools (**Figure 2.12E**). Therefore, we concluded that αV-expressing DC in the lymph node, but not spleen, were functionally specialized in conditioning naïve $CD8^+$ T cells for eT_{RM} formation.

Conditioning of naïve T cells by TGF-β is mediated through migratory dendritic cells

A key distinguishing factor of the LN over the spleen is the presence of a population of $CD11c^{int}MHCII^{hi}$ migratory DC (mDC) which continually traffic at steady state from non-lymphoid tissues to draining LNs via the lymphatic system (**Figure 2.13A**). While both mDCs and lymphoid tissue resident DCs (rDCs) and their further distinguishing subsets actively transcribed αV, only the mDCs co-expressed the β8 integrin chain (**Figure 2.13B**) that endows them with the capacity to activate latent TGF-β[173,194]. To further test whether naïve T cell conditioning through TGF-β depends on mDCs, we analyzed the SLO of CCR7-deficient ($CCR7^{-/-}$) mice, in which DC trafficking from the peripheral tissues into the draining LNs is impaired [195], resulting in an absence of mDC not only from the spleen but also from the LNs (**Figure 2.13C**). As we

Figure 2.12 Lymphotoxin alpha deficient mice have impaired naïve CD8$^+$ T cell conditioning and eT$_{RM}$ formation. (**A**) CD103 expression on thymic CD8 SP cells from LT$\alpha^{-/-}$ or age-matched WT controls. (**B**) CD103 expression in naïve CD8$^+$ T cells in spleens of WT and LT$\alpha^{-/-}$ mice. (**C**) CD103 expression of naive CD8$^+$ T cells from LT$\alpha^{-/-}$ or WT mice following 3 days of culture 5 ng/ml of IL-7, 100ng/mL IL-15 in the presence or absence of 0.3 ng/ml of activated murine recombinant TGF-β1. (**D**) Frequency of CD69$^+$ CD103$^+$ eT$_{RM}$ in in skin 4 weeks after 'prime and pull' immune challenge through i.v. injection with *Listeria monocytogenes* to produce a circulating T$_{EFF}$ pool ('prime') followed by DNFB$^+$ treatment of ear skin ('pull') of WT and LT$\alpha^{-/-}$ mice. (**E**) Frequencies of CD44^{lo}CD62L^{hi} naive, CD44^{hi} CD62L^{hi} central memory, and CD44^{hi} CD62L^{lo} effector memory phenotype CD8$^+$ T cells in spleens of LT$\alpha^{-/-}$ or WT mice. (**F**) Frequency of CD69$^+$ CD103$^+$ CD8$^+$ T cells in the intraepithelial lymphocyte (IEL) compartment and lamina propria (LP) of the small intestine (SI) and lungs of of LT$\alpha^{-/-}$ or WT mice, after exclusion of intravascular cells. Each experiment was performed at least twice with similar results. ****: p<0.0001, n.s.: not significant.

Figure 2.12 (Continued)



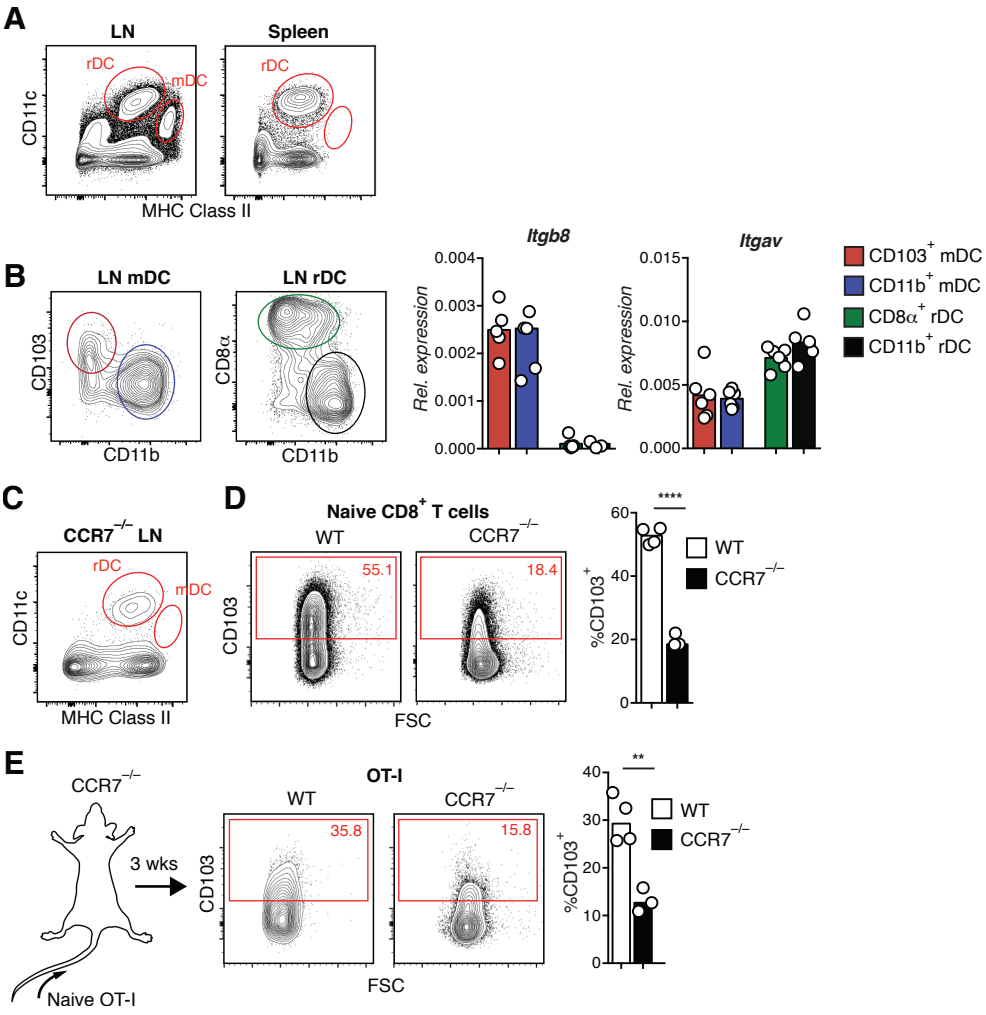


Figure 2.13 Naïve CD8$^+$ T cells are conditioned by migratory DC. (**A**) CD11c^{int} MHC II^{hi} migratory DCs in LNs are absent in spleens of C57BL/6 mice. (**B**) CD103$^+$ and CD11b$^+$ subsets of migratory DCs and CD8$^+$ and CD11b$^+$ subsets of resident DCs were purified by FACS and analyzed for expression of αV and β8 integrin mRNA by RT-qPCR. (**C**) CD11c^{int} MHC II^{hi} migratory DCs are absent in LNs of CCR7$^{-/-}$ mice. (**D**) CD103 expression in naïve CD8$^+$ T cells in pooled LNs and of CCR7$^{-/-}$ mice. (**E**) 10^6 naïve OT-I T cells were adoptively transferred into CCR7$^{-/-}$ or C57BL/6 hosts. CD103 expression on naïve OT-I cells from pooled LNs and spleen was analyzed 3 weeks later. Each experiment was performed at least twice with similar results. **: p<0.01, ****: p<0.0001.

observed in both the αV-ΔDC and LTα$^{-/-}$ mice, CD103 expression on naïve CD8^{+} T cells in the SLO was reduced in CCR7$^{-/-}$ mice as well (**Figure 2.13D**). However, considering that CCR7-deficiency in the T cell compartment of these mice also prevents their efficient localization in the lymph node and thereby may exclude them from TGF-β-dependent conditioning, we also adoptively transferred CCR7-sufficient OT-I T cells into CCR7$^{-/-}$ hosts. Three weeks following their transfer, analysis of the transferred OT-I revealed that they too were unable to maintain their CD103 expression (**Figure 2.13E**), confirming that CCR7-dependent migration of αVβ8-expressing DCs to the LNs at steady state is critical for the conditioning of naïve CD8^{+} T cell for eT$_{RM}$ formation in the skin.

Tonic interactions between naïve CD8^{+} T cells and migratory DC facilitate conditioning

At homeostasis, the survival and responsiveness of naïve CD8^{+} T cells critically relies on non-cognate, MHC-I-dependent interactions with DCs[191,196]. We considered whether resting CD8^{+} T cells may receive TGF-β conditioning during these MHC-I dependent physical interactions with αVβ8-expressing DCs, akin to what has been observed *in vitro* with CD4^{+} T cell differentiation[174], or if DCs may indiscriminately provide a pool of active TGF-β to all naïve CD8^{+} T cells. To test this *in vivo*, we generated mixed irradiation bone marrow chimeras (BMCs) consisting of MHC-I-deficient β2m$^{-/-}$ and αV-ΔDC bone marrow, which in theory would effectively segregate the expression of MHC-I and αV integrin on any given radiation-sensitive DC (**Figure 2.14A**). This setting would allow us to test whether antigen-presenting capacity and TGF-β activation would need to come from the same DC to effectively condition naïve CD8^{+} T cells during homeostasis. Our strategy was only partially effective, as roughly one third of all DCs in our chimeras still co-expressed MHC-I and αV (**Figure 2.14A**), perhaps as a result of "cross-dressing" where membrane-bound MHC-I can be transferred to MHC-I deficient DC from MHC-I sufficient DC[197]. Nevertheless, even a partial dissociation of MHC-I and αV on DC resulted in a

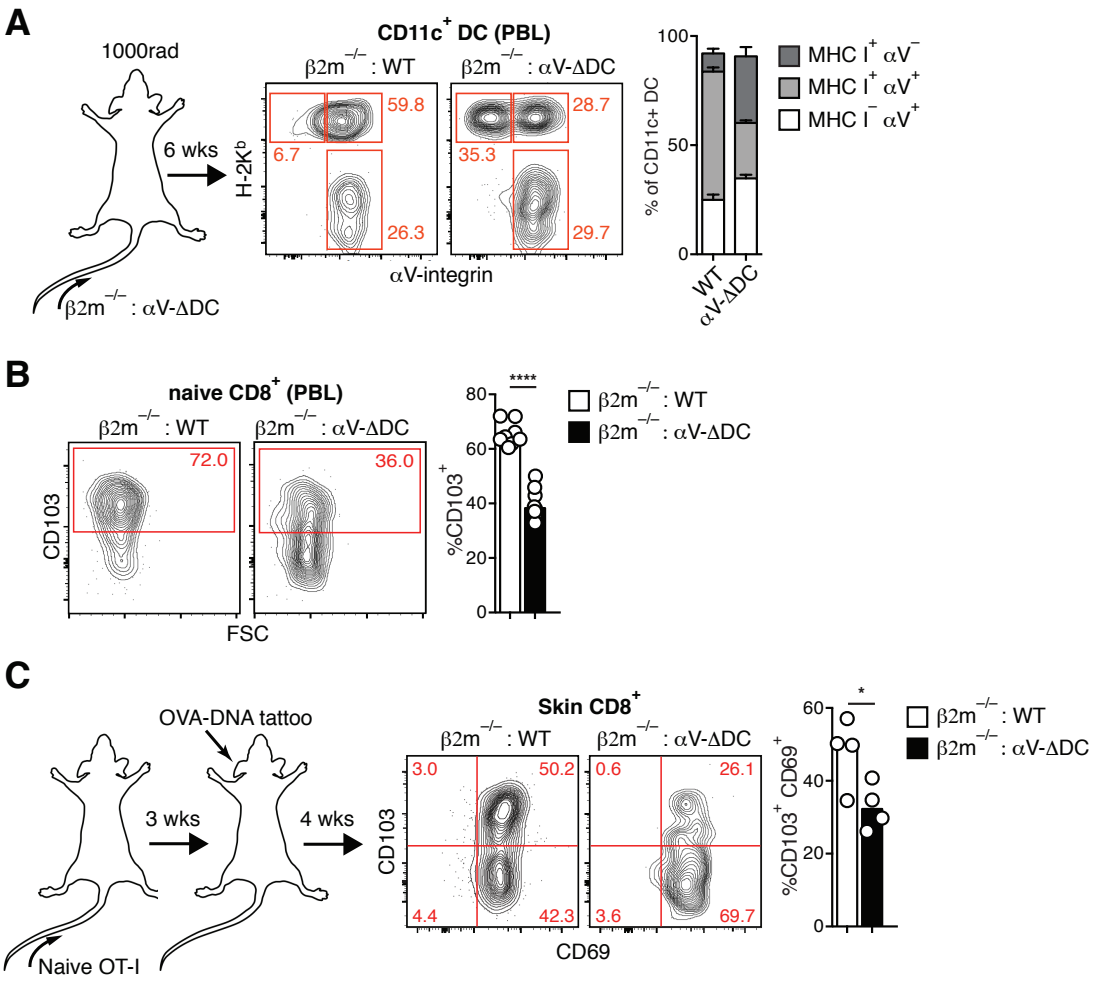


Figure 2.14 Tonic interactions between naïve CD8$^+$ T cells and DC facilitate conditioning.

(**A**) Lethally irradiated CD45.1 congenic mice were injected with 1:1 mixtures of either WT or αV-ΔDC and MHC I-deficient β2m$^{-/-}$ bone marrow cells. 8 week later, expression of H-2K^b and αV on CD11c$^+$ cells in peripheral blood was assessed to determine the frequency of cells that express either one of both proteins. (**B**) CD103 expression on naïve CD8$^+$ T cells in peripheral blood of αV-ΔDC : β2m$^{-/-}$ mixed bone marrow chimeras, in which expression of αV and MHC I on DCs is segregated. (**C**) 10^6 naïve OT-I cells were adoptively transferred into BMCs described in (A). 3 weeks later, mice were vaccinated by ear tattoo with OVA-encoding plasmid DNA. After 4 weeks, CD69 and CD103 expression was assessed on transferred OT-I T cells in skin. *: p<0.05, ****: p<0.0001.

significant decrease in CD103 expression on naïve CD8$^+$ T cells (**Figure 2.14B**) as compared to control BMCs. Accordingly, upon transfer and incubation of naïve OT-I into the β2m$^{-/-}$:αV-ΔDC BMCs, their capacity to form eT$_{RM}$ in the skin upon vaccination was impaired (**Figure 2.14C**), as we observed in αV-ΔDC mice. Therefore, αVβ8-expressing DCs in LNs discretely present active TGF-β to naïve CD8$^+$ T cells during MHC-I dependent interactions during homeostatic conditioning for eT$_{RM}$ formation.

Migratory DC preferentially condition naïve T cells with lowest self-reactivity

Considering that non-cognate T-DC interactions drive the discrete presentation of TGF-β during conditioning, we wondered whether there was a particular subset of naïve CD8$^+$ T cells that may be selectively conditioned. While prior studies indicate that there is some predicted overlap of clones between the most abundant T$_{RM}$ in the skin and those memory T cells circulating through lymph nodes[109], there is an even greater proportion of clones that are unique to each tissue site. Our study suggests not only that homeostatic conditioning may not impact circulating memory formation, but also that cells conditioned for eT$_{RM}$ fate do not necessarily lose the capacity to become circulating memory subsets. This would explain the observed overlap of some abundant clones between lymph node ("T$_{CM}$") and skin memory T cells in this study ("T$_{RM}$"). However, upon our own analysis of this TCR sequencing data[109], we found less than 1% overlap of unique TCR clones between the "T$_{CM}$" and "T$_{RM}$" (**Figure 2.15A**), and a nearly 20-fold increase in TCR diversity found in the "T$_{CM}$" compartment as compared to the "T$_{RM}$" compartment (**Figure 2.15A**). This would suggest that not all naïve T cells may have equal propensity to differentiate into T$_{RM}$. Therefore, we hypothesized that conditioning may already help to "select" for clones that are better equipped for long-term residence in the tissue. Curiously, we tend to observe bimodal expression of the conditioning marker CD103 on the population of naïve CD8$^+$ T cells in the SLO ranging over more than an order of magnitude,

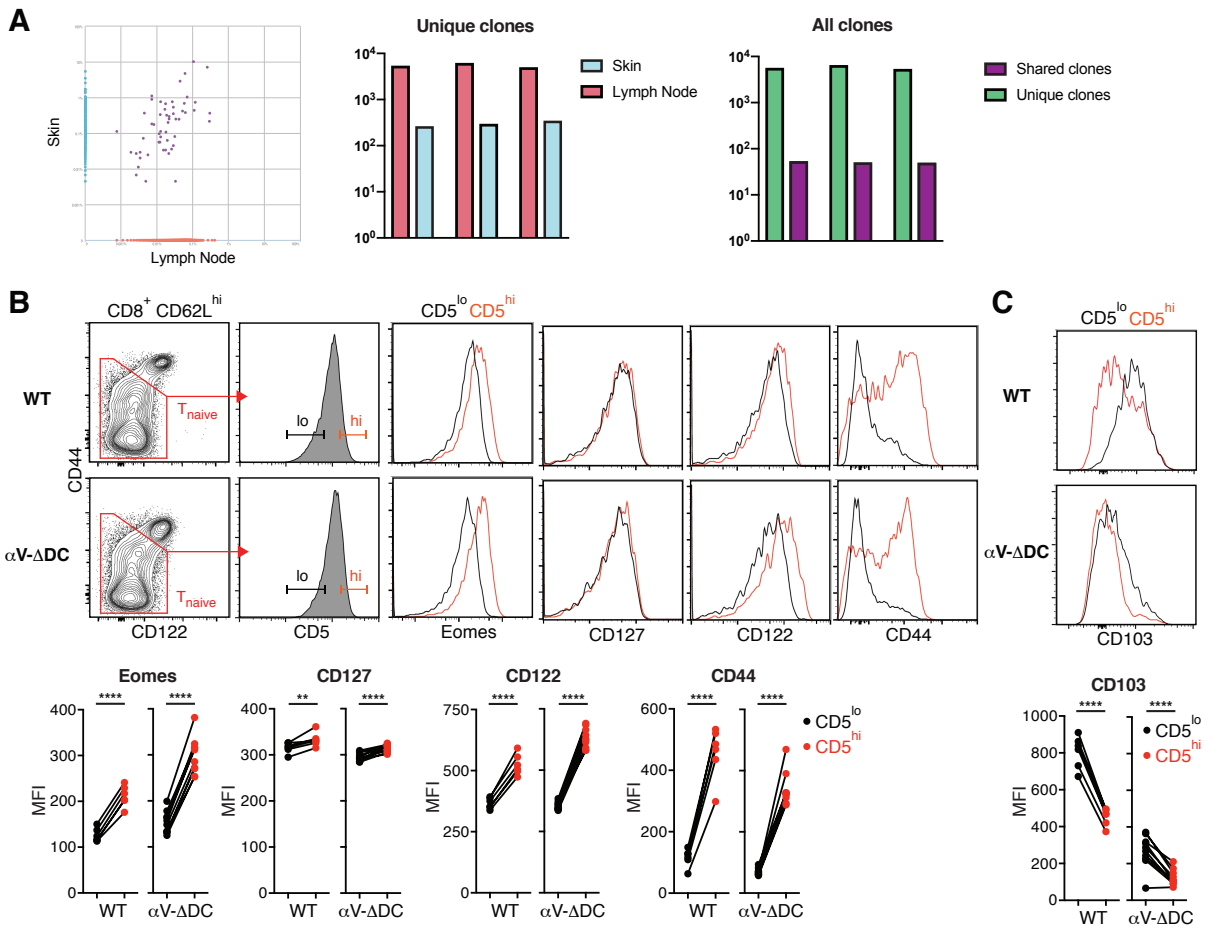


Figure 2.15 CD5^{lo} cells are preferentially conditioned for eT$_{RM}$ formation. (**A**) TCR sequencing from Gaide et. al. Nat. Med. 2016, from mice treated with DNFB. T cells were sorted from the ear skin and draining lymph nodes and overlap between DNA rearrangements is presented. (**B**) Expression of indicated markers on the CD5^{hi} and CD5^{lo} subsets of CD62L^{hi} CD122^{lo} CD44$^{lo/int}$ naive CD8^{+} T cells from peripheral blood of αV-ΔDC and WT littermate control mice. (**C**) Expression of CD103 on the same populations as shown in B. Each experiment shown was performed at least twice with similar results. **: p<0.01, ****: p<0.0001, n.s.: not significant.

suggesting that not all naïve T cells are efficiently conditioned. We wondered what type of existing heterogeneity in the naïve T cell pool would potentially contribute to certain clones being more selectively conditioned for eT_{RM} formation.

While TGF-β is thought to restrain TCR activation[163], strong TCR or co-stimulatory signaling can also restrain TGF-β signaling, for example, via ERK-mediated inhibitory phosphorylation of Smads that mediate TGF-β effects on gene expression[198]. As TGF-β-dependent conditioning of naïve T cells occurs in the context of self-peptide-MHC-I dependent interactions with DC, we hypothesized that the population of cells selected for conditioning may be predicated on varying levels of self-reactivity. Expression of the phosphatase CD5 is a well-validated indicator of the strength of TCR binding to self-antigen. Previous studies[97] have described that a more strongly self-reactive $CD5^{hi}$ naïve $CD8^+$ T cell population, representing roughly the top 20% of the CD5 distribution, has elevated levels of T-box transcription factors, reliance on IL-15 due to increased CD122, and is seen to be the population of cells that is more skewed for early effector expansion upon infectious challenge[97,98]. While the $CD5^{hi}$ population has been well-characterized in these studies, little is known about the lowest 20% of CD5 expressing cells ($CD5^{lo}$). As higher self-reactivity indicates stronger TCR signaling during homeostatic interactions, and is therefore more likely to inhibit TGF-β-dependent transcription[198], we thought perhaps that the $CD5^{lo}$ cells may be preferentially conditioned for eT_{RM} differentiation. Indeed, upon characterization of naïve $CD8^+$ T cells in WT mice, we found that while the $CD5^{hi}$ cells had increased expression of T-bet, Eomes, and CD122, they fell under the spectrum of $CD103^{neg}$ cells, while CD5lo cells preferentially expressed CD103 (**Figure 2.15B-C**). When we examined αV-ΔDC mice, we still observed a distinction in self-reactivity between $CD5^{hi}$ and $CD5^{lo}$ cells based on other markers, but as expected, the $CD5^{lo}$ failed to express the conditioning marker CD103 (**Figure 2.15B-C**). Therefore, the MHC-dependent

interactions of migratory DC with naïve $CD8^+$ T cells facilitate the selective conditioning of lower self-reactive $CD8^+$ T cells for eT_{RM} formation.

DISCUSSION

We demonstrate, for the first time, that homeostatic events prior to priming can influence the differentiation of T cells by modifying their epigenetic state. The lasting impact of an epigenetic state on the differentiation and function of T cells is becoming increasingly evident. T cells in aging or even those that are exhausted, for example, have a distinct chromatin landscape that is thought to translate into impaired downstream responsiveness and functionality[199-201]. Changes occurring in "stem-like" cells, such as naïve T cells, are therefore particularly impactful especially when downstream differentiation processes have a tightly controlled window for development. Intravital imaging of events during T cell activation in lymph nodes have demonstrated extremely tight architectural and spatiotemporal control of the differentiation of T cell subsets upon activation[4,202-204]. During the differentiation of eT_{RM}, the timing of this process becomes even more critical, as the "window of opportunity" is not only sensitive to the generation of precursors in secondary lymphoid organs, but also the inflammation in the tissue that allows for the precursors to be seeded in the epithelium. This may also explain why we observe a pronounced defect in epithelial tissue resident memory cells, but no significant change in the circulating memory pools in αV-ΔDC mice.

Our data also reveal a previously unappreciated division of labor between the secondary lymphoid organs, as lymph nodes are the site at which the pre-immune repertoire for barrier adaptive immunity is selectively instructed. Lymph nodes, often likened to the splenic white pulp, are an evolutionary adaptation in terrestrial vertebrates such as mammals and even some birds[205]. While the white pulp of the spleen and even some mucosal-associated lymphoid tissue-like (MALT) structures even in amphibians and reptiles are thought to be similar to lymph nodes

in their structure and support of adaptive immune responses, their ontogeny is quite distinct, coincident with the evolutionary acquisition of TNF family genes, and may serve somewhat different functions, as we show here. Mechanisms underlying immunosurveillance of the blood and mucosal tissues may therefore be distinct from those of organs surveying the environment that terrestrial animals are exposed to, such as the skin. An aspect of this environment are of course the microbial species that colonize these tissues, and are known to be vastly different between tissues, for example, the skin as compared to the gut. Distinct compartmentalization of adaptive immune responses to microbial species in the skin compared to those in the gut have, in fact, been previously highlighted[206].

This cross-talk with the external environment is mediated in lymph nodes by mDC, which we demonstrate are critical to condition naïve T cells for their downstream fate upon activation. While previous studies have shown a critical dependence on tissue-derived mDCs during priming for imprinting of homing capacity[101], few studies have explored the homeostatic contributions of these populations to adaptive immunity. Elegant work has demonstrated that homeostatic immunity to skin commensals impacts downstream responses to invasive species, mediated through mDCs that prime protective IL-17-skewed $CD8^+$ responses to commensals during homeostasis [47]. mDCs could therefore, even at homeostasis, provide feedback to the naïve T cell pool through sensing of the rapidly changing microbial environment in the skin [207,208], thereby promoting plasticity and diversity during homeostatic conditioning.

Our proposed mechanism of how naïve T cell conditioning impacts eT_{RM} generation may also offer an explanation for findings demonstrating that neonatal T cells have diminished capacity to become T_{RM}[209]. Recent studies describe neonatal naïve T cells as having a chromatin landscape that is skewed towards effector-like programs[98], which is reinforced by transcriptomics of effector cells from neonates revealing an enrichment for T-box-dependent

transcripts as compared to those from adult mice. We also observe hints of this signature in the ATACseq of naïve T cells in αV-ΔDC mice, where some biological processes linked to the DARs are enriched for effector programs and the open chromatin regions are enriched for T-box binding sites. From an evolutionary perspective, it is reasonable for T cells to be skewed towards an effector-like function early in life to fend off threatening infections while the immune system is still developing. However, through repeated exposure over a lifetime to barrier antigens through interactions with mDC, naïve T cells may actively acquire a state that is permissive to a more balanced and diverse phenotypic repertoire upon activation. While there is limited exploration of neonatal DC homeostasis, studies of neonatal responses to RSV indicate severely limited DC migration to the draining LNs [210]- a defect that is seen to be rescued rapidly with age or by enhanced stimulation of neonatal DC with TLR agonists. Rigorous studies of human DC also suggest an increase in DC migration from tissues to draining LNs with age[211]. It is therefore conceivable that neonatal T cells may lack the capacity to form T_{RM} due to lack of sufficient interactions at steady state with migratory DC that would adequately mold their epigenetic state.

We also show that the expression of our conditioning marker CD103 is actively maintained by homeostatic TGF-β signaling in the lymph node, and not simply a residual marker of recent thymic emigrants. We find that the bimodal expression pattern maintained in the lymph nodes by migratory DC facilitates the selective conditioning of naïve $CD8^+$ T cells with lower self-reactivity based on self-peptide-MHC dependent interactions. Elegant studies of human T_{RM} indicate an increase in their tissue seeding over a lifetime[85]. While the concern over self-reactivity in the $CD8^+$ memory pool is mitigated by other tolerance mechanisms, such as by existing thymic-derived T_{reg} populations and the generation of peripheral T_{reg} populations. Interestingly, conditioning of $CD8^+$ T cells for eT_{RM} formation is driven by the same DC

population that drives peripheral T_{reg} generation[194]. During aging, it is thought that the number of induced T_{reg} actually decreases[85,89,212] and that there is a higher degree of inflammation in the aged milieu. Therefore, a mechanism to limit self-reactivity that is intrinsic to $CD8^+$ T cells, in order to limit the risk of autoimmunity, is plausible. Further studies characterizing the contributions of $CD5^{hi}$ and $CD5^{lo}$ cells to pools of eT_{RM}, and any potential skewing of these in humans with eT_{RM} mediated pathologies such as vitiligo and psoriasis, would help inform vaccines that select effective clones for eT_{RM} formation that limit autoimmune side-effects. Future efforts to dissect these naïve populations by single cell characterization in combination with fate-mapping studies will be critical to gain further understanding of any clonal or phenotypic "selection" of cells for long-term fitness in various lymphoid or non-lymphoid tissues.

Here, we have reported a novel mechanism by which the pre-immune T cell repertoire is poised for downstream eT_{RM} differentiation via homeostatic interactions with dendritic cells in lymph nodes. Our findings uncover an unexpected role for TGF-β signaling during homeostasis which may have significant consequences for boosting or targeting protective immunity at barrier sites.

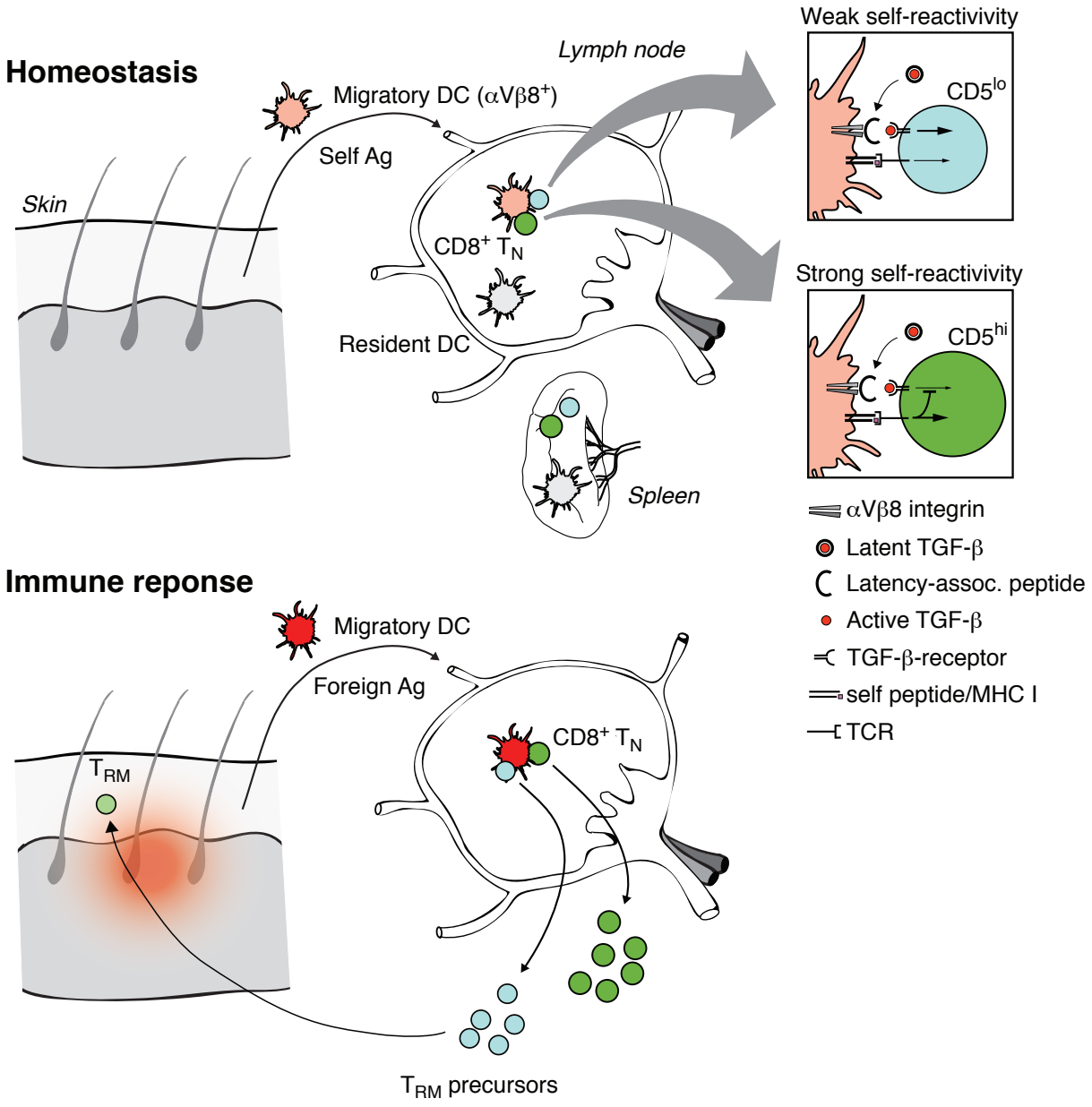


Figure 2.16 Graphical summary of proposed mechanism of naïve CD8$^+$ T cell conditioning for eT$_{RM}$ formation.

MATERIALS AND METHODS

Mice

Mice with floxed αV alleles were previously described[213] and crossed to CD11c^{Cre} BAC-transgenic mice[188] obtained from The Jackson Laboratory. C57BL/6, CD45.1 or Thy1.1 congenic C57BL/6, OT-I x TCRα$^{-/-}$, β2m$^{-/-}$, CCR7$^{-/-}$, and LTα$^{-/-}$ mice were purchased from The Jackson Laboratory and further bred in-house. Animals were housed in specific pathogen-free facilities at the Massachusetts General Hospital (MGH) and all experimental studies were approved and performed in accordance with guidelines and regulations implemented by the MGH Institutional Animal Care and Use Committee (IACUC).

Irradiation bone marrow chimeras

Recipient mice were i.p. injected with 200 μg α-NK1.1 mAbs (clone PK136, BioXCell) one day prior to irradiation to transiently deplete NK cells and enhance engraftment of β2m$^{-/-}$ bone marrow. Bone marrow was dislodged from femurs and tibiae of donor mice by flushing with 10 mL PBS w/o Ca^{2+}/Mg^{2+}, and filtered once through a 40 μM filter. Cells from either WT or αV-ΔDC mice were mixed at a 1:1 ratio with cells from β2m$^{-/-}$ mice and re-suspended at 50 x 10^6 total cells/mL. Recipients were irradiated at 1000 rad (Cesium) in a rotating chamber and injected retro-orbitally with 5 x 10^6 total donor bone marrow cells/mouse in 100 μL of PBS w/o Ca^{2+}/Mg^{2+}. Mice were given Sulfamethoxazole in drinking water for 4 weeks post-irradiation.

DNA Vaccination, mechanical skin irritation, DNFB treatment

Mice were anesthetized using isofluorane, ear or flank skin was shaved and epilated through treatment with Nair hair removal cream, and a droplet of H_20 containing 3 μg of ovalbumin (OVA)-expressing plasmid DNA was tattooed into the skin using a sterile disposable 11-needle bar mounted on a rotary tattoo device, as previously described.[74] Plasmids expressing OVA

under control of a modified CMV or ubiquitin promoter were kindly provided by Dr. J. J. Moon (unpublished data). To create a transient inflammatory reaction through mechanical skin irritation only, plasmid DNA was omitted from this procedure. Alternatively, mice were treated with 10 μl of 0.5% DNFB in 4:1 acetone:olive oil emulsion to inflame ear skin.

Prime and pull immune challenge

The attenuated OVA-expressing Listeria monocytogenes strain L.m.-OVA ΔactA was provided by Dr. James J. Moon (MGH), and was grown in brain heart infusion (BHI) medium containing 34 μg/mL of chloramphenicol to an absorbance of ~0.1 at 600 nm. 2×10^7 colony-forming units were injected intravenously into mice to induce a systemic T_{EFF} response ('Prime'). 4 days following infection, mice ears were treated with DNFB, as described above, to produce skin inflammation ('Pull') and enable seeding by eT_{RM} from the circulating pool of T_{EFF}.

Adoptive T cell transfers, in vitro activation of T cells

Naïve $CD8^+$ T cells were purified from LN and spleen single cell suspensions by immunomagnetic negative cell selection using the Miltenyi naïve $CD8^+$ T cell isolation kit and adoptively transferred by retro-orbital injection. For *in vitro* activation of T cells, OT-I x $TCR\alpha^{-/-}$ splenocytes were pulsed with 1 μM SIINFEKL peptide in 1 mL of T cell medium (RPMI, 10% FCS, 1% HEPES, 1% Sodium Pyruvate, 1% GlutaMAX, 1% non-essential amino acids, 55 μM 2-mercaptoethanol) for 1 hr at 37°C, diluted in 9 mL of T cell medium, and cultured at 37°C in 5% CO_2. 2 days later, 20 ng/mL IL-2 was added and cell density was maintained at 10^6 cells/mL. OT-I cells were adoptively transferred on day 5 after activation by retro-orbital injection.

FTY720 treatment

Mice received intraperitoneal injections of 1 mg/kg BW FTY720 (Fingolimod) in 150 μL H_20 every 2-3 days.

Naïve T cell treatment with TGF-β1

$CD44^{lo}$ naïve $CD8^+$ T cells were purified from LNs and spleens by immunomagnetic negative cell selection using the Miltenyi naïve $CD8^+$ T cell isolation kit. Cells were cultured in 96-well flat-bottom plates at a density of 2 x 10^6 cells/mL in serum-free XVIVO10 medium supplemented with 100 ng/mL recombinant mouse IL-15 and 5 ng/mL recombinant mouse IL-7 (Biolegend). Titrated amounts of acid-activated recombinant mouse TGF-β1 (Cell Signaling) were added as indicated and cells cultured at 37°C in 5% CO_2 for 72 hours before analysis of CD103 surface expression.

Isolation of cells from tissues and staining for flow cytometry

For cell isolations from non-lymphoid tissues, mice were i.v. injected with 3 μg of Alexa Fluor 700- or FITC-labeled α-CD45.2, or PE-Cy7-labeled α-CD8β antibody 3 min prior to euthanasia in order to label intravascular leukocytes for exclusion from analysis. All organs were harvested into ice-cold FACS buffer (PBS w/o Ca^{2+}/Mg^{2+} with 0.5% BSA and 2 mM EDTA).

Skin was processed as previously described[214]. Briefly, separated dorsal and ventral halves of ear skin or flank skin was minced into small pieces and placed in 2.5 mL digest buffer A (DMEM; 2% FBS; 1% HEPES; 25 U/mL Collagenase IV) or digest buffer B (DMEM; 2% FBS; 1% HEPES; 125 μg/mL Liberase TM; 0.5 mg/mL Hyaluronidase Type I-S from Bovine Testes) for 1 hr at 37°C under agitation, then quenched with 10% FBS and 1.5 mM EDTA and blended using a gentleMACS tissue blender (Miltenyi, C tubes, m_impTumor_01 protocol).

Small intestine was flushed free from feces, opened longitudinally, Peyer's patches were carefully removed, and the remaining tissue cut into pieces that were incubated in RPMI 5%

FCS, 1.5 mM EDTA, 1 mM DTT for 20 min on a nutating mixer at 37°C to separate intraepithelial lymphocytes (IEL) from lamina propria (LP). The LP was then further digested in RPMI 5% FCS, 1 mg/mL Collagenase IV for 30-45 min on a nutating mixer at 37°C. Leukocytes were enriched from both compartments using a Percoll gradient.

Spleen, thymus, and LNs were minced, passed through a 40 μm cell strainer, and red blood cells lysed with ACK lysis buffer as necessary. For isolation of dendritic cells, minced spleen and LNs were digested in digest buffer A for 20 min under agitation at 37°C and before passing through a 40 μm cell strainer.

Cell surface epitopes were stained in FACS buffer (PBS, 0.5-1% BSA or 5-10% FBS) in the dark at 4 °C for 15 min, followed by staining with fixable viability dye (Zombie Dyes, Biolegend) at room temperature for 15 min. For detection of intracellular epitopes, cells were fixed and permeabilized (eBioscience Fixation/Permeabilization kit), and stained with antibodies for 30 min. in the dark at room temperature.

Histology

Mice were epilated (Nair) and excised ear skin was fixed for 15min at room temperature in 4% PFA (freshly diluted from 16%), washed 6 times for 30 min each time in PBS w/o Ca^{2+}/Mg^{2+}, and stored overnight in 30% sucrose. For the small intestine, a small portion was excised, flushed, opened longitudinally, rolled, pinned and fixed as described for skin. 18h later, tissues were immersed in OCT, snap frozen in a dry ice/2-methylbutane bath and stored at -80°C until sectioning. 10-20 μm thick frozen cross sections were air-dried at room temperature for 5min and loaded into a Shandon Immunostaining Chamber with 1mL PBS w/o Ca^{2+}/Mg^{2+}. Tissues were fixed again with 100 μl 4% PFA for 10 min, washed twice with PBS w/o Ca^{2+}/Mg^{2+}, and then washed with 200 μl 0.3% PBST. Tissues were then blocked with 100 μl blocking buffer (5%

normal donkey serum, 1% BSA, 2% cold water fish gelatin, 0.3% Triton X-100 in PBS w/o Ca^{2+}/Mg^{2+}), for 30 min at room temperature. Sections were incubated overnight at 4°C with primary antibodies (purified α-$CD8^{+}$α clone 53-6.7 and α-CD3 clone 145-2C11, diluted 1:100 in blocking buffer, 100 µl total per slide), washed with 1 mL 0.3% PBST, and secondary antibodies (donkey anti-rat Cy3 and goat anti-hamster Alexa Fluor 647, Jackson ImmunoResearch, diluted 1:200 from stock in blocking buffer) were added for 1 hr at room temperature. Slides were washed with 1 ml 0.3% PBST, then 2 ml PBS w/o Ca^{2+}/Mg^{2+}, mounted with 100 µl DAPI fluoromount (Southern Biotech), and cured overnight in the dark at room temperature. Images were acquired on a Zeiss LSM800 confocal microscope and processed using Imaris Software (Bitplane) and ImageJ software (NIH).

Density of immune cells in skin was determined by manual counting and extrapolation of the number of cells per cm^2 skin surface based on the number of cells per section and the skin surface represented by the section (10 µm thickness x measured length of the epithelium in the field of view). Non-consecutive sections were analyzed for quantification to avoid overlapping of cells between sections.

Preparation of genomic material and ATAC-seq data analysis

30,000 $CD44^{low}$ naïve $CD8^{+}$ T cells per replicate from LNs were sorted by FACS into PBS containing 10% FBS in DNA loBind Eppendorf tubes. Pelleted cells were lysed in 50 µl of reaction mix (25 µl of 2 x TD, 2.5 µl of Tn5 enzyme, 0.25 µl of 2% digitonin, 22.25 µl of nuclease-free water). The mix was incubated at 37°C for 30 minutes with agitation at 300 rpm. DNA was purified using a QIAgen MinElute Reaction Cleanup kit and Nextera sequencing primers ligated using PCR amplification. Agencourt AMPure XP bead cleanup (Beckman Coulter/Agencourt) was used post-PCR and library quality was verified using a Tapestation

machine. Samples were sequenced on an Illumina HiSeq 2000 sequencer using paired-end 5' bp reads.

For analysis of sequences, first, adapters were trimmed using AdapterRemoval (v. 2.2.1a) (*29*). Second, the paired-end ATAC-seq were aligned to the mouse reference genome (mm10) using Bowtie2 (2.3.4.1) (*30*) with the following parameters --no-discordant --no-unal --no-mixed -X 2000. Third, the reads were shifted (Tn5 insertion) after removing the reads from mitochondrial DNA and duplicated reads.

ATAC-seq peak regions of the pooled sample were called using HOMER (v4.9.1) (*31*) with the following parameters -region -size 500 -minDist 50 -o auto -tbp 0. Then, the fragment counts for each peak region for each sample were obtained using bedtools (v2.24.0) (*32*). Differentially accessible peak regions between conditions using the two replicates per condition (adjusted p-value ≤ 1e-5) were called using DESeq (v1.30.0) (*33*) using the “pooled” dispersion estimation with the “local” fit. Then, overlapping differentially accessible peak regions (adjusted p-value ≤ 1e-5) per condition were merged using bedtools. For each merged peak region, we associate adjusted p and $\log_2$ fold-change values using the adjusted p and $\log_2$ fold-change values of the region of smallest adjusted p-value (among the regions prior to merging).

ATAC-seq coverage tracks were generated using deepTools (v.3.0.2) (*34*). The size factors estimated by DESeq were used to normalize ATAC-seq coverage tracks.

HOMER was used to annotate the merged differentially accessible regions per condition and to calculate distances from DARs to the closest transcription start sites in order to generate the heatmap. Additionally, HOMER was used to find enriched motifs in the merged differentially accessible regions of each condition with the following parameters (-size given -mask -nomotif) and using the default motif collection (364 motifs). To generate Appendix A3 we used the motif

family information included in the HOMER motif database and discarded the families with the "?" symbol in their identifier. Moreover, we separated the KLF motifs from the Zf family: the KLF family contains all the KLF motifs and the Zf (others) family contains all other Zf motifs.

The Genomic Regions Enrichment of Annotations Tool (GREAT) was used for gene ontology and molecular signature enrichment analysis (*35*). DARs were inputted into the GREAT and gene associations were defined by "basal plus extension" (basal as 5kb upstream of and 1kb downstream from the TSS, and extension to estimate gene regulatory regions 1Mb upstream and downstream of the TSS.)

Reverse transcription qPCR

2 x 10^5 dendritic cells each of indicated subsets were purified by FACS into TriZOL for RNA extraction and RNA was further purified using RNeasy Plus Micro or Mini Kit (Qiagen). RNA was reverse transcribed using a High Capacity cDNA Transcription Kit (Life Technologies) and RT-qPCR was performed using SYBR Green detection (Roche LightCycler 480 kit). Primer sequences used for amplification are as follows: αV integrin (Forward: CGGGTCCCGAGGGAAGTTA, Reverse: TGGATGAGCATTCACATTTGAG) and β8 integrin (Forward: AGTGAACACAATAGATGTGGCTC, Reverse: TTCCTGATCCACCTGAAACAAAA).

Statistical analysis

Two-tailed, paired or unpaired student's t-test (for normally distributed data) or Mann-Whitney test (for not normally distributed data) for was used for comparisons between two groups. The two-sample Kolmogorov-Smirnov was used to compare cumulative distributions. All statistical tests were performed with GraphPad Prism software, and $p < 0.05$ was considered statistically significant.

CHAPTER 3: TGF–β regulates the epidermal migration and lodgment of $CD8^+$ eT_{RM}

Contributors: Vinidhra Mani, Esteban Carrizosa, Debattama Sen, Thorsten R. Mempel

ABSTRACT

Tissue resident memory T cells represent a diverse subset of memory T cells residing long-term in non-lymphoid tissues such as the skin, gut and brain, providing compartmentalized antigen-specific protection against barrier invasion. Within these tissues, resident memory cells can further organize into those residing in epithelia and those found in the sub-epithelial layer, each subsisting on distinct survival factors[39] and exposed to a discrete set of antigens[47,48]. Epithelial T_{RM} (eT_{RM}), in particular, play a dominant role in protecting against barrier-disrupting pathogens[121]. While the importance of eT_{RM} is well-established, the determinants of their compartmentalization in epithelia have yet to be fully elucidated. Here, we sought to dissect the process by which T cells gain access to the epithelium, and therefore explored the role of critical factors in their epithelial lodgment, such as TGF-β. Through longitudinal multiphoton intravital microscopy (MP-IVM) of the ear skin, we find tight spatiotemporal regulation of T cell entry into the epidermis for terminal eT_{RM} differentiation. During epidermal migration, T cells initially cluster around hair follicles, the portals by which they eventually gain access to the epidermis, in a process strictly dependent on TGF-β. In the absence of TGF-β signaling in the skin, T cells fail to properly localize around hair follicles to gain epidermal access, as well as fail to be retained in the skin. Intriguingly, CD103, a well-known TGF-β target gene, was found to be dispensable for both the epidermal access of T cells as well as their long-term lodgment. Upon whole transcriptome analysis, we observed that TGF-β was regulating trafficking and localization-associated genes such as Klf2 and various chemokine receptors. While the epidermal entry and

lodgment of T cells was independent of sphingosine-1-phoshpate signaling, we uncovered a novel role for TGF-β in regulating the balance of chemokine receptors CCR6 and CCR2. We find that CCR6 is essential for the differentiation of eT_{RM}, attracting T cells to the infundibulum of the hair follicle, where they are able to access the epidermis. Thus, TGF-β is a multi-stage regulator of eT_{RM} compartmentalization and differentiation.

INTRODUCTION

Tissue resident memory T cells occupying distinct tissue sites have varying transcriptional profiles[41,42] that are thought to support their survival and function in the barrier tissue of their residence[119,121]. While there are shared signatures between all tissue resident lymphocytes, ranging from natural killer T cells and $CD8^+$ T tissue resident memory (T_{RM}) cells, the heterogeneity within each pool is gaining increased appreciation, revealing distinct functions in maintaining barrier function in both lymphoid and non-lymphoid tissues[38,39].

Even within a given tissue, T_{RM} can differentiate and reside in various sub-compartments, for instance in the sinusoids of the liver[53], or in the sub-epithelial and epithelial layers of other barrier tissues[85,215]. The T_{RM} in each of these compartments are distinct from one another not only in surface phenotype, but also cytokine production[47,49], metabolism[37,119] and other functionality. Epithelial tissue resident memory cells (eT_{RM}), in particular, are stationed at the cusp of host-pathogen interfaces, where they are constantly in contact with microbial species and serve as a first line of defense. eT_{RM} in the skin have been shown to be the dominant player during rapid recall responses to pathogens such as herpes simplex virus-1[121]. In addition to their long-term compartmentalization in the epithelium, eT_{RM} in most tissues can be distinguished by a surface phenotype consisting of dual expression of integrin αE (CD103) and CD69, both of which are thought to facilitate their maintenance in the tissue. While the functional importance of eT_{RM} is well-established, how they gain their distinct residence and phenotype in the epithelium is relatively unexplored.

As T cells enter the tissue after activation in the secondary lymphoid organs, they are exposed to a discrete cytokine milieu that is thought to facilitate their lodgment[116] and long-term survival[119] in the tissue. The differentiation process of eT_{RM} in particular is thought to rely on a number of different cytokine cues, including TGF-β. TGF-β is a critical cytokine that is known to

regulate a number of different steps in T_{RM} differentiation, such as the up-regulation of signature integrin αE (CD103)[40,130], which is thought to allow for interactions of eT_{RM} with E-cadherin-expressing epithelial cells to maintain their epithelial residence. Prior to T cell infiltration of non-lymphoid tissues, TGF-β is thought to influence the ratio of short lived effector cells and memory precursor cells as well as their homing potential to non-lymphoid tissues[130,131]. After T cells enter the skin, TGF-β is also known to regulate the balance of transcription factors such as T-bet and Eomes[121,124], which may play a role in their epithelial program. However, given the complexity of TGF-β signaling and its known intersection with many other pathways that regulate immune cell differentiation, is it unclear in what spatiotemporal context TGF-β may regulate the differentiation of eT_{RM}, and if this dependence on the cytokine also regulates the access of T cells to the epithelium to begin with, or simply influences other processes critical to their long-term residence.

Here, we first sought to understand the process by which $CD8^+$ T cells enter the epidermis during epithelial tissue resident memory (eT_{RM}) differentiation in the skin. Through longitudinal intravital microscopy studies, we find that upon entering the skin, $CD8^+$ T cells begin to accumulate around hair follicles, which serve as their entry point into the epithelium. In the absence of TGF-β signaling, skin-infiltrating T cells are unable to adequately position themselves around the perifollicular dermis for entry into epidermis. We find that TGF-β-dependent epidermal entry of $CD8^+$ T cells is independent of the S1P signaling axis and signature integrin CD103. Instead, we uncovered a novel role for TGF-β in the regulation of chemokine receptor CCR6 that facilitates the proper spatial organization of T cells for epidermal access and subsequent eT_{RM} terminal differentiation.

RESULTS

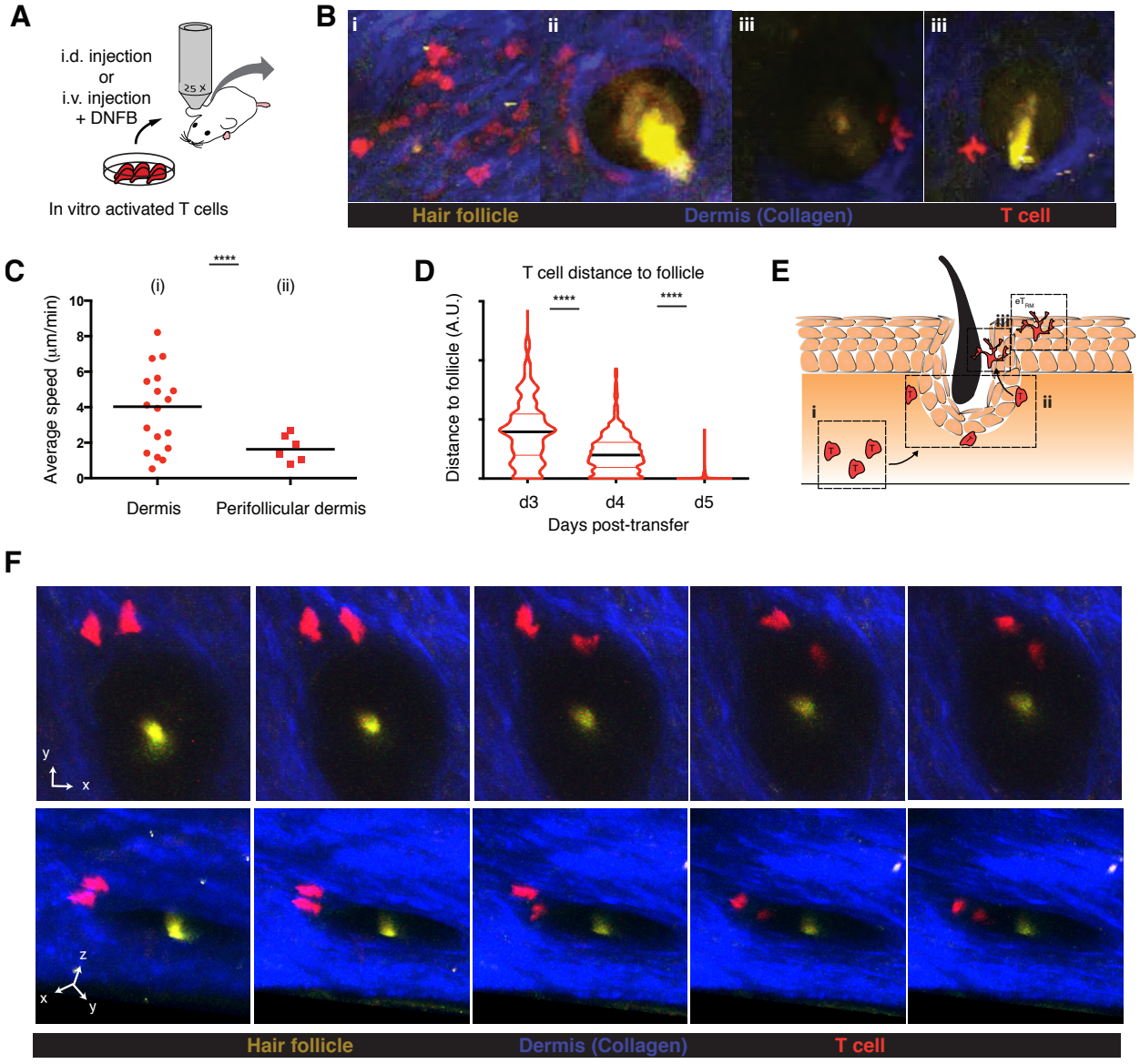
CD8$^+$ T cell migration into the epidermis during eT$_{RM}$ differentiation occurs in distinct stages via hair follicles

Most imaging-based studies of tissue resident memory cells have focused on their dynamics and localization in a homeostatic memory phase or during an antigen recall response [72-76,216]. These studies have described the physical attributes and limited homeostatic migration of CD8$^+$ T cells within the epithelium. Upon antigen encounter, eT$_{RM}$ are seen to become more motile, clustering near sites of invasion, and recruiting new T cells to expand the niche[75,76]. While the exploration of eT$_{RM}$ dynamics during the memory phase have yielded critical information on the behavior of these cells, how T cells make their way into the epithelium to begin with, whether in a stochastic or carefully guided fashion, is still unexplored. Thus, we set out to visualize the initial phases of eT$_{RM}$ differentiation that culminate in their epithelial seeding.

As the ear skin is a very accessible tissue for non-invasive longitudinal intravital imaging, we developed a setup where we would be able to visualize T cells in the ear skin during the process of eT$_{RM}$ differentiation. To this end, we transduced *in vitro* activated CD8$^+$ T cells with retrovirus encoding fluorescent protein tdTomato, and adoptively transferred them 4 days post-activation into mice either by direct intradermal injection into the ear skin[40] or by intravenous injection along with sensitizer 2,4-dinitrofluorobenzene (DNFB)-based inflammation of the ear skin to "pull"[106] the activated T cells into the skin for differentiation (**Figure 3.1A**). By multiphoton intravital microscopy (MP-IVM), we visualized the cells daily through the course of their epithelial migration. Within a few days post-transfer of T cells, we began to observe an accumulation of CD8$^+$ T cells around the hair follicles in the skin (**Figure 3.1B, D, E, Supplementary Video 3.1-3.2**). As cells approached the follicle, a number of them slowed their migration in the perifollicular area of the dermis (**Figure 3.1C, Supplementary Video 3.2**), prior

Figure 3.1 CD8$^+$ T cells enter the epidermis via hair follicles in distinct stages. (**A**) *Ex vivo* activated OT-I T cells were transduced with tdTomato (red) retrovirus and 10^5 cells were intradermally injected into the ear skin of C57BL/6$^{Tyrc\text{-}2J}$ for longitudinal imaging of eT_{RM} differentiation. Alternatively, 10^6 *ex vivo* activated OT-I transduced with tdTomato retrovirus were injected intravenously and cells were pulled into the ear skin by treatment with 2,4-dinitrofluorobenzene (DNFB). (**B**) Maximum intensity projections of cells visualized in the interfollicular dermis (i), perifollicular dermis (ii) and in the hair follicle following epidermal entry (iii). (**C**) Average speed of tracks of cells in the dermis (B, i) or perifollicular dermis (B, ii). Each point represents one cell in the selected field of view over time. (**D**) Distance to nearest hair follicles for any cell at a given time point, pooled, post-intradermal injection of cells, within one representative mouse. Black lines denote median. (**E**) Schematic of distribution presented in (B). (**F**) Progression of adoptively transferred T cells entering the epidermis via the hair follicle, 5 days following intradermal injection into the ear skin, as a maximum intensity projection viewed from the top of the skin (top panel) or the side (bottom panel). Each experiment shown was performed at least twice with similar results.

Figure 3.1 (Continued)



to entering the epidermis via the follicular epithelium (**Figure 3.1F, Supplementary Video 3.4-3.5**). 10-14 days post-transfer of cells, we observed very few T cells entering the follicle, suggesting a discrete window of epithelial access. This observed sequence of events hinted that epidermal migration of $CD8^+$ T cells is a guided process that may rely on intricate signaling events initiating in the sub-epithelial layer of the skin.

Factors regulating local eT_{RM} differentiation

As there looked to be a limited window of epithelial access for T cells, we sought to identify factors in the skin post-inflammation that may orchestrate the migration of T cells. We therefore inflamed the ear skin and screened for transcripts by RT-PCR in the whole skin in a time-course post-inflammation. While some inflammation-associated genes were, unsurprisingly, up-regulated (**Figure 3.2A**), we observed an up-regulation of TGF-β signaling associated genes shortly after inflammation – at time points around where T cells would enter the epidermis – (**Figure 3.2B-C**) including activating integrin chains such as *Itgav, Itgb6, Itgb8* as well as signatures of acute TGF-β exposure, such as the transcriptional activation of negative regulator *Smad7*. While this was a picture of the whole skin and not specifically T cells, given the known impacts of TGF-β on T_{RM} differentiation, we set out to test if and how T cell-intrinsic TGF-β signaling in the skin may impact the migration of T cells into the epidermis.

Model system for studying the role of effector-site TGF-β signaling during T_{RM} differentiation

Existing studies characterizing the contributions of TGF-β signaling to T_{RM} differentiation have been mostly restricted to the use of non-inducible models, such as the TGF-β$RII^{fl/fl}$ TCR transgenic mice crossed to mice expressing Cre driven by the distal Lck promoter[133], which is active in all T cells after thymic development[217]. In these animals, T cell responsiveness to the cytokine is disrupted even during homeostasis and activation, possibly regulating events prior to

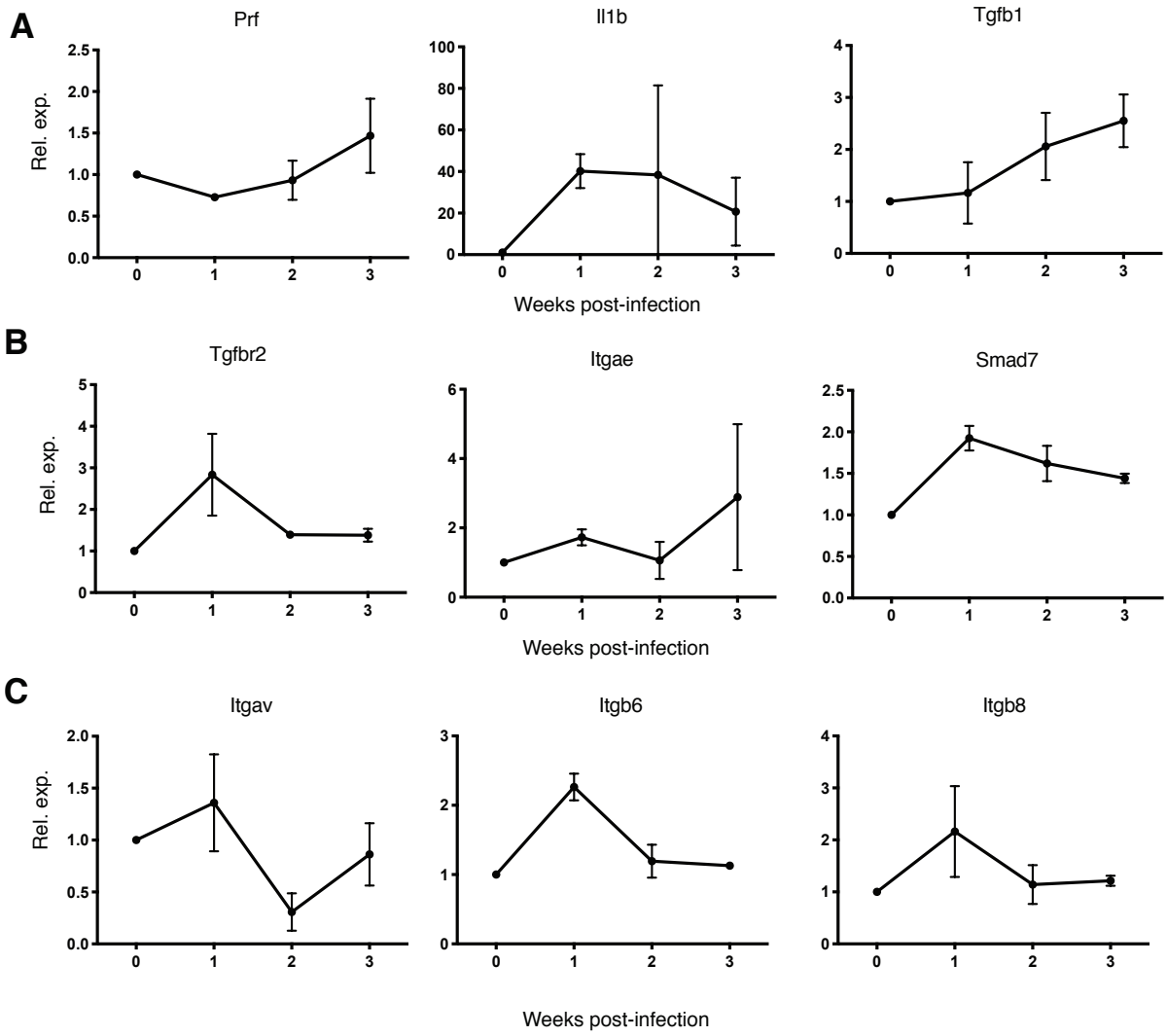


Figure 3.2 Inflammation and TGF-β associated genes are up-regulated following skin infection. Real-time PCR of transcripts from the whole skin following the indicated time point post-infection with 10^6 p.f.u. HSV-1 by skin scarification. Transcripts associated with (**A**) inflammation and wound healing, (**B**) active TGF-β signaling, and (**C**) TGF-β activation. Each experiment shown was performed at least twice with similar results.

the entry of T cells into the skin during inflammation, such as expression of homing and tissue entry receptors[130,189,218], as well as the ratio of short lived effector cells to memory precursors, as defined by KLRG1 expression[99,131]. While this model has provided great insights into TGF-β's role in regulating integrin αE (CD103) [40,130] and tissue homing capacities[130] of T cells, the limitations of this model keep us from strictly resolving the contributions of any key TGF-β-associated signaling events that occur in the effector site. Therefore, we wanted to develop a system where we would be able to selectively investigate the role of TGF-β at the effector site during terminal differentiation. To this end, we generated a strain of mice by crossing Rosa26-Cre-ER^{T2} to TCR transgenic (OT-I) TGF-βRII$^{fl/fl}$, a setting in which we would be able to induce deletion of the receptor upon treatment of mice or cells with tamoxifen or its metabolite 4-hydroxytamoxifen (4-OHT), inducing Cre recombinase-mediated deletion of TGF-βRII.

To test our system, we activated OT-I T cells *in vitro* by pulsing splenocytes from these animals with SIINFEKL peptide and subsequently deleted the receptor by addition of 4-OHT to the cultures two days post-activation. In a dose titration time-course, we found that at 64nM of 4-OHT in culture, beginning 2 days post-activation, and washed out after 24 hours to limit 4-OHT associated toxicity, yields near-complete deletion of the receptor in all cells within one day and complete deletion in all cells by two days post-treatment, as determined by genomic PCR of the TGF-βRII locus (**Figure 3.3A**). We also verified reduction of the protein on the cell surface, as well as non-responsiveness to TGF-β *in vitro* by surface expression of TGF-β target CD103 after exposure to the cytokine in culture (**Figure 3.3B-C**). Upon T cell activation, TGF-βRII is known to be downregulated from the surface of cells and reported to reappear around 5 days after activation[99]. Our Cre-mediated deletion of the receptor therefore fits within these kinetics, where, by the time the receptor is re-expressed, the gene has already been deleted. Thus, the cells are activated under TGF-β sufficient conditions, and rendered unresponsive to the cytokine

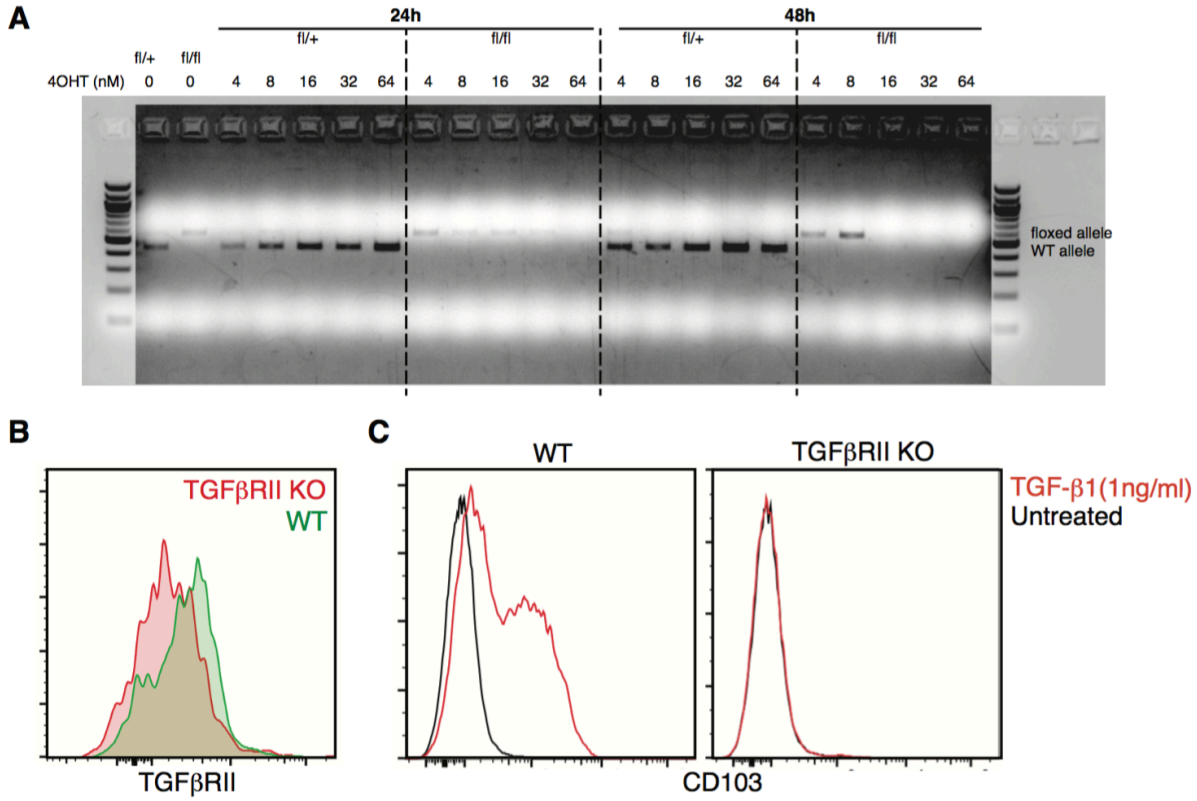


Figure 3.3 Characterization of TGF-βRII deletion in Rosa26-Cre-ER^{T2} x TGF-βRII$^{fl/fl}$ mice. (**A**) Activated CD8^{+} T cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-βRII$^{fl/fl\ or\ fl/+}$ mice were treated *in vitro* with 4-hydroxytamoxifen (4-OHT) at indicated doses beginning at two days post-activation of T cells, in T cell medium containing 20ng/mL IL-2. 4-OHT was washed out from the culture medium at 24 hours post-treatment. 100K cells were harvested for genomic PCR of the TGF-βRII locus at 24 hours and 48 hours post-treatment with 4-OHT using primers indicated in the Appendix. (**B**) Flow cytometry of TGF-βRII in T cells indicated in (A) 72 hours post-treatment with 4-OHT. (**C**) Cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-βRII$^{fl/fl\ or\ fl/+}$ were stimulated with TGF-β1 72 hours post-treatment with 4-OHT to induce receptor deletion. CD103 up-regulation as an indicator of productive TGF-β signaling was assayed by flow cytometry 48 hours post-stimulation with TGF-β1. Each experiment shown was performed at least twice with similar results.

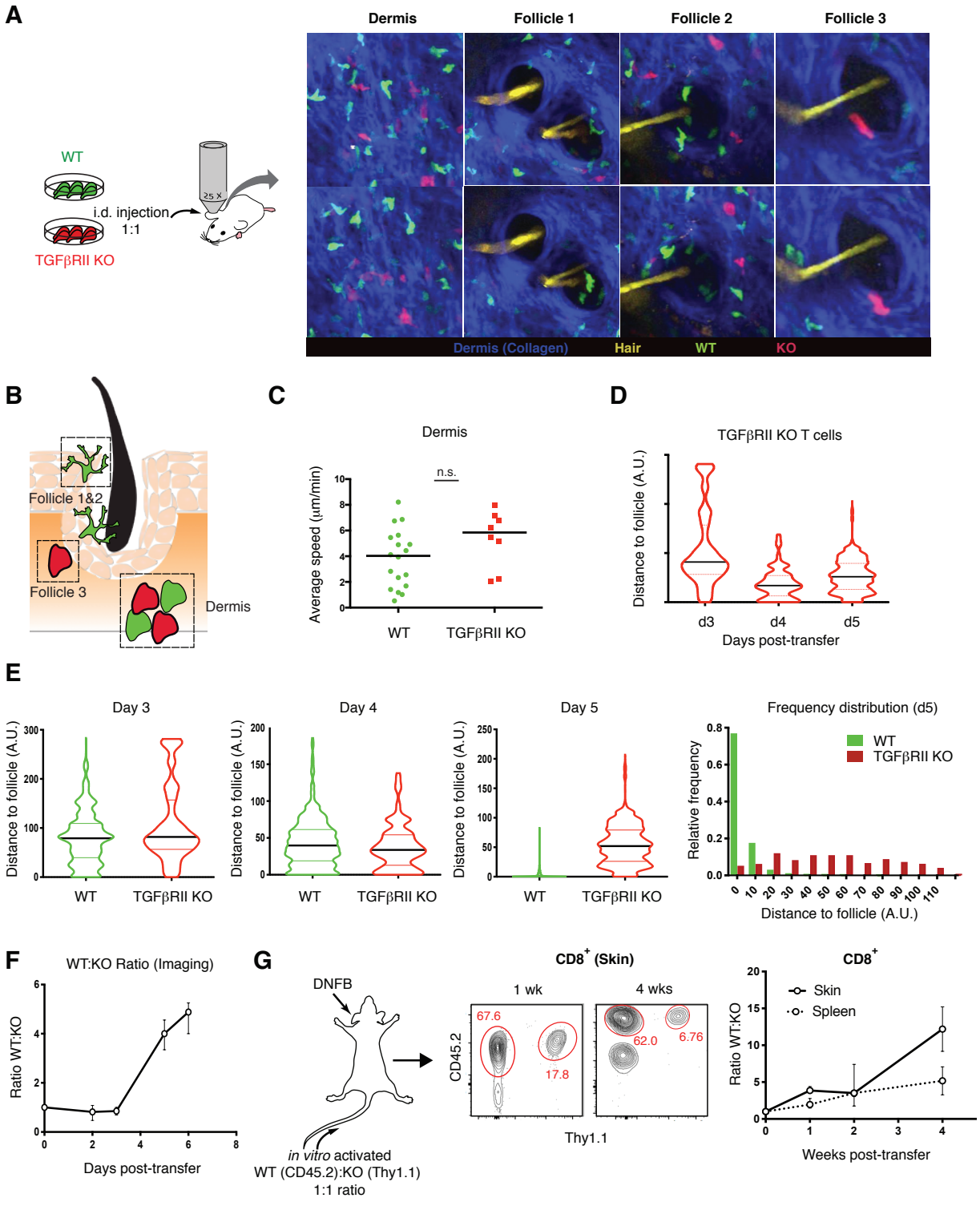
once they make their way to the effector site, upon adoptive transfer into mice 5 days after activation.

Loss of TGF-β signaling in activated T cells disrupts follicular accumulation and epidermal migration

In order to understand the spatiotemporal context of TGF-β signaling during terminal differentiation we next visualized the migratory behavior and patterns of TGF-β signaling-deficient T cells (Rosa26-Cre-ER^{T2} x TGF-βRII$^{fl/fl}$) *in vivo* alongside wild type cells (Rosa26-Cre-ER^{T2} x TGF-βRII$^{fl/+ \text{ or } +/+}$). After activation of the cells *in vitro*, we transduced the cultures with tdTomato or GFP encoding retrovirus to be able to visualize and distinguish the two populations from one another. We subsequently induced Cre activity in the cells *in vitro* by treatment with 4-OHT, and adoptively transferred in the two populations (hereafter referred to as TGF-βRII KO and WT) of cells in a 1:1 ratio five days post-activation into host mice for longitudinal imaging by MP-IVM (**Figure 3.4A**). In the first couple of days following transfer TGF-βRII KO and WT control cells into mice by intradermal injection, we observed no significant changes in their migratory behavior in the dermis of the ear skin, as measured by parameters such as migration speed of the cells (**Figure 3.4C, Supplementary Video 3.6**). However, by five days after transfer, at which time WT cells had started to preferentially localize near hair follicles, TGF-βRII KO cells remained relatively evenly distributed across both the follicular and interfollicular dermis (**Figure 3.4D-E**). We also observed divergent behavior of the WT and TGF-βRII KO cells around the hair follicles. Over time, while many WT cells traversing into the follicular epidermis, the TGF-βRII KO cells near the follicles were observed merely lingering, not slowing their migration or able to enter the epidermis. (**Supplementary Video 3.7-3.9**). These cells were then observed to migrate away from follicles after a short period of time. Additionally, while the WT and TGF-βRII KO cells were injected in a 1:1 ratio, the KO cells seemed to wane off within a

Figure 3.4 Loss of local TGF-β signaling in the skin impairs T cell epidermal entry and retention. (**A**) Activated $CD8^+$ T cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-βRII$^{fl/fl\ or\ fl/+}$ mice were treated *in vitro* with 64nM 4-OHT at 2 days post-activation, and transduced with either tdTomato (KO = fl/fl) or GFP (WT = fl/+) retrovirus. 72 hours after 4-OHT treatment (5 days post-activation), 10^5 cells each of "WT" GFP and "TGF-βRII KO" tdTomato cells were adoptively transferred at a 1:1 ratio. Images span day 3 to 5 post transfer of cells. (**B**) Schematic of distribution presented in (A). (**C**) Cells in the dermis were tracked over a 60 minute movie and average speeds were recorded. (**D**) Distance to nearest hair follicles for any cell at a given time point, pooled, post-intradermal injection of cells, within one representative mouse. Black lines denote median. (**E**) Distance to nearest hair follicles within a single representative mouse over time, comparing WT and TGF-βRII KO cells. (**F**) Ratio of WT to TGF-βRII KO cells after transfer in a 1:1 ratio as determined through an MP-IVM timecourse, using 3 representative animals. (**G**) Activated $CD8^+$ T cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-βRII$^{fl/fl\ or\ fl/+}$ mice were treated *in vitro* with 64nM 4-OHT at 2 days post-activation. 10^6 cells each of WT (CD45.2) and TGF-βRII KO (Thy1.1/1.2) were then adoptively transferred into CD45.1 hosts at a 1:1 ratio 5 days post-activation and the ear skin was treated with DNFB to pull cells into the skin. Ratios of $CD8^+$ T cells were determined by flow cytometry at indicated time points post-transfer. Each experiment was performed at least twice with similar results.

Figure 3.4 (Continued)



week post-transfer, suggesting that they were either actively migrating out of the skin to other sites or had impaired survival in the absence of TGF-β signaling in the tissue (**Figure 3.4F**). We also observed this loss of the TGF-βRII KO cells from the skin over time when we conducted a flow-cytometry based time-course (**Figure 3.4G**). Collectively, these observations led us to conclude that TGF-β signaling in the tissue site is not only critical for follicular accumulation and epidermal entry of T cells, but even the short-term retention or survival of T cells in the tissue during differentiation.

CD103 is neither necessary nor sufficient for T cell entry into the epidermis

A prevailing hypothesis in the field is that CD103, a TGF-β transcriptional target, is a critical mediator of T cell retention in the epidermis. While CD103 induction is known to occur shortly after T cell entry into the effector site[40], the compartment in which this CD103 up-regulation occurs upon exposure to TGF-β signals, whether the dermis or epidermis, is not clear. As all epidermal $CD8^+$ memory T cells seem to express CD103[121], we wondered whether CD103 was a pre-requisite for entry into the epidermis, and if the inability of TGF-βRII KO cells to up-regulate CD103 could explain the phenotype we observed upon imaging. We therefore hypothesized that TGF-β-dependent induction of CD103 served to license T cells for epidermal entry. To investigate this, we looked to complement TGF-βRII KO cells with CD103 in an attempt to rescue epidermal migration. To this end, we cloned a retroviral construct encoding integrin αE (CD103) driven by the 5' LTR, followed by an IRES and a surface protein selection marker ΔLNGFR (truncated human nerve growth factor receptor). After activation of cells *in vitro*, we transduced them with the CD103 encoding construct to force expression in the absence of TGF-βRII. As αE integrin requires pairing with the chain β7 for functional expression of the integrin on the surface of cells, we verified that activated T cells express high levels β7 and that upon transduction, T cells expressed functional surface CD103, as measured by an

antibody recognizing the intact integrin on the cell surface (**Figure 3.5A**). Upon initial validation of the construct, we proceeded to conduct longitudinal MP-IVM of TGF-βRII KO cells with ectopic expression of CD103 alongside WT cells as indicated (**Figure 3.5B, Supplementary Video 3.10-3.12**). Contrary to our hypothesis, we found that ectopic CD103 expression in TGF-βRII KO T cells did not change their migratory behavior towards the hair follicles (**Figure 3.5C**), nor did it rescue their ability to be retained in the skin or cross into the epithelium (**Figure 3.5D**). We therefore concluded that the role of TGF-β in regulating epidermal entry and tissue retention was independent of its induction of CD103.

In light of this outcome, we considered whether CD103 was even a necessary "license" for $CD8^+$ T cells to enter the epidermis. To test this, we harvested CD103-deficient $CD8^+$ polyclonal cells from a $CD103^{-/-}$ mouse, activated and transduced them with distinguishing fluorescent reporters alongside $CD8^+$ polyclonal cells from a WT mouse, and adoptively transferred the cells in a 1:1 ratio into host mice for longitudinal MP-IVM. Our imaging studies revealed that $CD103^{-/-}$ T cells not only migrated similarly to WT cells, but were also able to easily enter the epidermis (**Figure 3.6A, Supplementary Video 3.13**), revealing that CD103 was not even necessary for the epidermal migration of $CD8^+$ T cells during eT_{RM} differentiation. This finding is consistent with other reports showing that loss of CD103 did not affect morphology[216] of cells in the epithelium, where they may latch on to epithelial cells via E-cadherin-dependent interactions, or significantly impair retention[131]. Additionally, in a longitudinal flow cytometry-based adoptive transfer setting, when we transferred 1:1 ratio of *in vitro* activated $CD103^{-/-}$ and WT polyclonal $CD8^+$ T cells into mice with DNFB-inflamed skin, we observed a steady ratio of WT to $CD103^{-/-}$ cells in the skin (**Figure 3.6B**). This was in stark contrast to a similar experiment conducted with TGF-βRII KO cells alongside WT cells, where the TGF-βRII KO cells waned off within one to two weeks after transfer. These results further

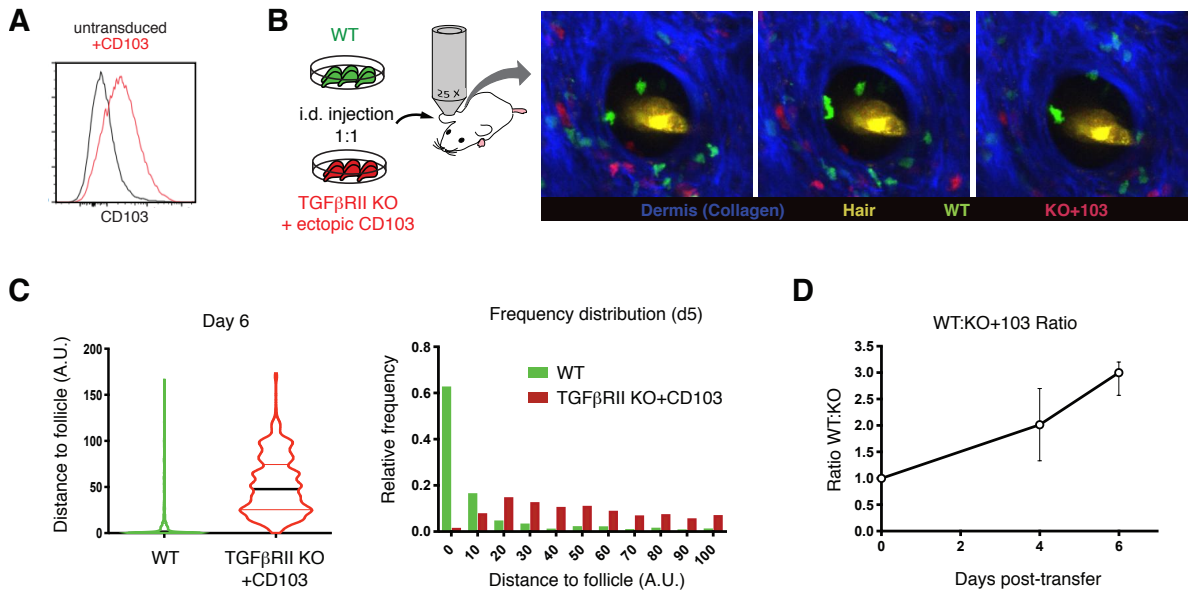


Figure 3.5 Ectopic CD103 does not rescue the ability of TGF-βRII KO cells to access the epidermis. (**A**) Activated CD8^{+} T cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-βRII$^{fl/fl\ or\ fl/+}$ mice were treated *in vitro* with 64nM 4-OHT at 2 days post-activation, and transduced with either tdTomato and CD103 (KO = fl/fl) or GFP (WT = fl/+) retrovirus. 72 hours after 4-OHT treatment (5 days post-activation), 10^5 cells each of "WT" GFP and "TGF-βRII KO+CD103" tdTomato cells were adoptively transferred at a 1:1 ratio. (**B**) Images span day 3 to 5 post transfer of cells. (**C**) Distance to nearest hair follicles for any cell 6 days post-intradermal injection of cells, within one representative mouse. Black lines denote median. (**D**) Ratio of WT to TGF-βRII KO+CD103 cells after transfer in a 1:1 ratio as determined through an MP-IVM timecourse, using 4 representative animals. Each experiment was performed at least twice with similar results.

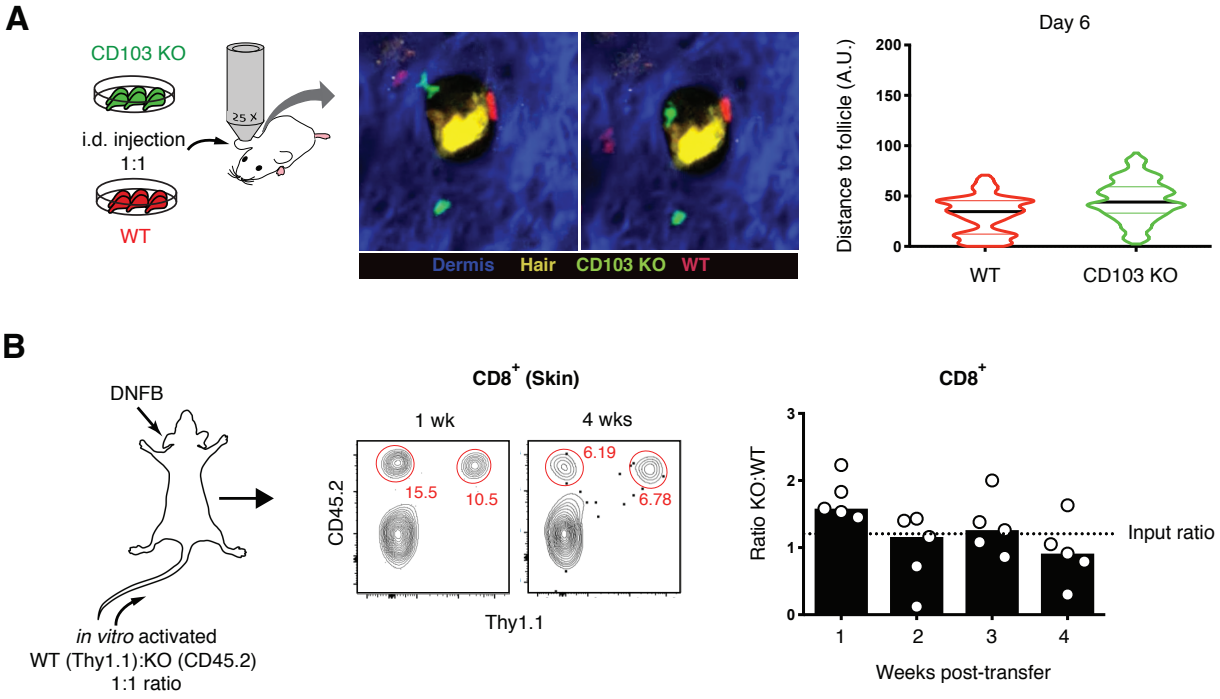


Figure 3.6 CD103 is not required for CD8$^+$ T cells to access the epidermis. (**A**) WT and CD103 deficient polyclonal cells from sex and age-matched controls were activated and transduced with either tdTomato (WT) or GFP (KO) retrovirus. CD8$^+$ T cells were enriched by negative selection. 10^5 cells each of WT tdTomato cells CD103 KO GFP cells were adoptively transferred at a 1:1 ratio. Distance to nearest hair follicles for any cell 6 days post-intradermal injection of cells, within one representative mouse. Black lines denote median. (**B**) 10^6 cells each of activated polyclonal CD8$^+$ WT (Thy1.1/1.2) or CD103 KO (CD45.2) were adoptively transferred into CD45.1 hosts at a 1:1 ratio 5 days post-activation and the ear skin was treated with DNFB to pull cells into the skin. Ratios of CD8$^+$ T cells were determined by flow cytometry at indicated time points post-transfer. Each experiment was performed at least twice with similar results.

solidified the conclusion that the role of TGF-β in regulating eT_{RM} terminal differentiation extends much further than its regulation of the integrin CD103.

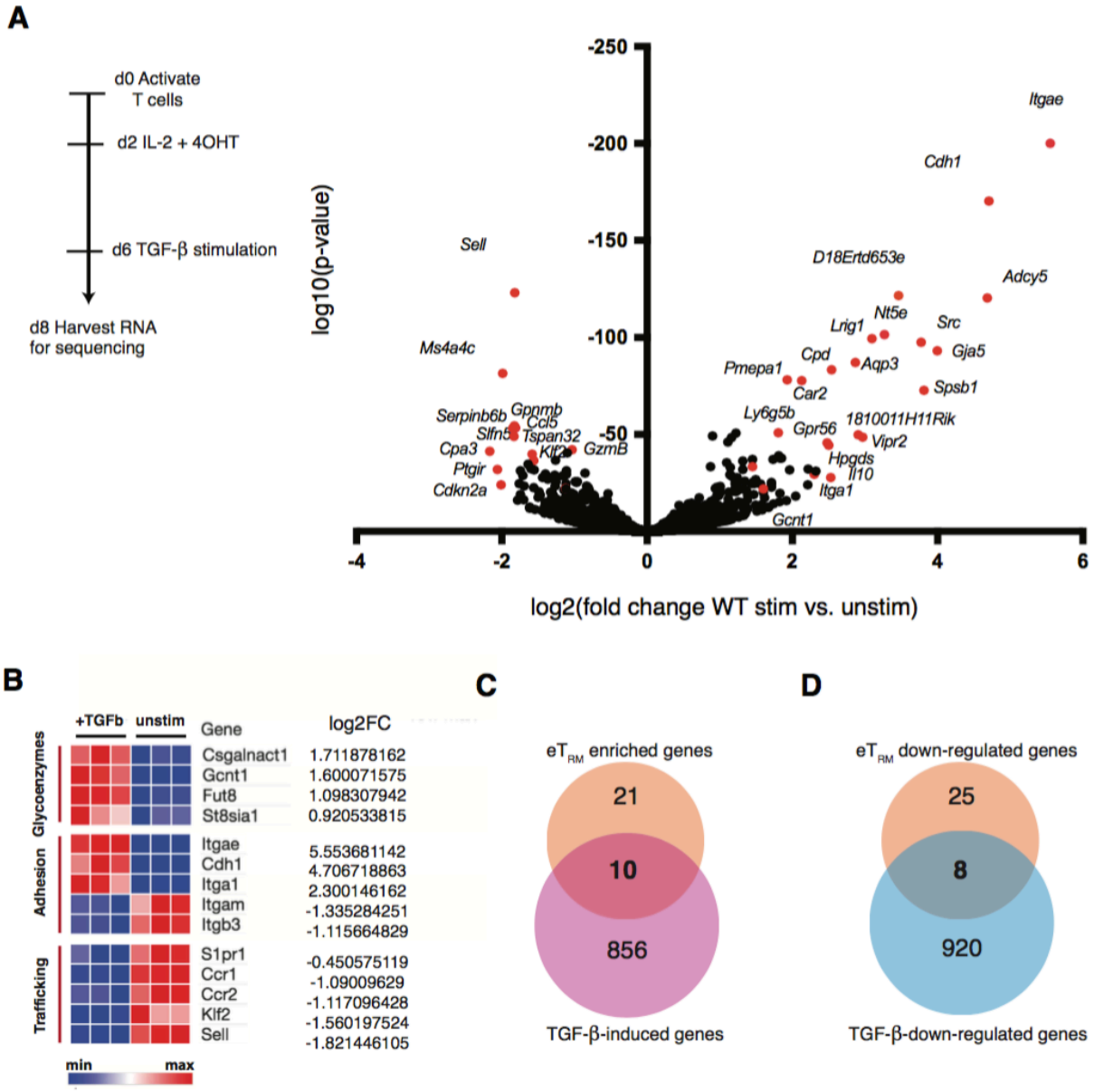
TGF-β signaling in activated T cells regulates a number of T_{RM} associated transcripts

Given our observation that loss of TGF-β signaling at the effector site leads to disruption of a multitude of aspects of eT_{RM} differentiation, we chose to conduct an unbiased transcriptome analysis to identify candidate targets that may regulate these functional axes. After activation of cells and tamoxifen treatment, we exposed TGF-βRII KO and WT cells to TGF-β *in vitro* six days after activation, relatively around a time where they may be exposed to TGF-β signals *in vivo* (**Figure 3.7A**). Two days after cytokine treatment, we prepared the cells for RNA sequencing. We then analyzed transcripts that were enriched or down-regulated in the WT treated with TGF-β, as compared to both TGF-βRII KO treated with TGF-β (as background) and WT not treated with cytokine. Many canonical TGF-β target transcripts were enriched, such as *Itgae* and *Il10*, as well as some notable trafficking and adhesion associated genes (**Figure 3.7A-B**). Intriguingly, we also found that within the core transcriptional signature of skin eT_{RM}[40], nearly 50% of enriched transcripts were also upregulated by TGF-β (**Figure 3.7C**), including *Itga1, Cdh1, Itgae, Rgs1,* and *Ahr*. While not as pronounced as the induced genes, TGF-β treatment also down-regulated (**Figure 3.7D**) a number of critical down-modulated transcripts in the eT_{RM} signature. This suggested that TGF-β is likely critical for inducing and even actively maintaining the transcriptional signature of eT_{RM} even beyond its previously described roles in CD103 induction[40] and T-box transcription factor regulation[121,124].

In searching for candidate targets from our screen that could potentially regulate the migration of T cells into the epidermis, and perhaps facilitate their retention or survival in the skin, we focused on genes involved in trafficking and adhesion. Integrins αE and α1, as well as E-cadherin, all of which implicated in tissue retention[33,49] and compartmentalization of T_{RM}, were

Figure 3.7 Transcriptome analysis of WT and TGF-βRII KO cells. (**A**) Activated $CD8^+$ T cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-$\beta RII^{fl/fl\ or\ +/+}$ mice were treated *in vitro* with 4-hydroxytamoxifen (4-OHT) at indicated doses beginning at two days post-activation of T cells, in T cell medium containing 20ng/mL IL-2. Cells were stimulated with 1ng/mL TGF-β1 4 days post-treatment with 4-OHT and mRNA was purified from 10^6 cultured cells 48 hours post-treatment with TGF-β1 for RNA sequencing. Experiment was performed in triplicates and log2 of the fold change between WT cells stimulated or not with TGF-β1 were plotted. (**B**) Selected genes from the RNA sequencing. Heat map represents maximum and minimum values within a given row. (**C**) Genes up-regulated by TGF-β were compared to transcripts from Mackay et. al. NI 2013 known to be enriched in eT_{RM}. (**D**) Genes down-regulated by TGF-β were compared to transcripts from Mackay et. al. NI 2013 known to be down-regulated in eT_{RM}.

Figure 3.7 (Continued)



induced by TGF-β. A handful of chemokine receptor axes also seemed to be differentially regulated by TGF-β, including up-regulation of *Rgs1,* a regulator of G-protein coupled receptor signaling that can globally impact sensitivity to chemokine gradients[219], coupled with a down-regulation of chemokine receptor *Ccr2*, and the transcription factor *Klf2,* which is involved in the regulation of *S1pr1*[117], a critical determinant of T_{RM} lodgment[116]. These data indicate that TGF-β may play a role in the proper localization or trafficking of cells within the skin during eT_{RM} differentiation.

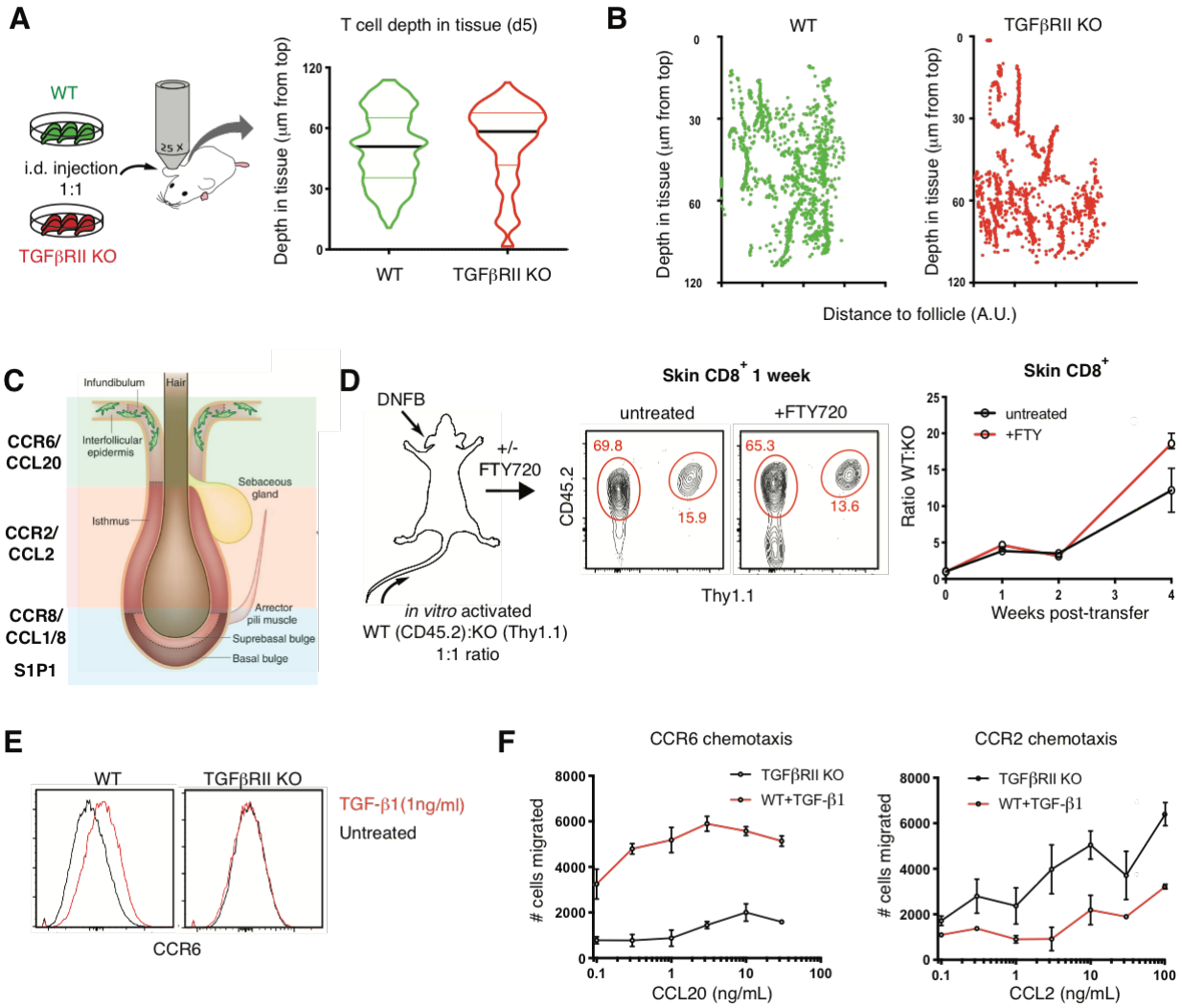
TGF-β regulates the spatial organization of T cells in the skin

Working from the basis that TGF-β regulates *Ccr2* and *Klf2*, we considered that perhaps these two factors may regulate the 3D spatial organization of $CD8^+$ T cells as they prepare to traverse the epithelium. We therefore analyzed the z-axial positioning of WT and TGF-βRII KO cells from our imaging experiment. Interestingly, we found that a greater density of TGF-βRII KO cells were found deeper in the skin as compared to WT cells (**Figure 3.8A**). When we plotted the relative depth of T cells in the tissue against distance to the nearest hair follicle, we found an even greater difference, where WT T cells closer to the follicle clustered closer to the epidermis (**Figure 3.8B**, left), while the TGF-βRII KO cells that appeared to migrate near the follicle were actually positioned deeper in the tissue (**Figure 3.8B**, right). We therefore hypothesized that cells may enter the epidermis through the infundibulum or upper isthmus, the more superficial regions of the hair follicle, thereby avoiding the bulge where the epidermal stem cell niche resides. This led us to investigate which factors impacted by TGF-β signaling may determine cell positioning that facilitates epidermal entry of $CD8^+$ T cells.

The down-regulation of *Klf2* by TGF-β (**Figure 3.7**), taken in conjunction with existing literature demonstrating a role for Klf2 in regulating the sphingosine-1-phosphate (S1P) signaling axis during T_{RM} differentiation[117] led us to hypothesize TGF-β may, in part, exert its

Figure 3.8 TGF-β regulates the localization of cells by modulating balance of chemokine receptors. (**A**) Activated $CD8^+$ T cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-β$RII^{fl/fl\ or\ fl/+}$ mice were treated *in vitro* with 64nM 4-OHT at 2 days post-activation, and transduced with either tdTomato (KO = fl/fl) or GFP (WT = fl/+) retrovirus. 72 hours after 4-OHT treatment (5 days post-activation), 10^5 cells each of "WT" GFP and "TGF-βRII KO" tdTomato cells were adoptively transferred at a 1:1 ratio. After 5 days post-transfer, depth of cells in the tissue was measured and also (**B**) plotted as a function of the distance away from the follicle. (**C**) Schematic adapted from Heath and Carbone NI 2012 preview of Nagao et. al. NI 2012. (**D**) Activated $CD8^+$ T cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-β$RII^{fl/fl\ or\ fl/+}$ mice were treated *in vitro* with 64nM 4-OHT at 2 days post-activation. 10^6 cells each of WT (CD45.2) and TGF-βRII KO (Thy1.1/1.2) were then adoptively transferred into CD45.1 hosts at a 1:1 ratio 5 days post-activation and the ear skin was treated with DNFB to pull cells into the skin. Over the course of 4 weeks, mice were either treated or not with FTY720. Ratios of $CD8^+$ T cells were determined by flow cytometry at indicated time points post-transfer. Each experiment was performed at least twice with similar results. (**E-F**) Activated $CD8^+$ T cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-β$RII^{fl/fl\ or\ +/+}$ mice were treated *in vitro* with 4-hydroxytamoxifen (4-OHT) to induce receptor deletion. Cells were stimulated with 1ng/mL TGF-β1 4 days post-treatment with 4-OHT and 48 hours post-treatment with TGF-β1 (**E**) CCR6 expression was determined by flow cytometry and (**F**) TGF-β1 treated cells were placed into a chemotaxis assay with a 1:1 ratio (1.25 x 10^5 cells each) of WT:TGF-βRII KO cells in the top chamber and increasing doses of CCL20 or CCL2 in the bottom well of a 3.0μM pore transwell plate in T cell medium. 4 hours later, the number of migrated cells was determined using a volumetric flow cytometer. Each experiment was performed at least twice with similar results.

Figure 3.8 (Continued)



function on T cells by down-regulating S1P signaling. As S1P is known to be important for mediating the egress of lymphocytes into the bloodstream from the tissue, we hypothesized that the absence of TGF-β signaling may target cells towards an S1P gradient, which is highest in the vasculature, and may facilitate their deeper positioning in the tissue as well as the inability of TGF-βRII KO cells to be retained in the skin long-term. Therefore, we thought to inhibit the S1P signaling axis to see whether this might rescue the inability of TGF-βRII KO cells to be maintained in the skin, in order to receive access to the necessary signals for epithelial entry. We activated both WT and TGF-βRII KO cells *in vitro* and adoptively transferred them intravenously in equal ratios into recipient mice, whose ears were DNFB-inflamed, to pull the cells into the ear skin. To block S1P signaling, we treated mice with FTY720, an S1P receptor antagonist, from the time of T cell transfer through the end of the time-course of 4 weeks. We monitored the ratios of WT to TGF-βRII KO cells over time as a measure of tissue retention that could facilitate the receipt of epidermal entry signals. To our surprise, FTY720 treatment did not rescue the ratio of WT:TGF-βRII KO in the skin over time (**Figure 3.8D**), suggesting that effects of TGF-β on T cells in the skin were independent of its regulation of the S1P signaling axis.

We then considered other factors that may regulate the positioning of cells in the skin to facilitate epidermal migration. In addition to its role in regulating S1P receptor, KLF2 has been shown to both directly and indirectly regulate the expression of various chemokine receptors on T cells[220], such as CXCR3[111], which could affect the positioning of cells based on sensitivity to chemokine gradients in a given tissue. In the skin, there is a gradient of chemokines produced in the hair follicle during inflammation that allow for the migration of myeloid precursor cells into the epidermis during their differentiation into Langerhans cells [221]. Notably, CCR6 ligand CCL20 is produced higher up in the follicle in the infundibulum, while CCR2 ligand CCL2 is produced deeper in the follicle, in the isthmus. As WT cells seemed to enter the epidermis through the

infundibulum we asked whether TGF-β regulated the balance of these two chemokine receptors to influence localization of T cells for epidermal migration. While our RNAseq revealed a distinct transcriptional down-regulation of *Ccr2* in T cells upon TGF-β exposure (**Figure 3.7**), CCR6 was not differentially expressed in TGF-β-responding cells at the transcriptional level. Noting that CCR6 protein expression may be regulated by TGF-β post-transcriptionally, we treated WT cells with TGF-β and measured expression of the receptor at the surface protein level, which was seen to increase after treatment (**Figure 3.8E**). As chemokine receptors require other intricate cellular machinery in order to sensitively respond to chemokine gradients, we also conducted an *in vitro* chemotaxis assay to verify functional differences between WT and TGF-βRII KO cells. Indeed, in comparison to TGF-βRII KO cells, WT cells treated with TGF-β were significantly more responsive to CCR6 ligand CCL20 (**Figure 3.8F**) and less sensitive to CCR2 ligand CCL2 (**Figure 3.8G**). These data indicate that TGF-β regulates the balance of chemokine receptors CCR2 and CCR6 in activated T cells, which may, in turn, impact their spatial organization in the tissue.

CCR6 is required for CD8$^+$ T cell epidermal access and eT$_{RM}$ differentiation

Based on our observation that TGF-β dependent T cell spatial distribution around the hair follicle is linked to their ability to enter the epidermis, we wanted to test whether T cells were licensed for epidermal access through a specific region of the follicle. Previous reports of lymphocyte migration have implicated CCR6 in the seeding of gamma delta T cells in the epidermis [222]. This is consistent with our observations showing that the positioning of TGF-β-responsive T cells closer to the infundibulum is linked to CCR6 expression and responsiveness to CCL20. Therefore, we thought to test whether cells impaired in their ability to access this "portal" would be less equipped to access the epidermis. To this end, we conducted longitudinal MP-IVM, as previously described, of CCR6-deficient polyclonal CD8$^+$ T cells alongside WT

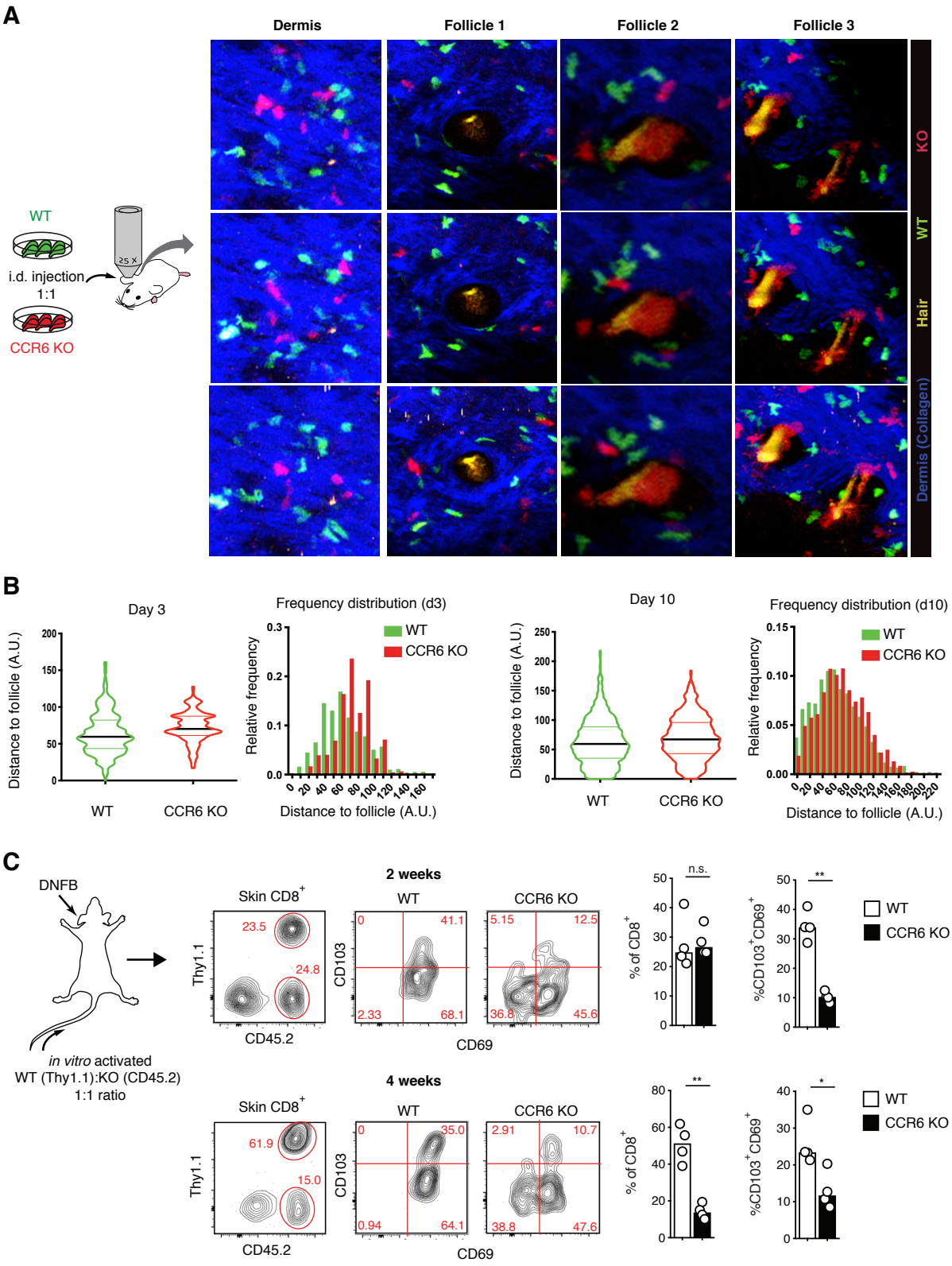
polyclonal $CD8^+$ T cells, transduced with distinguishing fluorescent reporters. We observed over time that $CCR6^{-/-}$ cells clustered inefficiently around hair follicles (**Figure 3.9A-B**) and were not very often observed to cross into the epidermis (**Figure 3.9A, Supplementary Video 3.14-3.17**), suggesting that CCR6 expression and follicular positioning was important for cells to migrate into the epidermis. We further corroborated our imaging studies with longitudinal flow cytometry-based adoptive transfer studies. Two weeks following transfer of *in vitro* activated $CCR6^{-/-}$ and WT polyclonal $CD8^+$ T cells in a 1:1 ratio into mice with DNFB-inflamed skin, we observed a steady ratio of the $CCR6^{-/-}$ and WT cells in the skin. However, $CCR6^{-/-}$ cells did not begin to up-regulate CD103 as the WT cells did (**Figure 3.9C,** top), suggesting that the $CCR6^{-/-}$ cells failed to reach the epidermis. By 4 weeks post-transfer, the $CCR6^{-/-}$ cells failed to be retained in the skin as well as WT cells, and the ones that did remain were mostly $CD103^-$, unlike their WT counterparts (**Figure 3.9C,** bottom). Taken together, these results confirm that CCR6 is required for epidermal entry and terminal eT_{RM} differentiation, through its role in regulating the spatial distribution of T cells in the skin for follicular entry.

DISCUSSION

Here, we present the first visualization of the process by which $CD8^+$ T cells enter the epidermis during eT_{RM} differentiation. We found a critical dependence on T cell-intrinsic TGF-β signaling that licenses T cells to enter the epidermis by aptly positioning them near the infundibulum of the hair follicles, away from the epidermal stem cell niche that sits near the bulge of the follicle. This process orchestrated through TGF-β-dependent control of chemokine receptors, facilitating the up-regulation of CCR6 on T cells to make them more sensitive to CCL20 gradients in the infundibulum. We also uncovered a novel requirement for CCR6 in the epidermal lodgment of $CD8^+$ T cells during eT_{RM} differentiation.

Figure 3.9 CCR6 is required for CD8 epidermal access and eT_{RM} formation. (**A**) WT and CCR6 deficient polyclonal cells from sex and age-matched controls were activated and transduced with either tdTomato (KO) or GFP (WT) retrovirus. $CD8^+$ T cells were enriched by negative selection. 10^5 cells each of WT GFP and CCR6 KO tdTomato cells were adoptively transferred at a 1:1 ratio. Images are shown from 6 days post-intradermal injection of cells, within 3 representative mice (by column). (**B**) Distance to nearest hair follicles for any cell 3 or 10 days post-intradermal injection of cells, within one representative mouse. Black lines denote median. (**C**) 10^6 cells each of activated polyclonal $CD8^+$ WT (Thy1.1/1.2) or CCR6 KO (CD45.2) were adoptively transferred into CD45.1 hosts at a 1:1 ratio 5 days post-activation and the ear skin was treated with DNFB to pull cells into the skin. Ratios of $CD8^+$ T cells and frequency of $CD103^+CD69^+$ $CD8^+$ T cells (eT_{RM}) were determined by flow cytometry at indicated time points post-transfer. Each experiment was performed at least twice with similar results. * = $p < 0.05$, ** = $p < 0.01$, n.s. = not significant.

Figure 3.9 (Continued)



While there is literature linking CCR6 to various tissue resident cell subsets[49], it is curious that this receptor was not previously described as a critical component of the general eT_{RM} transcriptional signature[40]. This could be due to the fact that many of these defined signatures focus mostly on terminally differentiated T_{RM}, and may not capture any dynamic changes throughout differentiation. It is quite plausible that the window of epidermal entry is very tight, during which CCR6 expression on T cells and CCL20 expression in the follicle is short-lived and not easily reflected in existing transcriptional analysis. While our transcriptomics looks at this narrow window, it did not reveal a transcriptional change in CCR6 in the presence or absence of TGF-β, which may also be due to the kinetics by which CCR6 is induced. TGF-β, along with RORγT, is implicated in the transcriptional profile of IL-17 producing T cells, which also feature an enrichment of CCR6. In fact, the T_{RM} cells which do express CCR6 in the long-term, as reflected in the literature, may actually be associated with a Tc17 phenotype[47,49] and possibly different localization, as opposed to interferon gamma-skewed populations that we have focused on. Given the distinct functions and compartments of these different cell types, it will become more important in the future to dissect the potential lineage determinants that promote or inhibit the formation of these subsets, as well as understand the dynamics by which these determinants are modulated.

Another observation of note is that upon the loss of CCR6, which results in impaired epidermal entry, CD103 fails to become up-regulated on these cells. This suggests that CD103 may only be induced after T cells traffic into the epidermis and is more likely a reflection of sustained TGF-β signaling and an epidermal residence marker of $CD8^+$ T cells, rather than a requirement for maintenance of T cells in barrier sites through interaction with E-cadherin. This is supported by studies of T_{RM} in the brain, which are also seen to sustain CD103 expression, even in the absence of E-cadherin or other putative ligands[32].

Furthermore, our data, along with other longitudinal studies of CD103-deficient $CD8^+$ T cells, demonstrate that the loss of CD103 does not significantly impact T cell epidermal access, residence[216] or retention in the tissue, unless in the very long term[40]. This is in contrast to studies of CD103 on regulatory T cells (T_{reg}), where the integrin is seen to be critical for suppressive function in the skin by way of mediating T_{reg} retention in the tissue during *Leishmania major* infection[223]. Kinetics may play a role in this juxtaposition, as T_{reg} could up-regulate CD103, a known T_{reg} activation marker, prior to their entry into the tissue, conferring upon them the ability to be retained. On the other hand, CD103 induction on $CD8^+$ T cells seems to occur, based on our studies, only after T cells gain access to the epithelium. This difference in CD103 requirement may also be due to the fact that $CD8^+$ T cells with sustained TGF-β signaling may have compensatory mechanisms at play for their epithelial retention. In our transcriptome analysis, we also observed TGF-β-dependent up-regulation of *Cdh1,* coding for E-cadherin. While CD103 is thought to allow cells to interact with E-cadherin in epithelia for their maintenance, there is also evidence that homotypic interactions between E-cadherin-expressing cells can serve a similar function[224]. Therefore, in the absence of CD103, yet presence of active TGF-β signaling, E-cadherin may serve to compensate in functionality. Studies of T_{RM} in the salivary gland and their reliance on E-cadherin for maintenance and function during MCMV infection also support this theory[33]. Thus, while attempting to block CD103 may be thought of as a targeted therapy for eT_{RM} depletion in the epithelium, for example in the context of autoimmune diseases to deplete pathology-driving T_{RM}, a more broad strategy targeting TGF-β, for instance by blocking epithelial TGF-β activating integrins[172], may be a more robust therapeutic option.

It is plausible that, while this is a phenomenon we observe in the skin for the time being, TGF-β-dependent control of trafficking and localization may serve a mechanism by which T cells

enter the epithelium in other barrier sites, while also avoiding penetration through the stem cell niches in these tissues[225]. Resident memory cells in various barrier sites are transcriptionally distinct, yet still share a dependence on TGF-β signaling to varying degrees. For example, although TGF-β is seen to be somewhat dispensable for the formation of liver T_{RM}[53], which are intravascular and therefore non-epithelial, TGF-β is extremely critical for inducing transcriptional programs associated with mucosal and other epithelial barrier T_{RM}[132] (L. K. Mackay, communication). The regulation of chemokine receptors and other trafficking mediators by TGF-β may play a particularly significant role in driving compartmentalization within large, heterogeneous tissues such as the gut [70], for precise positioning of T cells upon pathogen encounter or other barrier insults. Of note, the CCL-20-CCR6 axis has been shown to be induced upon inflammation in the gut and lungs[192,226], though this may not be the only chemokine axis regulated by TGF-β that can direct localization within a given tissue.

In comparison to other tissues that allow the constant seeding of activated T cells for prolonged residency[23,35], the skin is thought to be a less “permissive” tissue with a more defined window of opportunity for T_{RM} formation. Notably, even in our hands, inflammation of the skin in some capacity is required for long-term seeding by activated T cells. Our observation that the hair follicles serve as a primary portal of entry for T cells into the epidermis supports this idea. Hair follicle cycles are re-booted upon epilation of hair from the follicle, or during infections or sterile inflammation. These cycles also regulate the structure of the epithelia, perhaps making them more permissive at certain times to allowing the seeding of other cell types in the keratinized, stratified epithelium. This structural change combined with the stress or cycle-induced production of chemotactic factors[221] that are thought to allow for the re-building of the epithelium upon damage or innate protection of the barrier, are likely to mediate this window by which T cells gain access to the epidermis, as we observe in the case of CCR6. The fact that

TGF-β, a critical factor in the rebuilding of tissues, mediates this circuit in T cells as well suggests a “soft” adaptation of these cells to incorporate into what may be construed as a “foreign” barrier site for T cells.

In considering T_{RM} vaccine strategies for cancer or infectious agents, it is important to understand the factors within various tissue sites that may expand the permissivity or “window of opportunity” for T_{RM} seeding. For example, vaccine strategies for expanding the epidermal T_{RM} niche may include boosting CCL20 or other chemokine production by a topical agent, while at the same time, limiting the necessary inflammation for seeding of skin eT_{RM}. Furthermore, we observe quantitatively that not all T cells present in the skin even at a given time-point are able to access the epithelium. Whether this is simply a stochastic phenomenon or if there are specific T_{RM} precursors is a question yet to be answered. Studies addressing the heterogeneity of skin-infiltrating T cells and identifying a putative precursor are therefore essential in order to better elicit these subsets via vaccination.

Our studies have uncovered more critical roles for TGF-β in the regulation of eT_{RM} differentiation and migration in the skin. Given the complexity of this pathway and the number of stages at which this regulates acquisition of adaptive immunity in barrier sites, further studies are critical to dissect the tight contexts by which T cells may receive discrete TGF-β signals allowing for their differentiation.

MATERIALS AND METHODS

Mice

C57BL/6, C57BL/6$^{Tyrc\text{-}2J}$, CD45.1, and CD103$^{-/-}$ mice were obtained from JAX. OT-I TCRa$^{-/-}$ mice were obtained from JAX and bred in-house to Rosa26-CreER^{T2} and TGF-βRII$^{fl/fl}$ mice, obtained from Dr. Adam Lacy-Hulbert. CD45.1 and Thy1.1 mice were obtained from JAX and bred to OT-I or C57BL/6 mice to generate sources of congenic marked T cells. CCR6$^{-/-}$ mice were provided by Dr. Andrew D. Luster. Animals were housed in specific pathogen-free facilities at the Massachusetts General Hospital (MGH) and all experimental studies were approved and performed in accordance with guidelines and regulations implemented by the MGH Institutional Animal Care and Use Committee (IACUC).

Multiphoton intravital microscopy of the ear skin

Tyrosinase mutant B6 (C57BL/6$^{Tyrc\text{-}2J}$) “Albino B6” mice obtained from Jackson Laboratories were used as host mice for two-photon imaging so as to prevent melanin-dependent auto-fluorescence from interfering with the imaging of events in the ear skin. After adoptive transfer of cells as indicated, mice were prepared for longitudinal intravital imaging. Prior to the first imaging session, the ear skin was lightly epilated using Nair. For each imaging session, mice were anesthetized using isofluorane (Isothesia) delivered via a SomnoSuite MSTAT (Kent Scientific). Anesthesia was maintained at 1-2% isofluorane at a flow rate of 30-40mL/min. To prepare the ear skin for imaging, a custom-built stage to fix the mouse ear on was heated to 34°C using a feedback based heating apparatus (Warner Instruments). The body of the mouse was maintained at 37°C using a feedback-based rectal probe thermometer and heating pad. The ear pinnae was affixed to the imaging stage using double-sided tape (3M) on the ventral side of the ear. The lens (Olympus 25X) was immersed in Genteal Eye Gel (Alcon), covering the top of the ear skin and the lens was heated sufficiently to maintain the interface temperature at

34°C. Mice in each imaging session were under anesthesia for no more than 6 consecutive hours. An Olympus custom MPE-RS system was used for image acquisition in laser scanning mode, with Insight and DeepSee Broadband (Spectra Physics) lasers. Each z stack was acquired in Z-stacks with cycles lasting 30 or 60 seconds, varying depth and 3μm spacing between slices. Olympus FV30S software was used for acquisition and conversion of raw data files.

Analysis of intravital microscopy images

Movies were quantified, smoothed and edited using Imaris 8.3.1 software (Bitplane). Briefly, images were first stabilized for drift correction, then 3 dimensional “surfaces” were created for follicular regions (by manual approximation of follicular regions) and the dermis (automated, by intensity of second harmonic signal). A distance transformation was performed, yielding additional “channels” with arbitrary intensity values corresponding to the distance from hair follicles and dermis, that are applied to all defined objects. The “Spots” function was utilized to define and track cell migration, using automatic detection and thresholding. When information on morphology of cells was required, cells were defined using “Surfaces”. Z-stacks were then thresholded to reduce autofluorescence and background fluorescence intensity, and then converted into *.mov or *.mp4 movies for easy viewing. Graphs were plotted using Prism 8 software (GraphPad).

In vitro activation, retroviral transduction, 4-OHT treatment of T cells

OT-I splenocytes were pulsed with 1 μM SIINFEKL peptide in 1 mL of T cell medium (RPMI, 10% FCS, 1% HEPES, 1% Sodium Pyruvate, 1% GlutaMAX, 1% non-essential amino acids, 55 μM 2-mercaptoethanol) for 1 hr at 37°C, diluted in 9 mL of T cell medium, and cultured at 37°C in 5% CO_2. Polyclonal $CD8^+$ were activated by pulsing splenocytes with 1μg/mL anti CD3

(Biolegend) at 10^7 per mL in T cell medium. $CD8^+$ were negatively selected (Miltenyi) from the culture 2 days post-activation. 2 days post-activation, 20 ng/mL IL-2 was added and cell density was maintained at 10^6 cells/mL. 4-OHT (64nM) was added to cultures for induction of TGF-βRII deletion 2 days post-activation along with IL-2. Activated T cells were transduced with retrovirus for fluorescent proteins and ectopic CD103. Briefly, on day 1 and day 2 post-activation, cells were plated at 10^6/mL in viral supernatant containing 4μg/mL polybrene, and spin-infected for 90 minutes at 32°C at 1000g. After spin-infection, the viral supernatant was replaced with T cell medium.

Adoptive transfer of T cells

Activated T cells were adoptively transferred either by intravenous injection of 10^6 cells/100μL PBS and subsequently pulled into the ear skin by painting with 5μL (on both dorsal and ventral sides) 0.5% dinitrofluorobenzene (DNFB, Sigma), in a mixture of 4:1 acetone:olive oil. Alternatively, activated T cells were intradermally injected 10^5 cells/10μL in PBS into the ear skin for intravital imaging using 30G needle, with the bevel facing up towards the dorsal side of the mouse and needle pointing towards the outer edge of the ear, at a manual rate of roughly 1μL per second to facilitate lateral spreading of cells.

Preparation of samples for RNA sequencing

OT-I x Rosa-CreER^{T2} x TGF-βRII$^{fl/fl\ or\ +/+}$ cells were pulsed with 1 μM SIINFEKL peptide in 1 mL of T cell medium for 1 hr at 37°C, diluted in 9 mL of T cell medium, and cultured at 37°C in 5% CO_2. 2 days post-activation, 20 ng/mL IL-2 and 64nM 4-OHT was added to induce Cre activity and cell density was maintained at 10^6 cells/mL. On day 4 post-activation, T cells were treated with 1ng/mL TGF-β-1 (Cell Signaling). 48 hours post-treatment, cells were harvested and processed using Qiagen RNeasy kit. Library was prepared using PolyA selection + NEBNext

Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). Sequencing was done by Illumina HiSeq paired-end 50bp high-output, with experimental conditions run on the same lane as controls.

Isolation of cells from tissues and staining for flow cytometry

For cell isolations from non-lymphoid tissues, mice were i.v. injected with 3 μg of Alexa Fluor 700- or FITC-labeled α-CD45.2, or PE-Cy7-labeled α-CD8$^+$β antibody 3 min prior to euthanasia in order to label intravascular leukocytes for exclusion from analysis. All organs were harvested into ice-cold FACS buffer (PBS w/o Ca2+/Mg2+ with 0.5% BSA and 2 mM EDTA). Skin was processed as previously described in Chapter 2. Briefly, separated dorsal and ventral halves of ear skin or flank skin was minced into small pieces and placed in 2.5 mL digest buffer A (DMEM; 2% FBS; 1% HEPES; 25 U/mL Collagenase IV) or digest buffer B (DMEM; 2% FBS; 1% HEPES; 125 μg/mL Liberase TM; 0.5 mg/mL Hyaluronidase Type I-S from Bovine Testes) for 1 hr at 37°C under agitation, then quenched with 10% FBS and 1.5 mM EDTA and blended using a gentleMACS tissue blender (Miltenyi, C tubes, m_impTumor_01 protocol). Spleen, and LNs were minced, passed through a 40 μm cell strainer, and red blood cells lysed with ACK lysis buffer as necessary. Cell surface epitopes were stained in FACS buffer (PBS, 0.5-1% BSA or 5-10% FBS) in the dark at 4°C for 15 min, followed by staining with fixable viability dye (Zombie Dyes, Biolegend) at room temperature for 15 min. TGF-βRII surface staining was conducted after using the BD Fixation Permeabilization kit, in a saponin based buffer, and then staining post-permeabilization for 20min at 4°C.

RNA sequencing analysis

Trimmomatic pipeline was used to trim data for quality with the following parameters: LEADING:15TRAILING:15 SLIDINGWINDOW:4:15 MINLEN:16. Bowtie2 was used to align data

to mouse mm10. HTSeq was used to map aligned reads to genes and to generate a gene count matrix. DESeq2 was used to normalize the counts for library depth and perform differential expression analysis between pairwise comparisons.

FTY720 treatment

Mice received intraperitoneal injections of 1 mg/kg BW FTY720 (Fingolimod) in 150μL H_2O every 2-3 days where indicated.

In vitro chemotaxis assay

TGF-βRII KO and WT T cells that could be distinguished by congenic marker were prepared as above described for RNA sequencing. 48 hours after TGF-β-1 treatment, 250K WT and KO cells were mixed at 1:1 ratio in 75μL T cell medium and placed in the upper chamber of a HTS Transwell-96 permeable support plate with 3.0μM pore. In the lower chamber chemokines were titrated in each well in 225μL T cell medium. Plate was incubated at 37°C for 4 hours, after which the lower chamber was harvested for flow cytometry analysis on a volumetric flow cytometry analyzer (Accuri) to obtain accurate cell counts.

Generation of retroviral constructs and packaging cell lines

Mouse CD103 was cloned from a cDNA (Origene) by Gibson Assembly into a MiNW plasmid (construct in Appendix) linearized by BglII enzymatic digestion, using the following primers sequences: F 'ttctctaggcgccggaattaCCACCATGAAGTGGCTCTTC' and R 'ggaattcgttaacctcgagaCTTGGAGCAGACTGTCAG'. The plasmid construct was then used to create a stable packaging line by first Calcium Phosphate transfection to create VSVg-pseudotyped virus in Platinum E cells (HEK293 containing *gag, pol, env*), and then subsequently transducing Platinum E cells with the VSVg pseudotyped virus. Single clones

were selected by limiting dilution and then subsequently for NGFR and CD103 dual surface marker expression.

Additional materials

Retroviral construct maps, primers for RT-PCR and antibodies for flow cytometry are included in the Appendix.

Statistical analysis

Two-tailed, paired or unpaired student's t-test (for normally distributed data) or Mann-Whitney test (for not normally distributed data) for was used for comparisons between two groups. The two-sample Kolmogorov-Smirnov was used to compare cumulative distributions. All statistical tests were performed with GraphPad Prism software, and $p < 0.05$ was considered statistically significant.

ACKNOWLEDGEMENTS AND CONTRIBUTIONS

V.M. performed most experiments and analyzed all of the data. E.C. generated the OT-I ROSA Cre-ER^{T2} x TGF-βRII$^{fl/fl}$ mice and performed initial characterization of TGF-βRII deletion kinetics *in vitro*. D.R.S. assisted with normalization of RNA sequencing data. V.M. and T.R.M. designed the study.

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CHAPTER 4: Intravital microscopy tools to visualize cytokine signaling dynamics

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ABSTRACT

Cells of the immune system are poised to sensitively communicate with their neighbors via a discrete exchange of cytokines and cellular interactions. While the importance of immunological synapses and various cytokines to the differentiation of immune cells and maintenance of their programs is established, our current understanding of these communications fails to shed light on discrete spatiotemporal contexts that ultimately regulate functionality *in vivo*. For instance, it remains unknown exactly how T cells acquire TGF-β signals *in vivo* and if heterogeneity in the receipt of signals may better serve to explain the pleiotropism of the cytokine. On the other hand, it is also unknown exactly how the delivery of effector cytokines by T cells, such as TNFα and IFNγ, is triggered and mediated *in vivo.* Dissecting any nuances in signaling contexts may help increase specificity in the therapeutic targeting of these pathways. Therefore, we sought to generate tools for use *in vivo* that would enable us to determine the contexts by which T cells receive and deliver cytokine signals in disease and immune homeostasis. In particular, we looked to measure the activity of the cytokines TGF-β and IFNγ *in vivo*, in order to better understand the terms of their successful receipt on target cells. Here, we validate the use of a GFP-Smad2 reporter that can report TGF-β signaling *in vivo* in skin eT_{RM}, as well as a STAT1-GFP reporter that can be used to sensitively and acutely measure receipt of IFNγ signals by tumor cells *in vivo*. We aim to utilize these tools in future

studies to dissect the spatiotemporal contexts of cytokine signaling as they pertain to critical determinants of effector function and differentiation in the adaptive immune system.

INTRODUCTION

The innate and adaptive immune systems are finely tuned to communicate with their environments to facilitate their optimal function in maintenance of homeostasis or during an immune response. These fine-tuned communications can consist of prolonged cellular interactions that facilitate downstream signaling occurring in isolation or in combination with a number of different cytokine signals that instruct transcriptional and other functional programs within a cell. While the importance of such communications is established, much of our investigation of these communications is restricted to snapshots of cells in time, limiting the amount of information we can draw from such studies.

The growing use of intravital microscopy (IVM) to probe the dynamic nature of immune processes has paved way for interesting revelations surrounding the critical immune interactions and trafficking events that give rise to a particular function in the innate or adaptive immune system[227-230]. However, IVM can also have its pitfalls, where presumed "interactions" of cells may or may not actually reflect functional signaling. To begin to skirt the issues of rogue reporting of events, reporters of signaling have been adapted for use *in vivo*, such as a GFP-tagged NFAT to report TCR triggering in T cells upon translocation of the reporter from the cytoplasm into the nucleus, visualized by two-photon IVM[231]. Here, we have followed suit to build and validate tools that will allow us to map and thereby understand the complex contexts of TGF-β signaling in T cells as well as the terms of IFNγ release by T cells, in order to dissect the spatiotemporal dynamics of these critical immune cytokines for the first time *in vivo.*

RESULTS

Validation of a GFP-Smad2 as a reporter for TGF-β signaling in T cells

TGF-β plays pleiotropic roles in its regulation of the immune system. One layer of regulation of the cytokine's activity is its activation from a latent form, mediated predominantly

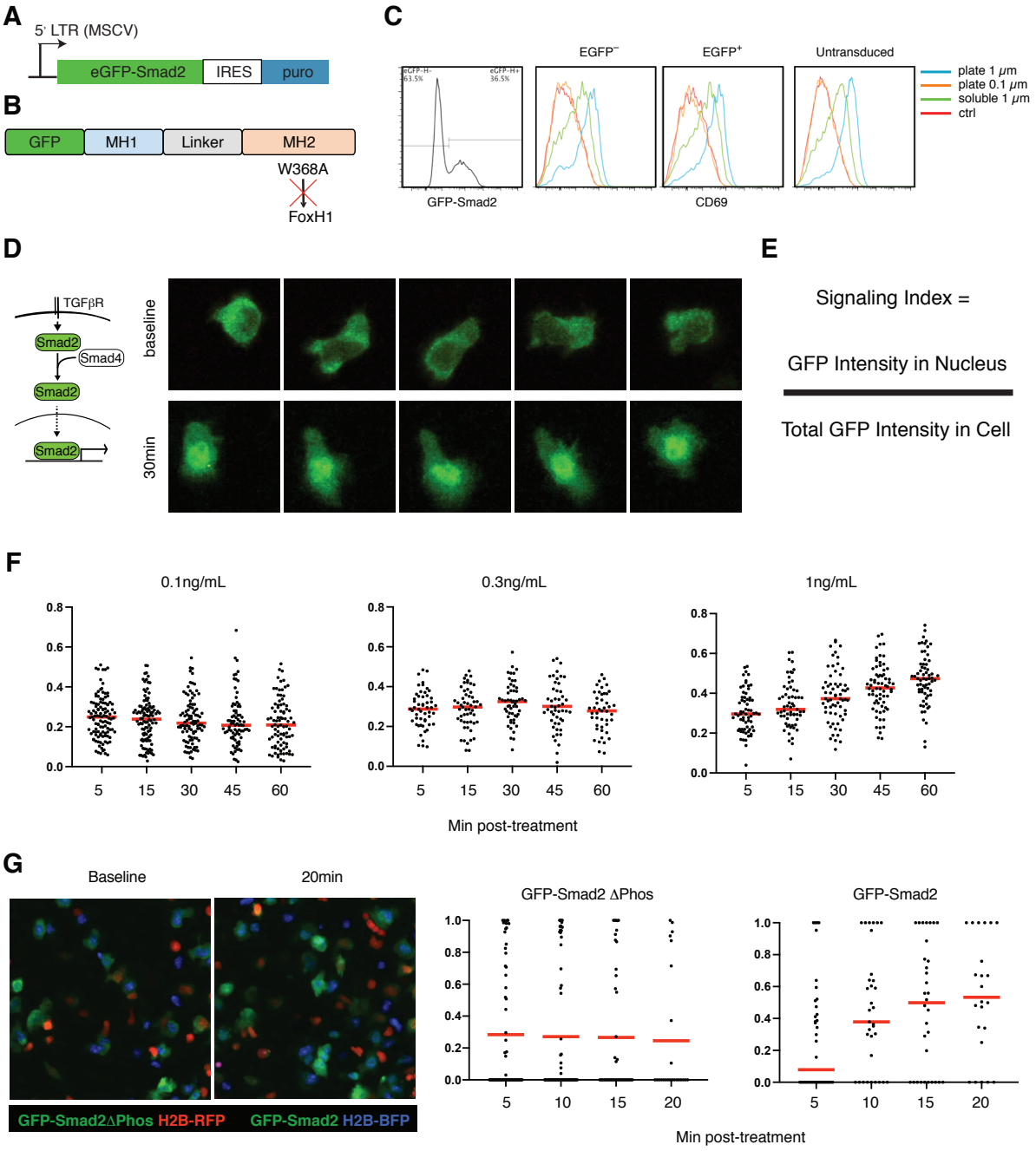
through the activity of αV integrins *in vivo.* While the importance of TGF-β activation in the differentiation of many T cells subsets is established in prior studies as well as the ones we present here, the fundamentals behind how T cells actually receive these signals *in vivo* is still unknown. Studies of TGF-β activation by dendritic cells, including our own (Chapter 2), suggest that receipt of productive TGF-β signals may be restricted to instances of cellular interactions that may drive, by mechanical force, the presentation of active TGF-β at the immunological synapse. However, there is little concrete evidence of this occurring *in vivo*, due to the limited tools at our disposal. We therefore sought to create and validate tools for this purpose.

Canonical TGF-β signal transduction relies on phosphorylation of the receptor-regulated Smads (R-Smads) Smad2 and Smad3, which then form a complex with Smad4 to shuttle from the cytoplasm to the nucleus. As nuclear translocation of the Smads occurs rapidly following a TGF-β stimulus, we thought that we would be able to harness this property to adapt a reporter of acute TGF-β signaling. Studies of TGF-β and Activin signaling have demonstrated that upon cytokine stimulus of human, mouse or xenopus cells, a constitutively expressed fluorescent protein-tagged Smad fusion protein can translocate from the cytoplasm to the nucleus, serving as a reporter of TGF-β signaling[148,232]. As Smad2 is thought to accumulate in the nucleus more selectively as compared to Smad4 upon its phosphorylation in a TGF-β dependent fashion[148], we decided to adapt this reporter for use in T cells.

We first obtained a GFP-Smad2 cDNA and cloned the construct into an MSCV backbone plasmid containing a puromycin selection marker following an IRES site where GFP-Smad2 expression is driven by a 5'LTR (**Figure 4.1A**). Upon retroviral transduction of this reporter into our cells, we initially observed toxicity in our cultures that we attributed to the overexpression of transcriptionally active Smad that renders T cells hypersensitive to TGF-β signaling. Therefore, we introduced a W368A mutation in Smad2 (hereafter referred to as GFP-

Figure 4.1 GFP-Smad2 reports TGF-β signaling in T cells *in vitro.* (**A**) Schematic of retroviral construct and (**B**) W368A mutation which abrogates Smad2 association with FoxH1 transcription factors. (**C**) $CD8^+$ T cells transduced with GFP-Smad2 were re-stimulated with plate bound or soluble CD3/28. CD69 expression was assayed by flow cytometry 24 hours after re-stimulation. (**D**) GFP-Smad2-expressing $CD8^+$ cells were treated with 1ng/mL TGF-β1 and live cells were imaged by confocal microscopy in a chamber at 37°C, 5% CO_2. (**E**) Signaling index represents the fraction of GFP signal intensity detected in the nucleus. (**F**) GFP-Smad2 expressing $CD8^+$ T cells were treated with increasing doses of TGF-β1 *in vitro* and live cells were imaged for 1 hour by confocal microscopy in a chamber at 37°C, 5% CO_2. Signaling index was determined on cells at each given time-point. Red line denotes median. (**G**) $CD8^+$ T cells were transduced with either GFP-Smad2ΔPhos and H2BtagBFP or GFP-Smad2 and H2BmRFP. Reporter-bearing cells were treated with 1ng/mL TGF-β1 and live cells were imaged by confocal microscopy in a chamber at 37°C, 5% CO_2. Red line denotes median.

Figure 4.1 (Continued)



Smad2) that prevents its interaction with FoxH1 transcription factors (**Figure 4.1B**), thereby reducing its transactivation activity without altering its nucleocytoplasmic shuttling capacity and kinetics[148]. After retroviral transduction of OT-I (T cell receptor transgenic recognizing the MHC-I restricted epitope for chicken ovalbumin) T cells with this mutant reporter construct, we verified that cells expanded normally in culture and that cells that were transduced with the reporter appeared phenotypically similar to un-manipulated cells *in vitro*. Of note, we observed no changes in CD62L down-regulation, indicating minimal alteration to cell homing capacity to tissues *in vivo*, as well as no difference in transduced cell CD69 up-regulation upon re-stimulation with anti-CD3/28 (**Figure 4.1C**).

In order to test whether our construct reported sensitivity of transduced $CD8^+$ T cells to TGF-β signals, we plated GFP-Smad2 transduced T cells on ICAM-coated coverslips and recorded time-lapse movies before and after addition of 1ng/mL of activated TGF-β1. While GFP-Smad2 was observed to be mostly cytoplasmic before addition of TGF-β1, within 30min of treatment with cytokine, the reporter began to accumulate in the nucleus (**Figure 4.1D, Supplementary Video 4.1**). We quantified this based on a measurement called the signaling index[231], which represents the fraction of reporter found in the nucleus (**Figure 4.1E**). An *in vitro* dose titration revealed a modest increase in the signaling index with increased doses of TGF-β *in vitro* (**Figure 4.1F**). Therefore, GFP-Smad2 nuclear accumulation can report a graded response to TGF-β signaling.

Notably, in comparison to previously described reporters of signaling in T cells, such as NFAT-GFP[231] which are essentially binary in their nuclear vs. cytoplasmic residence, we consistently observed some baseline level of GFP-Smad2 in the cytoplasm after stimulus, as well as in the nucleus without stimulus with TGF-β, and a dynamic range of the signaling index between 0.2 and 0.7 *in vitro*. While this may be due to residual TGF-β in culture from serum

possibly resulting in desensitization of the signaling pathway, it could also be an effect of Smad2 over-expression in cells causing nuclear accumulation of the reporter, or even a property of endogenous Smad2 itself that is also reflected in our reporter. We therefore thought to design and utilize a control reporter to use alongside the GFP-Smad2 reporter that would allow for us to estimate the background signaling index. To this end, we introduced two new mutations (S465/7A) in GFP-Smad2, resulting in a variant of Smad2 that is unable to get phosphorylated for translocation into the nucleus upon TGF-β stimulus (GFP-Smad2ΔPhos). Upon co-culture of cells expressing either reporter, distinguished by two different nuclei, and subsequent treatment of the culture with TGF-β1, we indeed observed no change in the signaling index of the GFP-Smad2ΔPhos expressing cells, but did observe a steady increase in the signaling index of GFP-Smad2 expressing cells over time (**Figure 4.1G, Supplementary Video 4.2**). Therefore, GFP-Smad2ΔPhos can be used as a control reporter that does not translocate into the nucleus upon stimulus with cytokine.

GFP-Smad2 reports sustained TGF-β signaling in eT_{RM} in vivo

After validation of our reporter systems *in vitro*, we moved forward with *in vivo* validation. While the surface phenotype of $CD8^+$ T cells transduced with our reporter was similar to those that did not bear GFP-Smad2, we also wanted to ensure that reporter-bearing cells were able to undergo a normal differentiation process *in vivo*. We therefore thought to test our GFP-Smad2 reporter during eT_{RM} differentiation, a context where cells require TGF-β signaling for their lodgment and epidermal maintenance and that we could monitor cells longitudinally to pinpoint any potential abnormalities in the process. We also sought to visualize T cells alongside other immune cells in this process, to give us a landmark of where they may migrate and signal. While we have shown in Chapter 2 that DC were not critical for the activation of TGF-β in the skin during eT_{RM} differentiation, we thought that CD11c-mCherry reporter bearing mice would give us

a relatively stable point of reference in addition to hair follicles. Therefore, we chose to use CD11c-mCherry mice as hosts for the intravital imaging experiment. To monitor GFP-Smad2 signaling dynamics *in vivo*, we activated OT-I T cells *in vitro* and transduced them either with GFP-Smad2 and H2B-mRFP, to mark the nucleus, or as a control, GFP-Smad2ΔPhos and H2B-tagBFP to distinguish the two reporters from each other *in vivo* (**Figure 4.2A**). We then sorted reporter-bearing cells from culture by magnetic separation and adoptively transferred them at a 1:1 ratio *in vivo*, intradermally injecting 10^5 of each of the GFP-Smad2-H2B-mRFP bearing cells or control GFP-Smad2ΔPhos H2B-tagBFP bearing cells, in the ear skin. We then performed longitudinal multiphoton intravital microscopy (MP-IVM) of the ear skin of mice to monitor GFP-Smad2 signaling dynamics and cell behavior. Over time, we observed normal migratory behavior of the cells, as well as eventual migration into the epidermis where they adopted a dendritic morphology (**Figure 4.2B, Supplementary Video 4.3**). Reporter-bearing cells were seen to be migrating in a characteristically confined area upon reaching the epidermis[72], above the Langerhans cells (marked by CD11c-mCherry reporter). T cell entry into the epidermis and adoption of a stable morphology and migratory phenotype reassured us that the GFP-Smad2 reporter did not significantly interfere with T cell differentiation.

To determine whether the GFP-Smad2 reporter was functional *in vivo*, we quantified the signaling index of epidermal $CD8^+$ T cells bearing the reporter, based on evidence of their sustained TGF-β signaling in the epidermis[172]. In a representative 30min movie, we found reporter-bearing cells (red nucleus) signaling with between 30 to 60 percent GFP-Smad2 in the nucleus (**Figure 4.2C-D**). In the same region, we also quantified the signaling index of a GFP-Smad2ΔPhos (blue nucleus), which was seen to rest stably at around 20-30 percent of the reporter in the nucleus (**Figure 4.2D**, blue line). Considering that this background was lower than the signaling we observed in the GFP-Smad2 bearing cells, we concluded that our reporter

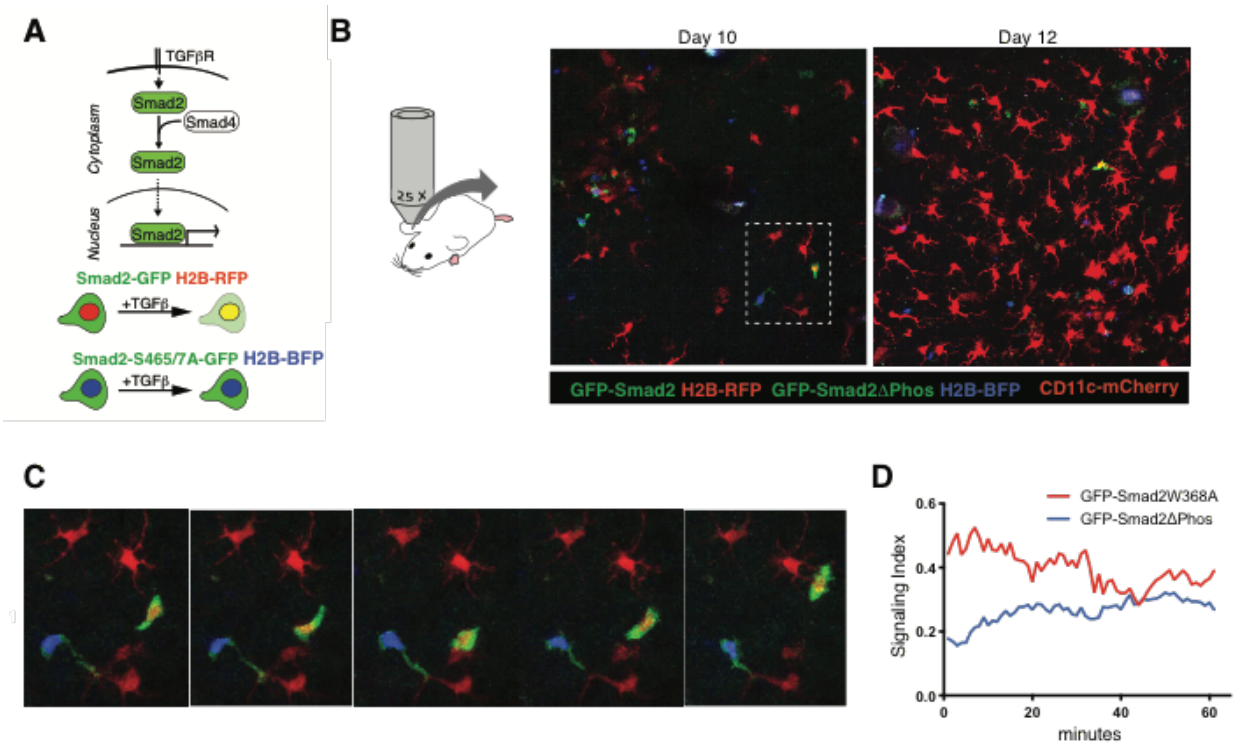


Figure 4.2 GFP-Smad2 reports TGF-β signaling in epidermal CD8$^+$ T cells *in vivo.* (**A**) Activated CD8$^+$ T cells were transduced with either GFP-Smad2ΔPhos and H2BmRFP or GFP-Smad2 and H2BtagBFP. 5 days after activation, 10^5 reporter-bearing cells were transferred intradermally into the ear skin at a 1:1 ratio into Albino CD11c-mCherry hosts. (**B**) Ear skin was longitudinally imaged for two weeks post-transfer. Representative images were selected. (**C**) Signaling index was quantified in cells bearing GFP-Smad2ΔPhos and GFP-Smad2 in the same field of view from day 10 post-injection of cells in the skin epidermis.

was indeed reporting moderate levels of TGF-β signaling *in vivo* in the epidermis, perhaps mediated through contact with epithelial cells that are able to activate TGF-β.

STAT1-GFP reports receipt of IFNγ signals by tumor cells

In effector T cells, TGF-β is known to limit not only their survival[99] but also their polyfunctionality by down-modulating the production of effector cytokines such as TNFα and IFNγ[165]. IFNγ in particular is known to play multiple roles in regulating the immune microenvironment, including the up-regulation of MHC Class I to promote antigen presentation and recruitment of effector cells by eliciting production of chemokines, or on the flip side, up-regulation of PD-L1 to dampen the inflammatory response. While IFNγ is well established as a potentiator of immune responses, the exact terms by which the cytokine is released (e.g. restricted to cellular synapses or effective in a soluble context) is still yet to be dissected. Existing tools to study IFNγ signaling *in vivo* are limited to transcriptional reporters such as GREAT or Yeti mice[233,234] which only report the transcription of the cytokine by cells, and therefore may not faithfully indicate the actual release of the cytokine. IFNγ-GFP fusion constructs have also been used to study the production of the protein[235]. However, *in vivo*, the GFP signal is not dense enough to quantitate the release of cytokine onto target cells with the existing microscopy tools. We therefore thought to build a reporter to read out the release of IFNγ that could be measured in cells responding to the cytokine *in vivo*.

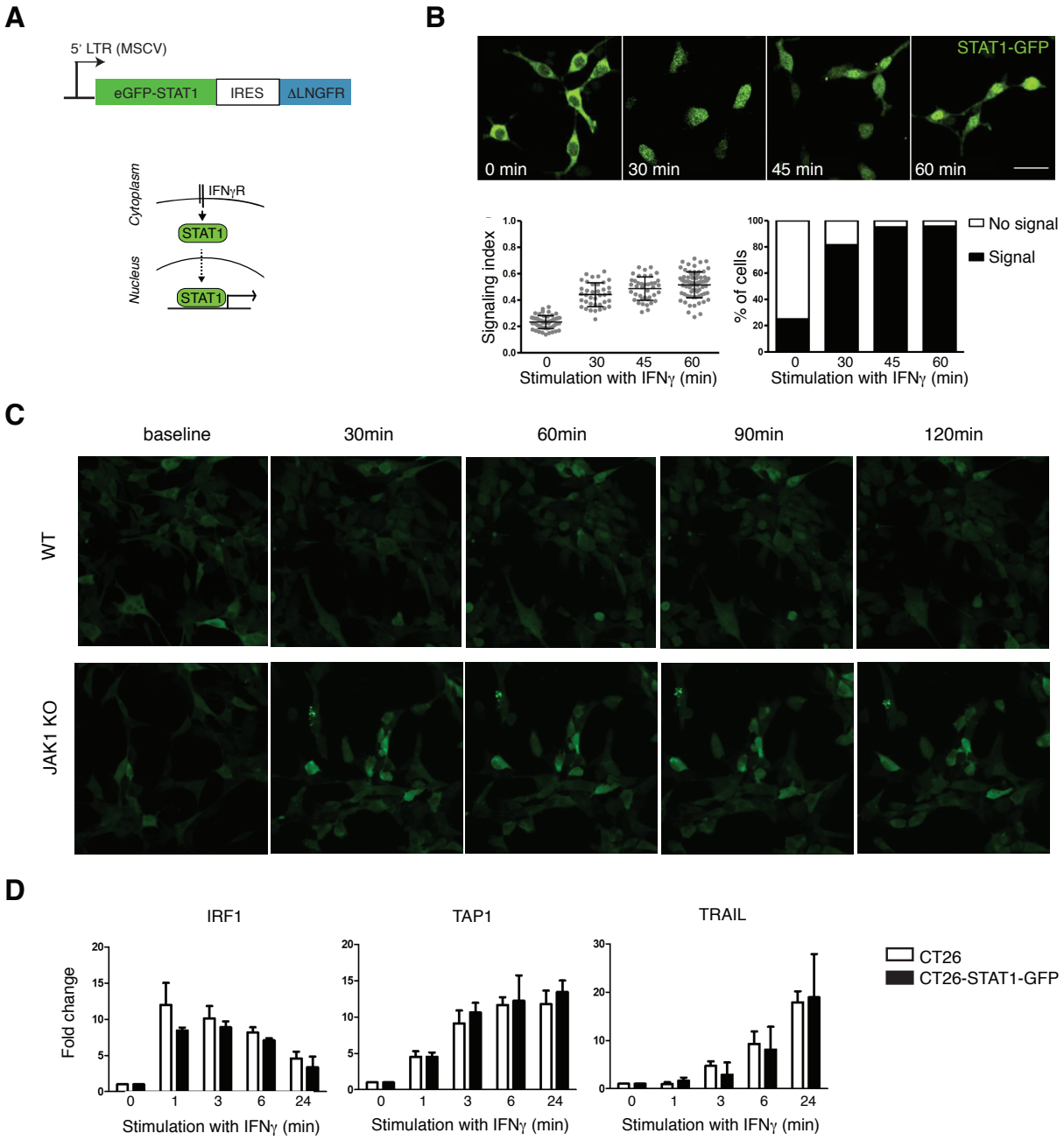
One particular context of IFNγ signaling that has rapidly gained interest is the role of cytotoxic T lymphocyte (CTL)-derived IFNγ in the anti-tumor response[68,69,236,237]. IFNγ in the tumor microenvironment can play a potentiating role on the endothelium[235], antigen presenting cells, or tumor cells themselves, on which IFNγ coordinates the up-regulation of both MHC Class I as well as the inhibitory receptor PD-L1. Given the complexity of IFNγ signaling in the tumor, as well as the emerging relevance of the pathway in anti-tumor responses, we wanted to

identify ways to read out IFNγ release in the tumor microenvironment. In order to read out IFNγ signaling in tumor cells, we decided to use a similar strategy as with the TGF-β signaling reporter, taking advantage of nucleocytoplasmic shuttling capacities of transcription factors. Upon binding of IFNγ to IFNγR1, kinase JAK1 phosphorylates transcription factor STAT1, causing its activation and translocation from the cytoplasm into the nucleus. This can be visualized using a C-terminal GFP tagged STAT1[238], which also translocates from the cytoplasm to the nucleus upon exposure of cells to IFNγ. We chose to adapt this reporter for use in a panel of tumor cell lines, including CT26 colon carcinoma, MC38 colon carcinoma, and the D4M.3A melanoma. We obtained a plasmid containing the STAT1-GFP reporter and cloned it into a vector that could drive its stable constitutive expression via the 5' LTR. We then transduced tumor cell lines with the reporter, sorted cells within a middle range of STAT1-GFP expression (so as to avoid toxicity and risk of overexposure during imaging), and proceeded to test the responsiveness of this reporter to IFNγ stimulus *in vitro*.

Upon treatment of reporter-bearing tumor cells with 1μg/mL of IFNγ *in vitro*, we observed a steady increase in the signaling index beginning within minutes of exposure to cytokine (**Figure 4.3B**). One hour following initial exposure to IFNγ, nearly all of the tumor cells had accumulated STAT1-GFP in the nucleus from baseline (**Figure 4.3B**). Furthermore, in our control isogenic tumor cell lines with a JAK1 deficiency, STAT1-GFP did not translocate to the nucleus upon IFNγ treatment (**Figure 4.3C**). As we were using a wild-type (WT) human STAT1 in our fusion protein, we wanted to ensure that the overexpression of the reporter in cells would not interfere with endogenous STAT1 transcriptional activity. To this end, we treated reporter-bearing or untransduced tumor cells with IFNγ *in vitro*, and performed qRT-PCR on known STAT1 target genes such as *Irf1, Tap1, Trail*. In a time-course after treatment of the cells, we observed no differences in the mRNA levels of STAT1-induced transcripts between reporter-

Figure 4.3 STAT1-GFP reports receipt of IFNg signals by tumor cells *in vitro*. (**A**) Schematic of STAT1-GFP reporter construct in a retroviral vector. (**B**) CT26 colon carcinoma cells were transduced with STAT1-GFP and stimulated with 1μg/mL of IFNg. Signaling index was measured in cells fixed at indicated time point post-stimulation. (**C**) WT or JAK1-deficient D4M.3A UV3 melanoma cells were transduced with STAT1-GFP reporter, stimulated with 1μg/mL of IFNg and live cells were imaged for 2 hours by confocal microscopy in a chamber at 37°C, 5% CO_2. (**D**) Tumor cells transduced or not with the STAT1-GFP reporter were stimulated with 1μg/mL of IFNg and mRNA was harvested at indicated time points for RT-PCR of target genes, normalized to beta actin.

Figure 4.3 (Continued)



bearing cells and the original parent cell line (**Figure 4.3D**). Therefore, we concluded that STAT1-GFP was a reliable reporter of IFNγ signaling in tumor cells and could be used as such without significantly impacting signaling pathways within the tumor cells.

STAT1-GFP reports IFNγ signaling in vivo shortly after anti PD-1 treatment

We then proceeded to identify contexts to study IFNγ signaling *in vivo*. Studies of human and mouse melanomas has revealed that successful PD-1 checkpoint blockade therapy relies on responsiveness to IFNγ and the STAT1 signaling axis[236,239]. We therefore thought to test the dynamics of our STAT1-GFP reporter *in vivo* in the context of anti-PD-1 therapy in a melanoma that is responsive to checkpoint blockade. To this end, we implanted D4M.3A.3 UV3 tumors (amelanotic $BRAF^{V600E}$/PTEN melanoma with UV-induced mutations rendering enhanced responsiveness to checkpoint blockade therapy by neo-antigen introduction) subcutaneously on the back skin of C57BL/6 mice and installed dorsal skin fold chambers (DSFC) in order to longitudinally visualize the tumor by MP-IVM (**Figure 4.4A**). One week after tumor implantation, we began to visualize STAT1-GFP reporter dynamics in tumor cells. Prior to treatment with αPD-1 antibody, we observed low levels of STAT1-GFP in the nucleus, perhaps attributable to microenvironmental triggers of STAT1 signaling, such as type I interferons. In the two days following treatment with αPD-1 antibody, we began to observe an increase in the signaling index *in vivo* (**Figure 4.4A**). As evidenced by the blebbing of nuclei (marked by H2B-mRFP), tumor cytotoxicity was detectable after αPD-1 treatment. By 4 days post-treatment with αPD-1, the tumor parenchyma was barely detectable by imaging. To understand whether this response to αPD-1 treatment was due to IFNγ signaling in the tumor, we also conducted the same timecourse experiment in mice implanted with D4M.3A UV3 isogenic cell lines lacking IFNγR1. While we also observed some baseline signaling prior to treatment in these tumors at relatively the same levels as in the WT tumors, upon treatment of the IFNγR1-deficient tumors with αPD-

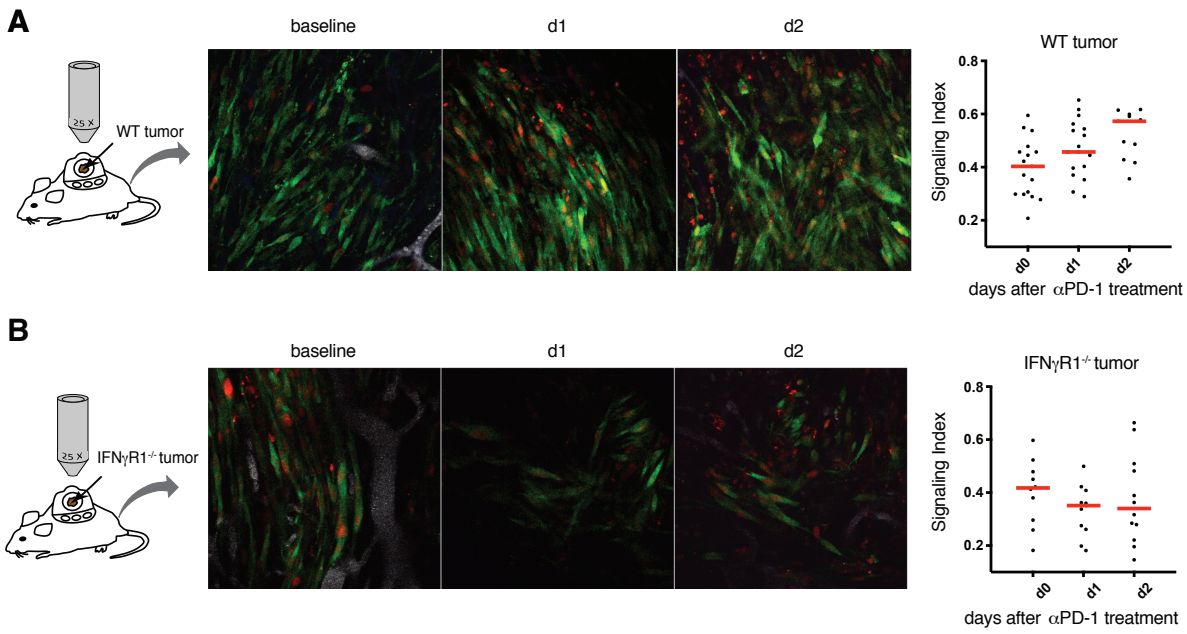


Figure 4.4 STAT1-GFP reports IFNg signaling in tumor cells following checkpoint blockade therapy. (**A**) 10^6 reporter-bearing D4M.3A.3 melanoma cells or (**B**) IFNgR1-deficient D4M.3A.3 were implanted into C57BL/6 mice and a dorsal skinfold chamber was implanted on the back of mice to visualize tumor longitudinally by intravital microscopy. One week following implantation of tumor, mice were imaged and injected with 200μg anti-PD-1 antibody intravenously. Signaling index was measured over time. Each point represents a distinct tumor region. Red line denotes median.

1, we did not observe any changes in the STAT1 signaling index of tumor cells (**Figure 4.4B**), suggesting that the increase we were seeing in STAT1-GFP signaling after αPD-1 treatment was due to the effects of IFNγ. Therefore, our reporter shows increased IFNγ signaling upon checkpoint blockade therapy *in vivo*.

STAT1-GFP activation in the tumor is dependent on Ag-specific CTL

As CTL are a predominant source of IFNγ in the tumor microenvironment we also looked to test our reporter dynamics *in vivo* alongside CTL in the tumor, in an attempt to understand the context under which IFNγ can be secreted by these cells. We have previously shown in a CT26 colon carcinoma model system that CTL isolated from these tumors have the capacity to produce and secrete IFNγ both in the presence and absence of their cognate antigen[240]. However, Ag recognition by CTL in the tumor is thought to be required for tumor regression[241]. Therefore, we hypothesized that local Ag recognition may also be required for IFNγ secretion by CTL in order to potentiate an anti-tumor response. We turned to this model again to test our hypothesis, implanting reporter-bearing CT26 tumors expressing model antigen hemagglutinin (HA) or not, into Balb/c mice and visualizing them with an installed DSFC. Each animal also received HA-specific CTL that were differentiated *in vitro* and transduced with a tdTomato reporter (red). As we hypothesized, over the few days following injection of HA-specific CTL, the fraction of signaling cells increased in the HA-expressing tumors, but not in the HA^- tumors (**Figure 4.5A-B**). Interestingly, while we did not necessarily observe many synapses between tumor cells and CTL that seemed to trigger STAT1-GFP translocation, the density of CTL in a given area also correlated with the percentage of cells that were signaling in the HA-specific tumor (**Figure 4.5C**). The use of the STAT1-GFP reporter *in vivo*, therefore, can also report Ag-dependent release of IFNγ by CTL within the tumor microenvironment.

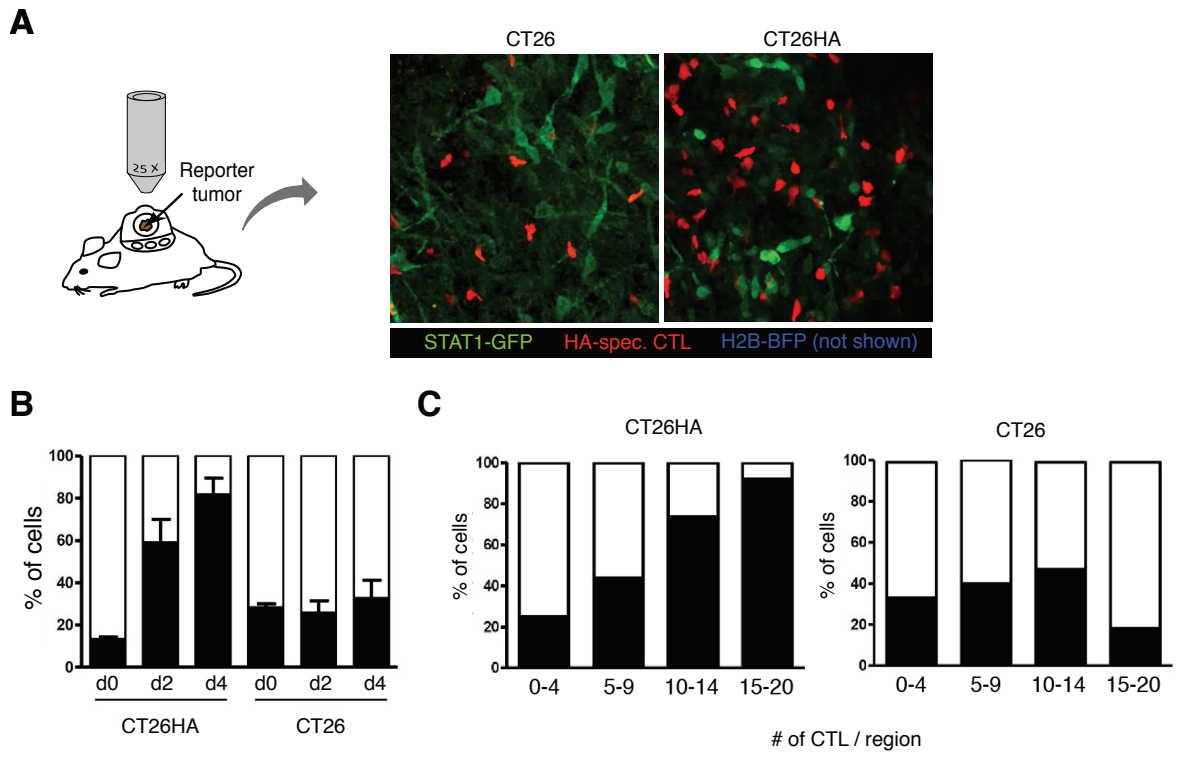


Figure 4.5 STAT1-GFP activation is induced by CTL recognizing tumor antigens (**A**) 10^6 reporter-bearing CT26 or CT26HA cells were implanted subcutaneously into Balb/c mice and a dorsal skin fold chamber was installed for longitudinal intravital imaging. Seven days after tumor implantation, 10^6 HA-specific CTL (CL4) transduced with tdTomato were injected intravenously and longitudinal imaging was conducted. Percentage of signaling cells (Signaling Index above 0.3) was quantified (**B**) on indicated days post-injection of CTL and (**C**) in a given region based on the number of CTL present.

DISCUSSION

Here, we have presented preliminary characterization and validation of tools to study the receipt and release of cytokines in $CD8^+$ T cells *in vivo*. Initial applications following the successful validation of these reporters will include expanding our characterization of STAT1 signaling dynamics in the tumor microenvironment upon exposure to various therapeutic agents, such as low dose radiation, other forms of checkpoint blockade and cellular therapies, as well as further dissecting how CTL-derived IFNγ may be delivered to target cells to allow for both anti-tumor responses as well as immunoregulatory responses, such as PD-L1 up-regulation. Additionally, we seek to understand the nuances of TGF-β activation and subsequent signal transduction in $CD8^+$ T cells. For example, expanding upon the studies we described in Chapter 2, we could determine where and how naïve $CD8^+$ T cells actually take in productive TGF-β signals at homeostasis in the lymph nodes, and if this is indeed restricted to contacts *in vivo* between T cells and specific subsets of DCs. This will offer insight into our initial questions as to how TGF-β may be activated and presented to T cells *in vivo*, a question that has remained elusive to many in the field for decades due to limited tools for *in vivo* study. While certain contexts of TGF-β signaling may be contact-dependent, there may also be other biological settings where this may not be the case, such as in the tumor microenvironment or other barrier tissues where active TGF-β may be released in inflammatory contexts, for example, by matrix metalloproteinases. Mapping and comparing signaling dynamics between these settings may offer us insights on how to more specifically target the pathway in various therapeutic settings, as well as more generally understand the intricacies of the biology *in vivo*.

In our initial characterization of our reporters for both TGF-β and IFNγ signaling, we have not yet addressed certain technical or biological caveats that would be critical to the interpretation of the data. There is the possibility that non-canonical signal transduction

occurring independently of Smad2 and STAT1, such as MAP kinase-driven signaling [171], would be missed in our use of these reporters. Therefore, selection of biological contexts where signaling predominantly occur through the canonical pathway, in combination with transient blockade of alternative transduction pathways will also be critical to draw a link between signaling reporter activity and ligands of interest *in vivo*. Additionally, both STAT1 and Smad2 can be activated by signals other than IFNγ and TGF-β, respectively. Smad2 can also be activated by activin signaling, as the receptor involved in the phosphorylation of Smad2 is also involved in the signal transduction of activin ligands[171]. To control for this, we could turn to conditional knockouts of signaling receptors, or small molecule and antibody based inhibitors of various ligands. While we have attempted to control for this in the tumor studies of STAT1-GFP using IFNγR-deficient tumor cells, in order to account for the variability between animals, internal controls may be more informative. For example, we could conduct imaging of IFNγR-deficient and WT cells distinguished by two distinct nuclear markers, mixed within the same tumor parenchyma in a given animal, may also be required for initial characterization of the reporter's dynamic range.

Future use of these reporters *in vivo* is also subject to additional technical hurdles. For example, the quantitation of signaling index in tumor cells *in vivo* in an automated fashion is not adequately representative, as computational programs have a limited ability to distinguish between the borders of tumor cells in a 3D setting. Therefore, much of the analysis we presented here of STAT1 signaling *in vivo* has been restricted to manual selection of representative slices and 2D automated quantification. While this is representative of the visual and biological changes we observe in these experimental settings, less reliance on manual measures is not only critical for reproducibility and consistency in data measurement, but also for uncovering intricacies in signaling and heterogeneity *in vivo*. In the tumor cells, for example,

we could begin to overcome the challenges of proper detection by the addition of membrane labels in distinguishing colors that would allow for better definition of cell borders in the tumor. Additionally, the application of deeper computational analyses, including computer vision-based models, would likely allow for more nuanced interpretation of the data at hand. This may even help us to determine whether the heterogeneity we observe in signaling index is truly a measure of differences in biological activity within a given system or simply an artifact of inconsistencies in measurement.

While being cognizant of the caveats, these studies can, of course, also be extended to other regulatory signaling pathways, such as NF-κB, and even metabolic pathways such as mTOR, potentially revealing previously unappreciated regulatory mechanisms. We have often considered transcription factors to react to ligands in a dose-dependent fashion, which we can refer to as "amplitude modulated" activity. However, drawing from lessons in the activities of bacterial transcription factors, there is an increasing body of literature that suggests that mammalian transcription factors can also operate in a pulsatile fashion, and that each given transcription factor may even have a set of transcriptional targets that can be modulated distinctly by their "amplitude" or by their "frequency" [242,243]. A deeper understanding of the correlation between signal "amplitude" and signaling index, as well as monitoring of nucleocytoplasmic shuttling using our reporter systems may reveal these distinctions *in vivo*. We have, in fact, already observed hints of this in the TCR signaling cascade, where the residence of the reporter NFAT-GFP in the nucleus in a frequency versus amplitude modulated fashion correlates with differential transcriptional output, such as IFNγ versus *Egr2* transcription[231]. In combination with other relevant analyses such as transcriptome and proteome profiling, our reporter systems can be extremely powerful in allowing us to uncover the intricacies of interactions and signals *in vivo* across the immune system.

MATERIALS AND METHODS

Mice

C57BL/6, C57BL/6$^{Tyrc-2J}$, CD45.1 and Balb/c mice were obtained from JAX. OT-I TCRa$^{-/-}$ mice were obtained from JAX. CD11c-mCherry mice were a gift from Dr. Kamal Khanna (UConn) and bred in house to C57BL/6$^{Tyrc-2J}$ mice. CL4 TCR transgenic mice (CD8^{+} specific for HA) were bred in-house. Animals were housed in specific pathogen-free facilities at the Massachusetts General Hospital (MGH) and all experimental studies were approved and performed in accordance with guidelines and regulations implemented by the MGH Institutional Animal Care and Use Committee (IACUC).

Tumor cell lines

D4M.3A.3 UV3 melanoma lines were obtained from Dr. David Fisher (Massachusetts General Hospital) and isogenic knockout lines of STAT1 signaling pathway were obtained from Dr. Nicholas Haining (Dana Farber Cancer Institute). CT26 tumor lines were originally obtained from ATCC and transduced in-house with hemagglutinin (HA). Tumor lines were cultured in DMEM, 10% FCS, 1% HEPES, 1% GlutaMAX, 1% Sodium Pyruvate, and Ciprofloxacin.

Multiphoton intravital microscopy of the ear skin and subcutaneous tumors

Tyrosinase mutant B6 (C57BL/6$^{Tyrc-2J}$) “Albino B6” mice obtained from Jackson Laboratories were used as host mice for two-photon imaging so as to prevent melanin-dependent auto-fluorescence from interfering with the imaging of events in the ear skin. After adoptive transfer of cells as indicated, mice were prepared for longitudinal intravital imaging. Prior to the first imaging session, the ear skin was lightly epilated using Nair. For tumor imaging, Balb/c or C57BL/6 mice obtained from Jackson Laboratories were used as hosts. 10^6 tumor cells were

injected subcutaneously on the flank and dorsal skin fold chambers were installed to visualize the tumors as previously described[244]. Mice were given buprenorphine as an analgesic as necessary. For each imaging session, mice were anesthetized using isofluorane (Isothesia) delivered via a SomnoSuite MSTAT (Kent Scientific). Anesthesia was maintained at 1-2% isofluorane at a flow rate of 30-40mL/min. To prepare the ear skin for imaging, a custom-built stage to fix the mouse ear on was heated to 34°C using a feedback based heating apparatus (Warner Instruments). The body of the mouse was maintained at 37°C using a feedback-based rectal probe thermometer and heating pad. The ear pinnae was affixed to the imaging stage using double-sided tape (3M) on the ventral side of the ear. The lens (Olympus 25X) was immersed in Genteal Eye Gel (Alcon), covering the top of the ear skin and the lens was heated sufficiently to maintain the interface temperature at 34°C. Dorsal skin fold chambers were also heated to maintain a tumor temperature of 37°C. Mice in each imaging session were under anesthesia for no more than 6 consecutive hours. An Olympus custom MPE-RS system was used for image acquisition in laser scanning mode, with Insight and DeepSee Broadband (Spectra Physics) lasers. Each z stack was acquired in Z-stacks with cycles lasting 30 or 60 seconds, varying depth and 3μm spacing between slices. Olympus FV30S software was used for acquisition and conversion of raw data files.

Analysis of intravital microscopy images

Movies were quantified, smoothed and edited using Imaris 8.3.1 software (Bitplane). Briefly, images were first stabilized for drift correction, and reporter-bearing cells were identified using "Cells" function, which distinguishes the nucleus from the cytoplasm. Signaling index in T cells in the skin was determined from 3D automated analysis of signal intensity from Imaris. Signaling index in tumor cells was determined using 2D analysis of nuclear GFP intensity in representative slices. Z-stacks were thresholded to reduce autofluorescence and background

fluorescence intensity, and then converted into *.mov or *.mp4 movies for easy viewing. Graphs were plotted using Prism 8 software (GraphPad).

In vitro activation, retroviral transduction

OT-I splenocytes were pulsed with 1 μM SIINFEKL peptide in 1 mL of T cell medium (RPMI, 10% FCS, 1% HEPES, 1% Sodium Pyruvate, 1% GlutaMAX, 1% non-essential amino acids, 55 μM 2-mercaptoethanol) for 1 hr at 37°C, diluted in 9 mL of T cell medium, and cultured at 37°C in 5% CO_2. Activated T cells were transduced with retrovirus for fluorescent proteins and reporters. Briefly, on day 1 and day 2 post-activation, cells were plated at 10^6/mL in viral supernatant containing 4μg/mL polybrene, and spin-infected for 90 minutes at 32°C at 1000g. After spin-infection, the viral supernatant was replaced with T cell medium. T cell cultures were supplemented with 20ng/mL IL-2 beginning two days post-activation. Tumor cells were plated at 80% confluency in a 24-well plate for retroviral transduction as indicated for T cells. VSVg pseudotyping was required for D4M.3A.3 cells, as they do not have the ecotropic receptor for retrovirus. Transduced cell lines were selected by FACS sorting.

Adoptive transfer of T cells

Activated T cells were adoptively transferred for tumor CTL experiments by intravenous injection of 10^6 cells/100μL PBS 7 days after activation. Alternatively, for skin studies, activated T cells were intradermally injected 10^5 cells/10μL in PBS into the ear skin 5 days post activation for intravital imaging using 30G needle, with the bevel facing up towards the dorsal side of the mouse and needle pointing towards the outer edge of the ear, at a manual rate of roughly 1μL per second to facilitate lateral spreading of cells.

ACKNOWLEDGEMENTS AND CONTRIBUTIONS

V.M. performed *in vivo* experiments with GFP-Smad2 and all *in vitro* and *in vivo* D4M.3A tumor experiments. V.M. and E.C. performed *in vitro* characterization of GFP-Smad2 reporters. V.M. cloned the STAT1-GFP reporter. F.M. and E.C. cloned the GFP-Smad2 reporters. S.M.W. performed all experiments with CT26 tumors. R.T.M. and H.W.P. generated isogenic knockout tumor lines on the D4M.3A.3 background. V.M., E.C., and S.M.W. analyzed the data. V.M., E.C., and T.R.M. designed the experiments.

I would like to thank Drs. David Fisher, Jennifer C. Lo, Nicholas W. Haining and Jeffrey Hammerbacher for helpful discussions and providing critical reagents for the STAT1 tumor studies.

CHAPTER 5: Conclusions and Future Directions

Our work has uncovered surprising and previously unappreciated functional consequences of TGF-β signaling as they pertain to the development of eT_{RM}. First, when we investigated the role of DC-mediated TGF-β activation on eT_{RM} differentiation, we found that naïve $CD8^+$ T cells require homeostatic conditioning prior to activation that impacts their ability to form eT_{RM}. Homeostatic conditioning occurs through non-cognate interactions of naïve $CD8^+$ T cells with migratory DC in lymph nodes that shapes chromatin accessibility for efficient eT_{RM} differentiation. This finding reveals a novel stage of $CD8^+$ T cell regulation during homeostasis that can ultimately select or enrich for low self-reactive TCR clones for subsequent eT_{RM} differentiation. While this mechanism may regulate the generation of memory precursors, the current literature strongly suggests that terminal eT_{RM} differentiation also relies on TGF-β signaling as cells make their way into certain tissues, such as the skin and gut. Therefore, we also wanted to understand the role of TGF-β in the skin during terminal eT_{RM} differentiation. In intravital microscopy-based studies, we found that TGF-β regulates the spatial organization of T cells within the skin, allowing T cells to properly position themselves near hair follicles to enter the epidermis. This is facilitated by TGF-β-dependent induction of CCR6. To begin to further investigate where and when T cells may pick up TGF-β signals in the skin, we developed and validated a GFP-Smad2 reporter that can be used *in vivo* to acutely read out TGF-β signaling. We hope to be able to utilize this reporter in various contexts *in vivo* to understand how T cells acquire TGF-β signals that instruct distinct functions.

In this chapter, we briefly discuss the future directions and implications of our efforts as they pertain to the study of eT_{RM} and TGF-β in immunity.

TGF-β activation, context and dose dependency in signaling

We have demonstrated that TGF-β may serve to drive or stabilize programs of $CD8^+$ T cell differentiation at various stages. While there may be shared programs when different subtypes of $CD8^+$ T cells are exposed to TGF-β, our own data and that of others also suggests a distinct functional outcome upon productive TGF-β signaling at distinct stages of differentiation. For example, while TGF-β may drive the contraction of short lived effector cells[99] and limit polyfunctionality[165] during infection, the same cytokine may drive memory differentiation and of subsets even better poised to release inflammatory cytokines. This dichotomy can be explained in a number of different ways. First, it is thought that rather than driving a particular transcriptional program by acting as a master transcription factor such as T-bet, GATA-3 or Runx3, Smad tends to act moreso in concert with other transcription factors[245]. The promiscuity of Smad association with various transcription factors[149] for its function makes TGF-β signaling all the more reliant on other transcription factors and signaling pathways that are active within the cell. Moreover, as cells migrate into new environments after activation, the exposure to various cytokine stimuli in those regions or tissues may better "drive" a particular transcriptional state. For example, high levels of STAT5 activation[99] or TCR triggering may allow a cell to "overcome" certain aspects of TGF-β signaling, as we have seen in our studies as well. Whether the dose of TGF-β itself elicits varying transcriptional outcomes, and if this is even relevant *in vivo*, is not yet known but may also be a useful area of investigation.

Additionally, while TGF-β1 is thought to be the predominant isoform regulating immune function, this view may be based exclusively on the immunosuppressive functions of the cytokine. In various tissues containing lymphocytes that depend on constant TGF-β signaling, there may be other isoforms at play, such as TGF-β2 and TGF-β3. In the salivary gland, for example, the tissue resident natural killer cells are thought to be sustained by non-canonical

TGF-β signaling which may be driven by the higher expression of TGF-β2 and 3 in the tissue as compared to TGF-β1[135], which results in a distinct transcriptional program. Further investigation of the TGF-β isoforms and other superfamily members in the regulation of lymphocyte programs is an interesting area of future investigation that may allow us to understand which TGF-β targeted transcripts may be uniquely associated with which isoforms.

Finally, the mechanism by which TGF-β is activated and presented to the cell may also influence the transcriptional outcome, for instance by increasing the "dose" of TGF-β presented to the T cell during a cellular interaction, or by facilitating the co-presentation of multiple signals at the same time to optimize downstream functions (e.g. TCR + TGF-β). We observe distinct functional outcomes of TGF-β signaling in naïve cells as compared to activated memory precursor cells. While the former is seen to rely on αVβ8 expressed by dendritic cells, this is certainly not the case for terminal eT_{RM} differentiation. The source of TGF-β signaling for the latter is not yet known, but can be a number of factors that either depend on cellular interactions, for instance with αVβ8 expressing T_{reg} or activated myeloid cells in the skin, αVβ6 in the epithelium, or even ECM-released TGF-β independent of cellular interactions. We look to be able to uncover this with further studies utilizing the GFP-Smad2 reporter to determine when and where T cells may pick up TGF-β signals in the skin. The responses to TGF-β signaling in distinct tissue compartments may also be a function of the patterning of the major activating integrins αVβ6 and αVβ8. αVβ6 is expressed in non-migrating epithelial cells, while αVβ8 is expressed on activated cells of the immune system that have migratory capacities. Both integrins are also structurally distinct from one another and the mechanism by which they activate TGF-β might be distinct[142]. As epithelial cell residence may require a different program than immune activation, the difference in the mechanism of TGF-β activation may also relay a different functional outcome in target cells.

TCR signal strength and TGF-β

In Chapter 2, we found that naïve T cells with lower self-reactivity, and therefore lower grade of homeostatic TCR signaling, seemed to be preferentially conditioned for eT_{RM} formation. This regulation of lower self-reactive clones by TGF-β has also been demonstrated during thymic selection and in the maintenance of $CD4^+$ T cells by regulation of IL-7R[163]. Mechanistically, this may be due to the phosphorylation of the Smad linker region by Erk, thereby blocking Smad nuclear translocation[198]. We hope to follow-through on our findings by sequencing TCRs of naïve cells and eT_{RM}, and comparing the enrichment of high vs low self-reactive naïve cell clones within this pool of eT_{RM}, both in mice and humans. If, indeed, we confirm that naïve $CD8^+$ T cells with lower self-reactivity are better represented in the pool of eT_{RM}, this could have therapeutic implications even beyond the targeting of T_{RM}. One context where TGF-β is abundant and may actually contribute to immune dysfunction is in the tumor microenvironment, where the cytokine and its signaling pathways are highly active. In fact, TGF-β targeting is being explored in many respects in the tumor microenvironment. Recent studies have demonstrated that PD-L1 therapy can be more efficacious if combined with TGF-β blockade to stimulate the extravasation of T cells into the tumor tissue[246,247]. If TGF-β signaling impacts T cells of lower TCR affinity (to self or neoantigens) moreso than cells of higher affinity, TGF-β targeting in combination with checkpoint blockade therapy may be best applied in a setting where $CD8^+$ tumor infiltrating lymphocytes are poorly reactive to the tumor. Initial studies in our lab have begun to address this very question (*E. Carrizosa, unpublished*).

Given this phenotype, it is also curious that TGF-β signaling is both active and critical to the induction, function and maintenance of T_{reg}, which exhibit high levels of antigen receptor and STAT5 signaling [178,248-250], both of which are known to interfere with TGF-β signaling in $CD8^+$ T cells . As postulated before, this may be due to the difference in transcription factors that

associate with Smad in $CD4^+$ T cells as compared to $CD8^+$ T cells. Additionally, the mechanism by which T_{reg} acquire active TGF-β for signaling may be different from $CD8^+$ T cells. While T_{reg} may depend on other cellular sources of TGF-β activation for their initial induction, it is very likely that they are able to entirely control the production and activation of TGF-β autonomously after activation, while $CD8^+$ T cells may rely entirely on exogenous sources of TGF-β activation to sustain their signaling. Activated T_{reg} (and tissue-resident T_{reg}) express the relevant machinery for their sustenance, including GARP, which sequesters latent TGF-β on the surface of T_{reg}, and αVβ8, the activating integrin, and finally, they are even able to produce high levels of their own TGF-β[178,181,182,185,251]. A recent study offers a structural basis for this to occur as well[252]. This might even be a mechanism by which T_{reg} maintain a higher dose of TGF-β signaling and can thereby override the high TCR signaling. This is supported by literature demonstrating that the targeting of GARP may limit the immunosuppressive capacity of T_{reg} *in vivo*[253-255]. Studies understanding the circuitry and potential effects of TGF-β dosing, perhaps comparing T_{reg} and $CD8^+$ T cells would be informative in identifying methods by which each cell type can be specifically targeted.

Evidence towards an eT_{RM}-specific precursor

In the introduction, we explored the evidence in favor of or against a specific precursor for T_{RM} as compared to circulating memory T cells, deeming that it was unclear whether a certain mechanism or feature of a devoted precursor existed within the naïve or activated T cell repertoire. In Chapter 2, we presented evidence that there may be characteristics of TCR clones (e.g. low self-reactivity) that would enrich for the ability to form eT_{RM}. However, we also demonstrate that the enhanced capacity to form eT_{RM} may not necessarily preclude the ability of T cells to also differentiate into circulating memory cells. Existing literature showing significantly fewer TCR clones for memory cells in the skin as compared to LN memory cells reveals

perhaps that within the pool of memory precursors, there is yet another additional set of additional set of criteria that distinguish those with the capacity to form eT_{RM}. As we mentioned, this may be due to the "window of opportunity" within the tissue for T cells to be able to acquire the appropriate signals to migrate into the epidermis. If timing is so critical, eT_{RM} precursors may also derive from a certain cycle of division after activation, or a certain time point following SLO egress after activation. We also seem to only observe this enrichment in skin eT_{RM}, but it is likely that similar homeostatic mechanisms may exist to select for clones that are better poised to seed T_{RM} in other tissues, using signals from the tissues to guide this process. Further analysis to dissect these questions, utilizing single cell sequencing with a combination of fate mapping approaches, is critical for our understanding as to how T_{RM} formation can be optimized through the more robust generation of precursors.

The microbiota and TGF-β activation as a part of the migratory DC program

The finding that migratory DC are the vehicles for dissemination of information to naïve T cells for their eT_{RM} conditioning is no surprise, given their critical role in fortifying adaptive immunity at barrier sites[47,211,256]. A curious feature of the migratory DC signature is the enrichment of *Itgb8*, with known, specific functions of TGF-β activation[257,258]. It is in fact one of the top 20 signature genes identified in skin migratory DC by expression level[259]. Therefore, it is almost certainly intentional that TGF-β is tasked to imprint knowledge from the tissues using migratory DC as the messenger. The induction of *Itgb8* and other components of the migratory DC signature has been demonstrated in elegant studies to be driven both by homeostatic sensing of the microbiota at barrier surfaces[194], as well as by sustained IFNγ signaling during activation[260]. That this can be driven by IFNγ signaling may reflect the existence of a feed-forward mechanism that harnesses the sensing capacities of adaptive immune cells like eT_{RM} which produce IFNγ upon stimulus, allowing not only for the further generation of eT_{RM}, but also

cross-protective T_H17 and T_{reg} that promote adaptive immune regulation in barrier tissues. It will also be interesting to explore both the naïve $CD8^+$ T cell and eT_{RM} repertoire in germ-free or otherwise dysbiotic settings, where there would likely be a significant disruption of the conditioning we observe in WT mice.

TGF-β signaling in the conditioning of naïve $CD8^+$ T cells

We have shown in Chapter 3 that TGF-β can control the spatial organization of T cells within a tissue through its effects on chemokine receptor regulation. In addition to differential chromatin accessibility, it is likely that one mechanism by which naïve $CD8^+$ T cells are better poised to differentiate into eT_{RM} is via proper positioning within the SLO during priming, for instance by finding the appropriate antigen presenting cell "partner". Many studies have now converged upon the concept that APC subsets with distinct functionalities in priming $CD4^+$ and $CD8^+$ T cell responses segregate asymmetrically, and possibly deliberately, in the LN[202,204,261]. Elegant intravital microscopy based studies of Herpes Simplex Virus (HSV) specific $CD4^+$ and $CD8^+$ T cells during priming have indicated that specialized subsets of DC, in a strict spatiotemporally regulated process, prime $CD8^+$ responses[203]. This is controlled by migratory DCs activating $CD4^+$ T cell help which then licenses other DCs to prime effective $CD8^+$ T cell responses. Though the exact process by which $CD8^+$ T cells are primed in the lymph node for eT_{RM} differentiation is not known, it is possible that this may even involve serial interactions with DCs or be restricted to certain areas within the lymph nodes. The tight spatiotemporal control of such processes would make the epigenetic conditioning all the more critical. Therefore, further studies determining the anatomical localization of conditioned cells during and throughout the process of priming may afford further insights into how naïve T cells may be selected and properly positioned for efficient eT_{RM} differentiation.

In our studies of naïve $CD8^+$ T cells in the SLO, we have utilized CD103 as a marker of TGF-β-dependent conditioning. While our studies of CD103 on eT_{RM} in the skin have shown that CD103 may be functionally dispensable in the short and maybe even long term, this may or may not also be the case in the SLO for naïve $CD8^+$ T cells. Although there is no known ligand for CD103 in the SLO, the integrin itself could have the capacity to regulate localization of cells. However, with the assumption that a similar mechanism may exist in humans, where CD103 does not seem to be expressed on naïve $CD8^+$ T cells, it is more likely than not that CD103 is simply a marker of TGF-β signaling. Very basic studies examining the localization of $CD8^+$ T cells in CD103 deficient mice, as compared to settings of impaired TGF-β signaling would allow us to distinguish between the role of the integrin and TGF-β signaling.

CD103 and naïve $CD8^+$ T cells in the SLO

Curiously, we see selective expression of CD103 on naïve $CD8^+$ T cells and not memory $CD8^+$ T cells which presumably have similar residence kinetics within the secondary lymphoid tissues as the naïve cells. It is known that TGF-β is also critical for the homeostasis of memory $CD8^+$ T cells[262], so the absence of CD103 expression on the memory $CD8^+$ found in the lymph nodes could perhaps indicate a distinct TGF-β dependent transcriptional or epigenetic program that does not include CD103 as a target in memory cells. One explanation for this could be the manner in which T cells receive these signals, even circling back to how TGF-β may be activated. For example, the activation of TGF-β by αVβ6 relies on cytoskeletal force to release the latent protein[139,141]. While the full mechanism by which αVβ8 activates TGF-β is still unknown[142] we hypothesize that a similar mechanism may mediate the release of active TGF-β during cellular interactions between $CD8^+$ T cells and DC.

Rigidity of cellular membranes at a synapse where TGF-β is activated can therefore impact the efficiency of activation, for example with more rigid membranes providing better

anchoring for the "straight-jacket-like" release of latent TGF-β to its active form. Naïve $CD8^+$ T cells are known to have more rigid membranes than effector cells or memory cells[263], a property that also impacts their immunological synapses, TCR signal strength and other actin-dependent signaling. It is therefore possible that naïve $CD8^+$ T cells may take in TGF-β signals in a context that depends on these mechanics, while memory T cells that circulate through the lymph nodes do not provide the membrane mechanics required for the activation and presentation of TGF-β at synapses. Therefore, while memory $CD8^+$ T cells can interact with DC during their homeostasis for IL-15 signals, they may pick up TGF-β signals from an alternative source of activation, for example through fibroblasts or regulatory T cells. Alternatively, this may even reflect a difference in the "dose" of TGF-β presented by the DC to the T cell during their interaction.

Another explanation for this phenomenon is of course the difference in active signaling pathways in naïve $CD8^+$ T cells as compared to memory $CD8^+$ T cells. The expression of CD103 in Jurkat cells has previously been linked to synergy between NFAT and Smad[264]. While this observation has yet to be tested *in vivo* and also in primary $CD8^+$ T cells, it is possible that differential TCR triggering during non-cognate interactions that maintain naïve $CD8^+$ T cells and memory cells contributes to the difference in CD103 expression on these subsets. Additionally, both naïve and memory $CD8^+$ T cells rely on the trans-presented cytokine IL-15 for their survival[3,265,266]. However, memory cells as compared to naïve cells may exhibit different levels of STAT5 signaling, which is postulated to interfere with signaling that results in the ultimate up-regulation of CD103. We have seen this in our hands, where CD103 induction on TGF-β treated effector cells *in vitro* is impaired by increasing doses of IL-2 (*E. Carrizosa, unpublished*). Future studies dissecting TGF-β signaling thresholds and contexts for naïve as compared to memory $CD8^+$ T cells in the SLO are critical to understanding the specificity of TGF-β/Smad dependent

transcripts and their downstream therapeutic targeting. These concepts could potentially be probed using our GFP-Smad2 reporter, attempting to correlate signaling index with transcriptional profiles.

Localization of naïve $CD8^+$ T cells as compared to memory $CD8^+$ T cells in the LN may also play a role in determining which cells subsets have "access" to the DC that can activate TGF-β at steady state. Studies of spatial organization within the LN have shown that memory $CD8^+$ T cells organize towards the outermost regions of the LN, away from naïve $CD8^+$ T cells, on the basis of CXCR3 expression, such that they are positioned to respond rapidly to cues in a recall response[267]. As a result, they may also interact more frequently with APC subsets such as $CD8\alpha^+$ DC that are also more abundant in that location[202] but lack the capacity to activate TGF-β at steady state.

Implications for the therapeutic targeting of TGF-β

Efficacious and specific therapeutic targeting of the TGF-β pathway has thus far been relatively lackluster, owing to a number of different factors. Initial strategies that were heavily explored, and perhaps still in clinical trials, in fibrosis and cancer focused on inhibiting binding of the active cytokine to the receptor or small molecule inhibition of receptor kinases[268]. While the *in vitro* data surrounding these strategies was quite formidable and some clinical trials have shown limited success, the widespread adoption has been limited. Moreover, as we have learned through our own studies and the emerging literature from other groups, it is not simply broad targeting of TGF-β and its signaling that may alleviate disease, but truly some level of specificity in targeting of a cell or activation method. Take for example tumor infiltrating lymphocytes (TILs). Some studies show that platelet-derived TGF-β[246] prevents the accumulation of TILs and therefore fuels the progression of cancer. On the other hand, we know that $CD103^+$ TILs that possess a T_{RM}-like phenotype also depend on TGF-β signaling for their

induction and, contrastingly, provide anti-tumor effects as have been shown in human and mouse studies[63,65,67]. It would therefore be more efficacious if it were possible to promote differentiation of the latter, while dampening the effects of the former. While the source of TGF-β activation is unknown for these cell types, further investigation of these nuances may allow more specific therapeutic targeting for desired outcomes. Recent studies have observed the relative promise of more specific approaches, such as the targeting of GARP/LAP *in vivo* that leads to reduced immunosuppression by Treg in the tumor microenvironment[269]. However, the design of antibodies to block specific integrins such as αVβ6 and αVβ8 *in vivo* has also been plagued by technical challenges in identifying a specific, yet targetable epitope. Other strategies such as targeted RNAi approaches to specific cell types may allow us to overcome some of these hurdles. Furthermore, a better understanding of which transcriptional or functional outcomes are desired and how each of them may be modulated by various contexts of activation or even doses or isotypes of TGF-β signaling may allow us to better enrich for these outcomes *in vivo* and identify differential targets in autoimmunity as compared to tumors or vaccine strategies for T_{RM}.

APPENDIX A: Antibodies, primers and constructs used in all studies

Table A1. Primers used for RT-PCR

Transcript	F	R
Ahr	AGCCGGTGCAGAAAACAGTAA	AGGCGGTCTAACTCTGTGTTC
Ccr8	ACGTCACGATGACCGACTACT	CCCAGCACAAACAAGACGC
Cd244	CTCGGGGCCATCATTTGTTTC	GCTAGAAGGGAGCTGAACATCA
Cdh1	CAGGTCTCCTCATGGCTTTGC	CTTCCGAAAAGAAGGCTGTCC
Dusp6	ATAGATACGCTCAGACCCGTG	ATCAGCAGAAGCCGTTCGTT
Eomes	GCGCATGTTTCCTTTCTTGAG	GGTCGGCCAGAACCACTTC
Hobit	CTCAGCCACTTGCAGACTCA	CTGTCGGTGGAGGCTTTGTA
Hprt	GGGGGCTATAAGTTCTTTGC	TCCAACACTTCGAGAGGTCC
Hspa1a	TGGTGCAGTCCGACATGAAG	GCTGAGAGTCGTTGAAGTAGGC
Id2	ATGAAAGCCTTCAGTCCGGTG	AGCAGACTCATCGGGTCGT
Id3	CTGTCGGAACGTAGCCTGG	GTGGTTCATGTCGTCCAAGAG
Il7r	GCGGACGATCACTCCTTCTG	AGCCCCACATATTTGAAATTCCA
Inpp4b	TCCAGAAGATTCCGAACGAGC	GTTTCCGATCACTGACAGGAG
Itgae	AGGGAGCCTTCAATTTGGATG	CTGGCTGGCAAGCAATTTCA
Itgav	CGGGTCCCGAGGGAAGTTA	TGGATGAGCATTCACATTTGAGA
Itgb3	CCACACGAGGCGTGAACTC	CTTCAGGTTACATCGGGGTGA
Itgb5	GCTGCTGTCTGCAAGGAGAA	AAGCAAGGCAAGCGATGGA
Itgb6	CAACTATCGGCCAACTCATTGA	GCAGTTCTTCATAAGCGGAGAT
Itgb8	AGTGAACACAATAGATGTGGCTC	TTCCTGATCCACCTGAAACAAAA
Klf2	CTCAGCGAGCCTATCTTGCC	CACGTTGTTTAGGTCCTCATCC
Litaf	ATGTCGGCTCCAGGACCTTA	GGTAGTAACTGTTGACACCCACT
Nfil3	GAACTCTGCCTTAGCTGAGGT	ATTCCCGTTTTCTCCGACACG
Nr4a1	TTGAGTTCGGCAAGCCTACC	GTGTACCCGTCCATGAAGGTG
Prdm1	TTCTCTTGGAAAAACGTGTGGG	GGAGCCGGAGCTAGACTTG
Rgs1	TCTGGGATGAAATCGGCCAAG	GCATCTGAATGCACAAATGCTT
Rgs2	GAGAAAATGAAGCGGACACTCT	GCAGCCAGCCCATATTTACTG
S1pr1	GTGTAGACCCAGAGTCCTGCG	AGCTTTTCCTTGGCTGGAGAG
S1pr5	GCTTTGGTTTGCGCGTGAG	GGCGTCCTAAGCAGTTCCAG
Sidt1	ATCGAAAGCGACAAAAACGTCA	GCGATGGTGATGATGTTCCAA
Sik1	TCATGTCGGAGTTCAGTGCG	ACCTGCGTTTTGGTGACTCG
Skil	AATGATGACCGACATTCATGCC	AGCGTGTGTTTCAGACTGGG
Tcf7	AGCTTTCTCCACTCTACGAACA	AATCCAGAGAGATCGGGGGTC
Xcl1	TTTGTCACCAAACGAGGACTAAA	CCAGTCAGGGTTATCGCTGTG

Table A2. Commonly used flow cytometry antibodies

Antigen	Clone	Vendor	Formats Used
(Viability)	-	Biolegend, eBioscience	Zombie Yellow, eFluor506
B220	RA3-6B2	Biolegend	FITC
CCR6	29-2L17	Biolegend	PE, BV421
CD103	2E7	Biolegend	APC, AF488, Pac Blue, PerCP-Cy5.5
CD11b	M1/70	Biolegend	PE-Cy7, APC-Cy7
CD11c	HL3	BD Biosciences	PE-Cy7
CD19	6D5	Biolegend	BV421
CD207	4C7	Biolegend	APC
CD3	17A2	Biolegend	Biotin, APC, FITC
CD4	RM4-5	Biolegend	PacBlue, APC-Cy7, AF700
CD44	IM7	Biolegend	BV605, PerCP-Cy5.5, AF700
CD45	30-F11	Biolegend	FITC, APC-Cy7, AF700
CD45.1	A20	Biolegend	FITC, PE-Cy7, BV605
CD45.2	104	Biolegend	FITC, PacBlue
CD51	RMV-7	Biolegend	PE
CD62L	MEL-14	Biolegend	AF700, APC, APC-Cy7, BV421
CD69	H1.2F3	Biolegend	PE-Cy7, BV421, PE
CD8a	5H10	Thermo Fisher	AF700, AF488, FITC
CD8b	YTS	Biolegend	PE, AF700, FITC, PE-Cy7
Eomes	Dan11mag	eBioscience	PE, AF488
F4/80	BM8	Biolegend	PE
FoxP3	FJK-15S	eBioscience	PE-Cy5
IA/IE	M5/114.15.2	Biolegend	FITC, AF700, APC-Cy7
KLRG1	MAFA	Biolegend	PE-Cy7, BV421, FITC
Ly6G	1A8	Biolegend	BV605, FITC
NK1.1	PK136	Biolegend	AF488
T-bet	4B10	Biolegend	BV421, PE-Cy7
Thy1.1	OX-7	Biolegend	AF700, PE-Cy7, PacBlue
Thy1.2	30-H12	Biolegend	AF700, PacBlue, BV605, FITC
TGF-βRII	AF532	R&D	PE, APC

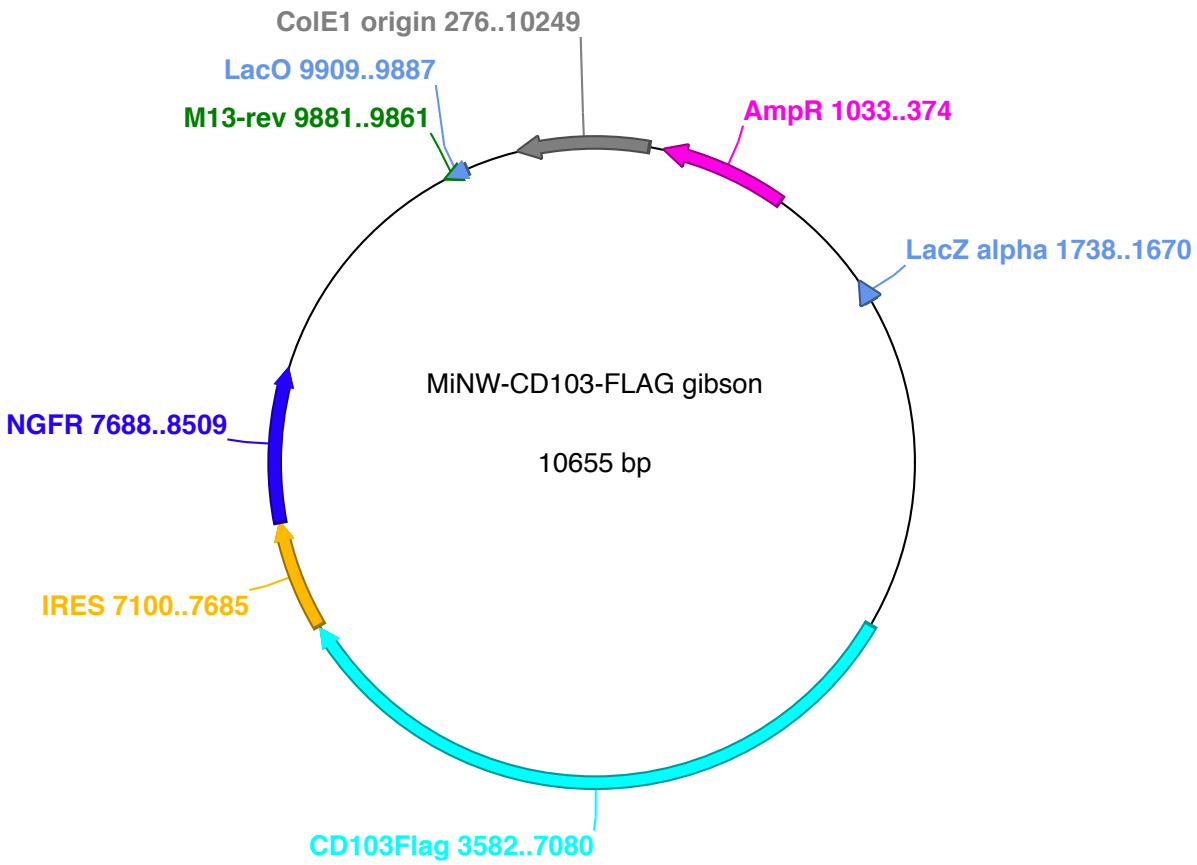


Figure A3. Plasmid map of CD103-Flag driven by 5' LTR as used in Chapter 3.

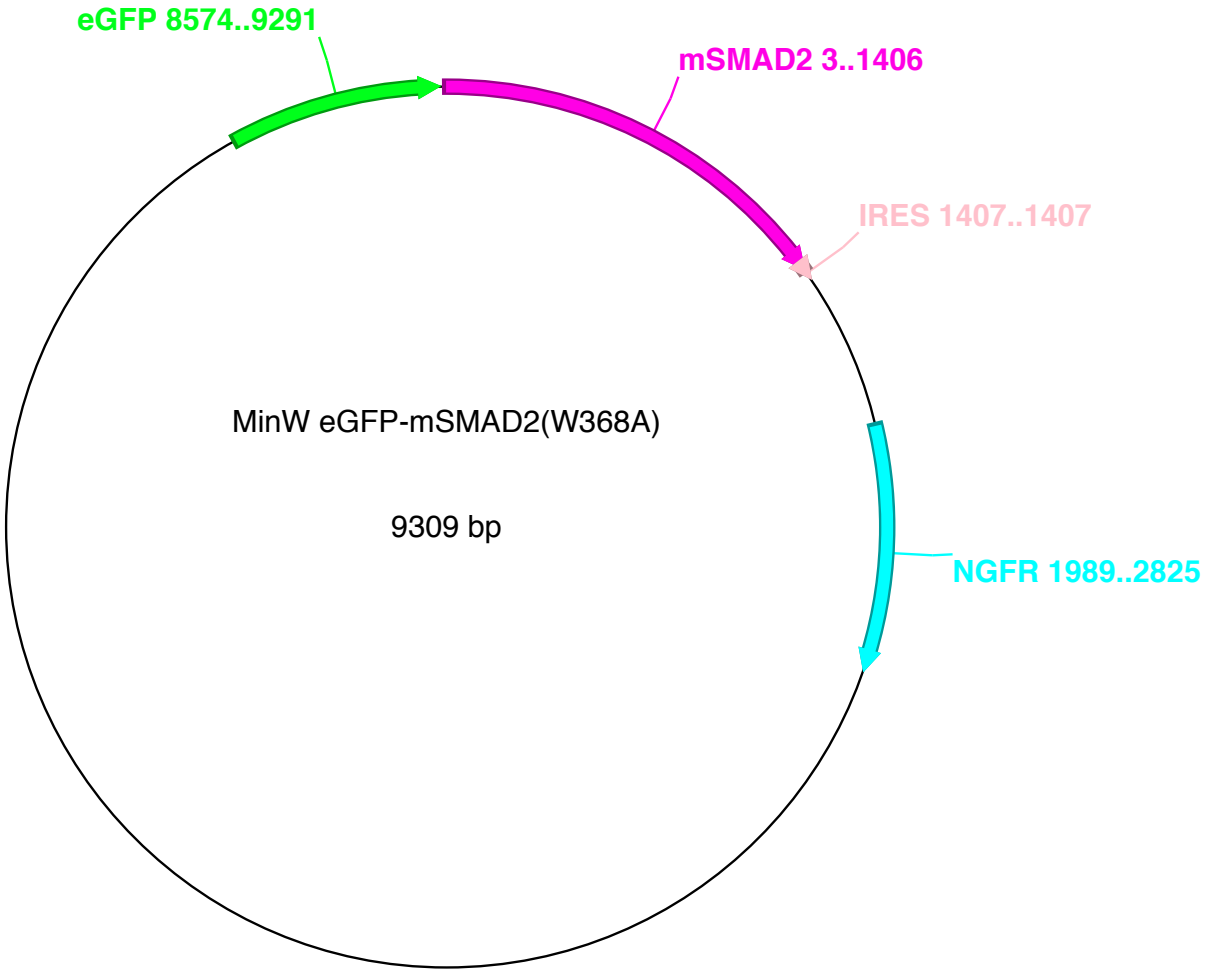


Figure A4. Plasmid map of GFP-Smad2 driven by 5' LTR as used in Chapter 4.

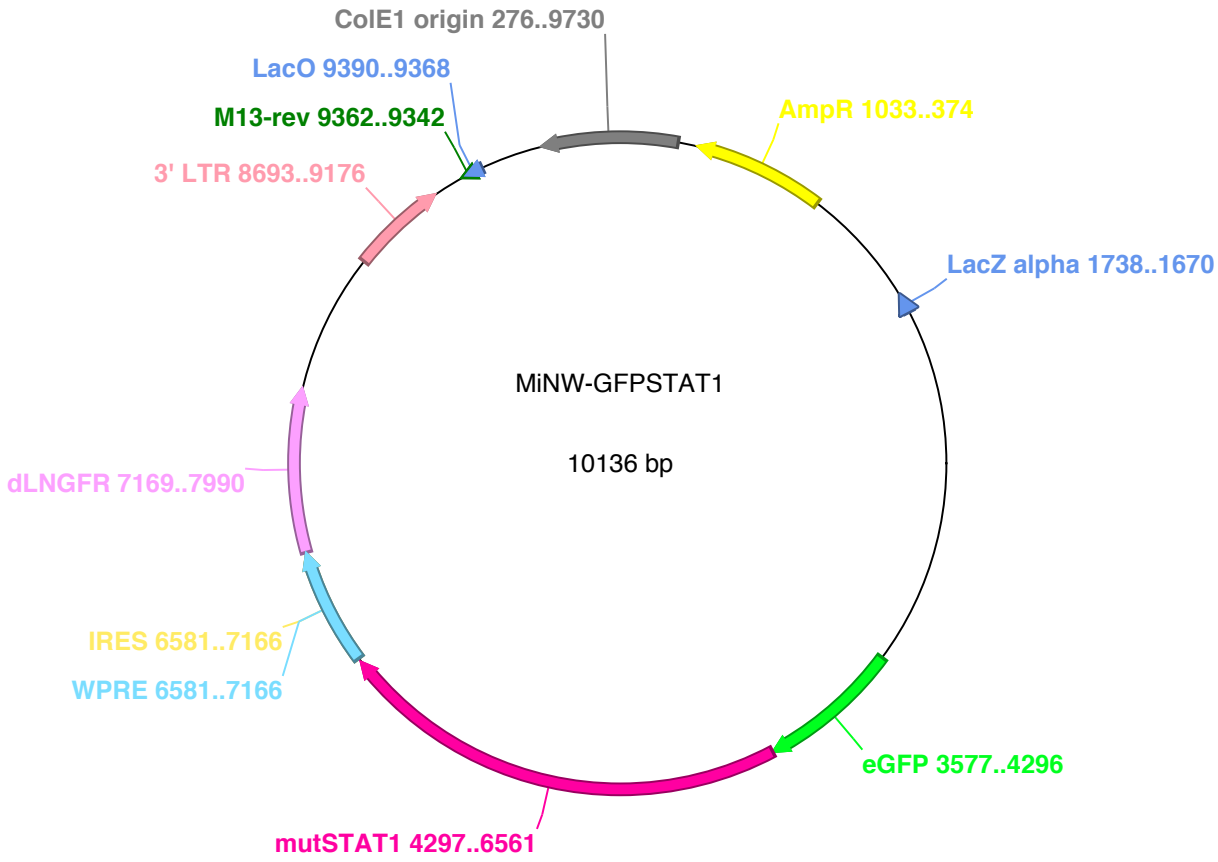


Figure A5. Plasmid map of STAT1-GFP driven by 5' LTR as used in Chapter 4.

APPENDIX B: Supplementary materials pertaining to Chapter 2

A

DARs ↑ in WT

Motif	Motif name	p-value	q-value (Benjamini)	% of target sequences with motif	% of background sequences with motif
	**Klf4(Zf)/mES-Klf4-ChIP-Seq(GSE11431)/Homer**	1.00E-21	0	31.20%	15.68%
	**KLF3(Zf)/MEF-Klf3-ChIP-Seq(GSE44748)/Homer**	1.00E-21	0	42.40%	24.77%
	**Klf9(Zf)/GBM-Klf9-ChIP-Seq(GSE62211)/Homer**	1.00E-17	0	35.52%	20.63%
	**RUNX1(Runt)/Jurkat-RUNX1-ChIP-Seq(GSE29180)/Homer**	1.00E-16	0	36.16%	21.31%
	**RUNX2(Runt)/PCa-RUNX2-ChIP-Seq(GSE33889)/Homer**	1.00E-16	0	31.04%	17.15%
	**RUNX(Runt)/HPC7-Runx1-ChIP-Seq(GSE22178)/Homer**	1.00E-16	0	28.80%	15.57%
	**RUNX-AML(Runt)/CD4+-PolII-ChIP-Seq(Barski_et_al.)/Homer**	1.00E-14	0	27.52%	15.11%
	Sp1(Zf)/Promoter/Homer	1.00E-13	0	34.40%	21.08%
	Sp5(Zf)/mES-Sp5.Flag-ChIP-Seq(GSE72989)/Homer	1.00E-10	0	56.80%	43.39%
	**KLF10(Zf)/HEK293-KLF10.GFP-ChIP-Seq(GSE58341)/Homer**	1.00E-10	0	32.48%	21.18%
	ETV1(ETS)/GIST48-ETV1-ChIP-Seq(GSE22441)/Homer	1.00E-09	0	50.56%	38.11%
	**KLF5(Zf)/LoVo-KLF5-ChIP-Seq(GSE49402)/Homer**	1.00E-09	0	59.84%	47.33%
	GABPA(ETS)/Jurkat-GABPa-ChIP-Seq(GSE17954)/Homer	1.00E-09	0	39.04%	27.78%
	**KLF6(Zf)/PDAC-KLF6-ChIP-Seq(GSE64557)/Homer**	1.00E-08	0	53.44%	41.58%
	Etv2(ETS)/ES-ER71-ChIP-Seq(GSE59402)/Homer(0.967)	1.00E-08	0	36.16%	25.40%
	EHF(ETS)/LoVo-EHF-ChIP-Seq(GSE49402)/Homer	1.00E-08	0	43.84%	32.61%
	**EKLF(Zf)/Erythrocyte-Klf1-ChIP-Seq(GSE20478)/Homer**	1.00E-08	0	12.48%	6.15%
	ERG(ETS)/VCaP-ERG-ChIP-Seq(GSE14097)/Homer	1.00E-07	0	50.88%	40.22%
	Tcf4(HMG)/Hct116-Tcf4-ChIP-Seq(SRA012054)/Homer	1.00E-07	0	15.68%	9.08%
	ELF3(ETS)/PDAC-ELF3-ChIP-Seq(GSE64557)/Homer	1.00E-06	0	26.40%	18.02%

Appendix B1. Motifs enriched in WT DARs.

B

DARs ↑ in αV-ΔDC

	**IRF2(IRF)/Erythroblas-IRF2-ChIP-Seq(GSE36985)/Homer**	1.00E-33	0	16.17%	2.61%
	**ISRE(IRF)/ThioMac-LPS-Expression(GSE23622)/Homer**	1.00E-31	0	12.47%	1.50%
	**IRF1(IRF)/PBMC-IRF1-ChIP-Seq(GSE43036)/Homer**	1.00E-25	0	16.63%	3.66%
	**IRF8(IRF)/BMDM-IRF8-ChIP-Seq(GSE77884)/Homer**	1.00E-23	0	25.17%	8.71%
	**IRF3(IRF)/BMDM-Irf3-ChIP-Seq(GSE67343)/Homer**	1.00E-18	0	23.33%	9.03%
	**PU.1:IRF8(ETS:IRF)/pDC-Irf8-ChIP-Seq(GSE66899)/Homer**	1.00E-15	0	16.63%	5.53%
	**Tbet(T-box)/CD8-Tbet-ChIP-Seq(GSE33802)/Homer**	1.00E-13	0	50.81%	33.22%
	**bZIP:IRF(bZIP,IRF)/Th17-BatF-ChIP-Seq(GSE39756)/Homer**	1.00E-11	0	25.87%	13.39%
	**IRF4(IRF)/GM12878-IRF4-ChIP-Seq(GSE32465)/Homer**	1.00E-11	0	24.48%	12.36%
	ETS1(ETS)/Jurkat-ETS1-ChIP-Seq(GSE17954)/Homer	1.00E-09	0	42.03%	28.39%
	ETS:RUNX(ETS,Runt)/Jurkat-RUNX1-ChIP-Seq(GSE17954)/Homer	1.00E-08	0	7.16%	2.08%
	**Tbr1(T-box)/Cortex-Tbr1-ChIP-Seq(GSE71384)/Homer**	1.00E-08	0	53.35%	39.80%
	PRDM1(Zf)/Hela-PRDM1-ChIP-Seq(GSE31477)/Homer	1.00E-07	0	26.33%	15.97%
	Tcf4(HMG)/Hct116-Tcf4-ChIP-Seq(SRA012054)/Homer	1.00E-07	0	24.25%	14.37%
	GABPA(ETS)/Jurkat-GABPa-ChIP-Seq(GSE17954)/Homer	1.00E-07	0	35.57%	24.27%
	**Tbx5(T-box)/HL1-Tbx5.biotin-ChIP-Seq(GSE21529)/Homer**	1.00E-06	0	82.91%	72.27%
	Eomes(T-box)/H9-Eomes-ChIP-Seq(GSE26097)/Homer	1.00E-06	0	68.13%	56.24%
	Tcf3(HMG)/mES-Tcf3-ChIP-Seq(GSE11724)/Homer	1.00E-06	0	15.24%	8.06%
	ETV1(ETS)/GIST48-ETV1-ChIP-Seq(GSE22441)/Homer	1.00E-06	0	47.81%	36.39%
	RUNX1(Runt)/Jurkat-RUNX1-ChIP-Seq(GSE29180)/Homer	1.00E-05	0	41.34%	30.45%

Appendix B2. Motifs enriched in αV-ΔDC DARs.

APPENDIX C: Supplementary Video Legends

Chapter 3

Supplementary Video 3.1-3.5 *Ex vivo* activated OT-I T cells were transduced with tdTomato (red) retrovirus and 10^5 cells were intradermally injected into the ear skin of C57BL/6$^{Tyrc\text{-}2J}$ for longitudinal imaging of eT_{RM} differentiation. Maximum intensity projections of cells migrating on day 5 post-injection in (3.1) dermis (3.2) perifollicular dermis and (3.3) follicular, migrating in the epidermis, (3.4) entering the epidermis via the hair follicle. (3.5) Side view of (3.4). Videos correspond to Figure 3.1. (**Links to Supplementary Videos 3.1, 3.2, 3.3, 3.4, 3.5**)

Supplementary Video 3.6-3.9 Activated CD8$^+$ T cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-βRII$^{fl/fl\ or\ fl/+}$ mice were treated *in vitro* with 64nM 4-OHT at 2 days post-activation, and transduced with either tdTomato (KO = fl/fl) or GFP (WT = fl/+) retrovirus. 72 hours after 4-OHT treatment (5 days post-activation), 10^5 cells each of "WT" GFP and "TGF-βRII KO" tdTomato cells were adoptively transferred at a 1:1 ratio. Maximum intensity projections of 1 hour long movies day 5 post transfer of cells into the ear skin in the (3.6) dermis, (3.7) follicle 1, (3.8) follicle 2, (3.9) follicle 3. Videos correspond to Figure 3.4. (**Links to Supplementary Videos 3.6, 3.7, 3.8, 3.9**)

Supplementary Video 3.10-3.12 Activated CD8$^+$ T cells from OT-I x Rosa26-Cre-ER^{T2} x TGF-βRII$^{fl/fl\ or\ fl/+}$ mice were treated *in vitro* with 64nM 4-OHT at 2 days post-activation, and transduced with either tdTomato and CD103 (KO = fl/fl) or GFP (WT = fl/+) retrovirus. 72 hours after 4-OHT treatment (5 days post-activation), 10^5 cells each of "WT" GFP and "TGF-βRII KO+CD103" tdTomato cells were adoptively transferred at a 1:1 ratio. Each video is from a representative follicle. Videos correspond to Figure 3.5. (**Links to Supplementary Videos 3.10, 3.11, 3.12**)

Supplementary Video 3.13 WT and CD103 deficient polyclonal cells from sex and age-matched controls were activated and transduced with either tdTomato (WT) or GFP (KO) retrovirus. CD8$^+$ T cells were enriched by negative selection. 10^5 cells each of WT tdTomato

cells CD103 KO GFP cells were adoptively transferred at a 1:1 ratio. Video corresponds to Figure 3.6. (**Link to Supplementary Videos 3.13**)

Supplementary Video 3.14-3.17 WT and CCR6 deficient polyclonal cells from sex and age-matched controls were activated and transduced with either tdTomato (KO) or GFP (WT) retrovirus. $CD8^+$ T cells were enriched by negative selection. 10^5 cells each of WT GFP and CCR6 KO tdTomato cells were adoptively transferred at a 1:1 ratio. Cells were visualized 6 days post intradermal injection in (3.14) dermis and (3.15-3.17) follicles. Videos correspond to Figure 3.9. (**Links to Supplementary Videos 3.14, 3.15, 3.16, 3.17**)

Chapter 4

Supplementary Video 4.1 GFP-Smad2-expressing $CD8^+$ cells were treated with 1ng/mL TGF-β1 and live cells were imaged by confocal microscopy in a chamber at 37°C, 5% CO_2. (**Link to Supplementary Video 4.1**)

Supplementary Video 4.2 $CD8^+$ T cells were transduced with either GFP-Smad2ΔPhos and H2BtagBFP or GFP-Smad2 and H2BmRFP. Reporter-bearing cells were treated with 1ng/mL TGF-β1 and live cells were imaged by confocal microscopy in a chamber at 37°C, 5% CO_2. (**Link to Supplementary Video 4.2**)

Supplementary Video 4.3 Activated $CD8^+$ T cells were transduced with either GFP-Smad2ΔPhos and H2BmRFP or GFP-Smad2 and H2BtagBFP. 5 days after activation, 10^5 reporter-bearing cells were transferred intradermally into the ear skin at a 1:1 ratio into Albino CD11c-mCherry hosts. Ear skin was longitudinally imaged for two weeks post-transfer. (**Link to Supplementary Video 4.3**)

Supplementary Video 4.4-4.5 10^6 reporter-bearing CT26 (4.4) or CT26HA (4.5) cells were implanted subcutaneously into Balb/c mice and a dorsal skin fold chamber was installed for longitudinal intravital imaging. Seven days after tumor implantation, 10^6 HA-specific CTL (CL4) transduced with tdTomato were injected intravenously and longitudinal imaging was conducted. (**Links to Supplementary Videos 4.4, 4.5**)

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