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_J Immunol_ 2013; 190:4483-4487; Prepublished online 29 March 2013;
doi: 10.4049/jimmunol.1300212
http://www.jimmunol.org/content/190/9/4483
Cutting Edge: Memory Regulatory T Cells Require IL-7 and Not IL-2 for Their Maintenance in Peripheral Tissues

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Thymic Foxp3-expressing regulatory T cells are activated by peripheral self-antigen to increase their suppressive function, and a fraction of these cells survive as memory regulatory T cells (mTregs). mTregs persist in nonlymphoid tissue after cessation of Ag expression and have enhanced capacity to suppress tissue-specific autoimmunity. In this study, we show that mTregs express specific effector memory T cell markers and localize preferentially to hair follicles in skin. Memory Tregs express high levels of both IL-2Rα and IL-7Rα. Using a genetic-deletion approach, we show that IL-2 is required to generate mTregs from naive CD4+ T cell precursors in vivo. However, IL-2 is not required to maintain these cells in the skin and skin-draining lymph nodes. Conversely, IL-7 is essential for maintaining mTregs in skin in the steady state. These results elucidate the fundamental biology of mTregs and show that IL-7 plays an important role in their survival in skin. The Journal of Immunology, 2013, 190: 4483–4487.

Regulatory T cells (Tregs) play an essential role in maintaining immune homeostasis in tissues. Both mice and humans harboring defects in these cells develop florid inflammation and autoimmunity, with a predilection for the gastrointestinal tract and skin (1, 2). Classically, two main subsets of Foxp3-expressing Tregs have been defined: those that develop in the thymus (thymus-derived Tregs; tTregs) and those that develop outside the thymus from naive CD4+ T cell precursors (peripherally derived Tregs; pTregs). Recently, we identified a population of Foxp3-expressing Tregs, termed memory Tregs (mTregs) (3). These cells are activated upon exposure to Ag in the periphery, persist in nonlymphoid tissues after Ag expression ceases, and have enhanced capacity to suppress autoimmune responses when tissue Ag is re-expressed. We showed that this population plays a major role in attenuating autoimmune responses in skin upon repeated exposure to self-antigen and other investigators recently showed that mTregs are required to maintain regulatory memory upon successive allo-antigen exposure expressed in the developing fetus (4). In addition, a similar population may exist in human blood (5). The fundamental biology of mTregs (i.e., how they are established and maintained in tissues) remains to be elucidated.

IL-2 plays an essential role in the development of Tregs in the thymus and for the maintenance of both tTregs and pTregs in secondary lymphoid tissues. Mice deficient in IL-2 have significantly reduced numbers of Foxp3-expressing cells and develop autoimmunity (6). Mechanistically, IL-2 is known to provide survival signals to Tregs and promote their functional activity (7).

IL-7 plays an essential role in the development and survival of both naive and memory T cells. Thymic T cell development is significantly impaired in IL-7-deficient mice and humans (8, 9). In addition, memory T cells fail to survive if they lack IL-7 receptor signaling or if they are adoptively transferred into IL-7-deficient hosts (10, 11).

Memory Tregs are a unique subset of regulatory cells that are maintained in nonlymphoid organs. Because they are both Foxp3-expressing cells and a subset of memory CD4+ T cells, we hypothesized that they would require IL-2, IL-7, or both for their maintenance in tissues. In this study, we showed that mTregs absolutely require IL-2 for their in vivo generation from peripheral naive CD4+ T cell precursors. However, IL-7, and not IL-2, is required to maintain this population in the skin. These results elucidate fundamental properties of mTregs in tissues and have potential therapeutic implications.

Materials and Methods

Mice

All animal studies were performed in compliance with institutional guidelines. K5/TGO and K5/TGO/DO11 mice were used as described (3). Hosts for adoptive-transfer experiments were created by crossing K5/TGO mice with TCRα–/– mice (TCRα−/−) on the BALB/c background. For adoptive...
transform of DO11 T cells, DO11.10 TCR-transgenic mice were crossed onto Rag2−/−/CD90.1+ or Rag2−/−/CD90.2−/IL-2−/− backgrounds.

Adoptive transfer of T cells
Single-cell suspensions were prepared from lymph nodes (LN) of DO11/Rag2−/−/CD90.1+ (i.e., DO11 wild-type [WT]) or DO11/Rag2−/−/CD90.2−/IL-2−/− (i.e., DO11−/IL-2−/−) mice. Approximately 5 × 10^3 LN cells were adoptively transferred i.v. into gender-matched K5/TGO/TCRα−/− recipient mice, and mice were started on doxycycline chow the same day as adoptive transfer. For experiments involving cotransfer of cells from WT-DO11 and DO11−/IL-2−/− mice, LN cells were premixed at a 3:1 ratio of DO11−/IL-2−/− to WT-DO11 cells. Sixty days later, mice were treated with a single injection of OX-7−SAP mAb (5 μg/mouse; Cat. #IT-2; Advanced Targeting Systems) to deplete CD90.1+ cells.

In vivo neutralization experiments
For IL-2–neutralization studies, mice were treated with i.p. injections of a 1:1 mixture of JES6-1A12 (University of California San Francisco mAb Core) and S4B6-1 (Bio X Cell, West Lebanon, NH) IL-2–neutralizing mAbs (50 μg each Ab/mouse, injected 3×/week for a total of 2–3 wk). For IL-7Rα–neutralization experiments, mice were treated with i.p. injections of A7R34 (Bio X Cell) monoclonal IL-7Rα Ab (500 μg/mouse, injected 2×/week for a total of 3 wk).

Statistics
Statistical analysis was done using GraphPad Prism software (GraphPad). The p values were calculated using a two-tailed unpaired t test.

Results and Discussion
Characterization of mTregs in murine skin
We recently established a mouse model of tetracycline-inducible self-antigen expression in the skin (3). In K5/TGO/DO11-transgenic mice, OVA is constitutively expressed in the thymus, leading to a modest deletion of OVA-specific CD4+ T cells (i.e., DO11 cells) and the generation of a significant number of Foxp3-expressing DO11 tTregs that populate secondary lymphoid tissues. Upon induction of cutaneous OVA expression with doxycycline, DO11 Tregs are activated, proliferate, acquire a more suppressive phenotype, and accumulate in the skin, where they play an obligatory role in resolving inflammation. When Ag expression is extinguished, a subset of Tregs stably resides in the skin and is capable of attenuating subsequent autoimmune responses when Ag is re-expressed.

Comprehensive phenotypic analysis of DO11 mTregs that remain in the skin of K5/TGO/DO11 mice >40 d after cessation of OVA expression (mTregs) reveals that these cells express high levels of effector memory markers, including IL-7R (CD127), CD44, CD27, and CCR6, and low levels of CD62L (data not shown). Compared with Tregs in the skin-draining LNs (SDLNs), mTregs in skin express significantly lower levels of the high-affinity IL-2Rα (CD25) and higher levels of the IL-7Rα (CD127) (Fig. 1). These results suggest that IL-2, IL-7, or both play a role in maintaining mTregs in skin.

Immunofluorescent confocal microscopy revealed that mTregs occupy a unique spatial niche in skin. Prior to Ag induction in K5/TGO/DO11 mice, there are very few DO11 cells in the skin (3). Approximately 10–14 d after induction of cutaneous OVA expression, K5/TGO/DO11 mice develop a florid inflammatory dermatitis. At the height of the autoimmune response, both effector DO11 cells and DO11 Tregs localize with CD11c+ cells diffusely throughout the papillary dermis and at the junction between the dermis and epidermis, with some cells infiltrating the epidermis (Supplemental Fig. 1). However, >40 d after cessation of OVA expression in the skin, mTregs localize preferentially to hair follicles (Supplemental Fig. 1). Notably, few mTregs were observed in the interfollicular epidermis or interfollicular dermis and were found preferentially in the dermis surrounding the lower segments of hair follicles. Some mTregs were observed in close proximity to hair follicle–associated CD11c+ cells. Interestingly, hair follicles have been called the “gatekeepers of the epidermis,” because keratinocytes that make up distinct regions of the follicular epidermis secrete specific chemokines that play a critical role in the migration of Langerhans cell precursors to hair follicles and subsequent entry into the epidermis (12, 13). It is possible that hair follicle keratinocytes and/or follicle-associated dendritic cells secrete chemokines that recruit mTregs to this anatomic niche.

IL-2 is required to generate pTregs in vivo
Given the well-defined role of IL-2 in Treg biology and the fact that mTregs express high levels of CD25, we sought to determine whether IL-2 plays a role in establishing mTregs. To do so, we used an adoptive-transfer approach. In this model, adoptive transfer of DO11 cells into K5/TGO mice crossed onto a naive CD4+ T cell precursor), because naive DO11 cells from DO11/Rag2−/− mice are devoid of thymus-derived Foxp3-expressing Tregs (Fig. 2B). Consequently, this model represents the most robust system available for pTreg generation in vivo. To elucidate the role of IL-2 in the generation of pTregs (and the subsequent formation of mTregs), LN cells from DO11/Rag2−/− or DO11/Rag2−/−/IL-2−/− mice were adoptively transferred into K5/TGO/TCRα−/− hosts, and OVA expression was induced in the skin. Compared with LN cells

FIGURE 1. Memory Tregs have reduced IL-2Rα expression and increased IL-7Rα expression. CD25 and CD127 expression was examined on mTregs isolated from the skin and SDLNs of K5/TGO/DO11 mice. Flow cytometry plots are gated on live CD4+DO11+ cells, and column graphs are gated on live CD4+Foxp3+DO11+ cells. Results are representative of four replicate experiments with more than three mice/group. MFI, Mean fluorescent intensity.
FIGURE 2. IL-2 is required to generate pTregs in vivo. (A) LN cells from DO11/Rag\(^{-/-}\) or DO11/Rag\(^{+/+}\)/IL-2\(^{-/-}\) mice were adoptively transferred into K5/TGO/TCR\(^{a-a}\) hosts, OVA expression was induced in the skin, and the severity of clinical skin disease was measured. (B) Skin and SDLNs were harvested at specific times after Ag induction, and DO11 cells were analyzed by flow cytometry. Live CD4\(^{+}\)DO11\(^{+}\) cells are shown. Error bars in (A) represent range. Results are representative of two replicate experiments with more than two mice/group.

from WT DO11/Rag\(^{-/-}\) mice, transfer of DO11/Rag\(^{+/+}\)/IL-2\(^{-/-}\)/IL-7\(^{-/-}\) LN cells resulted in a significant delay in the onset of cutaneous inflammation that failed to resolve (Fig. 2A). WT DO11 Tregs were observed in both the SDLNs and skin between 10 and 15 d after OVA induction and plateaued at \(>40\) d, where Foxp3-expressing cells accounted for 40 and 80% of DO11 cells in the SDLNs and skin, respectively (Fig. 2B). In contrast, Foxp3-expressing DO11 cells were never observed to account for \(>0.15\%\) in either the SDLNs or skin at all time points examined when DO11/Rag\(^{+/+}\)/IL-2\(^{-/-}\)/IL-7\(^{-/-}\) LN cells were used for adoptive transfer (Fig. 2B). Despite the fact that pTregs essentially failed to develop, DO11 cells lacking IL-2 were able to expand and differentiate into effector cells capable of inducing cutaneous inflammation, albeit with delayed kinetics compared with WT DO11 cells (Fig. 2A). These results suggest that, although cytokine(s) other than IL-2 can compensate for the expansion and differentiation of effector T cells in response to peripheral Ag (presumably IL-15 and/or IL-7), IL-2 is absolutely required for differentiation and expansion of pTregs in vivo.

**IL-2 is not required to maintain mTregs in the SDLNs and skin**

To determine whether IL-2 is required to maintain mTregs in peripheral tissues, we used both neutralizing-Ab and genetic-deletion approaches. To generate a stable mTreg population in the skin, cutaneous OVA expression was induced in K5/TGO/DO11 mice, and \(\sim30\) d later, Ag expression was extinguished. Mice were then maintained for \(>40\) d in the absence of OVA. We showed previously that OVA expression is not detectable 20–30 d after removal of doxycycline (3). To determine whether IL-2 is required to maintain mTregs in the skin and SDLNs, mice were treated with a well-characterized mixture of IL-2–neutralizing Abs (14, 15). Compared with mice treated with control Ab, there was no difference in the number of Foxp3-expressing DO11 cells in the skin and SDLNs in mice treated with IL-2–neutralizing Abs (Supplemental Fig. 2A). In these experiments, IL-2 neutralization was confirmed by downregulation of the IL-2R on Tregs in SDLNs (Supplemental Fig. 2B).

IL-2 neutralization with Abs most likely results in partial inhibition of all bioavailable IL-2. Thus, it is plausible that, in these studies, some IL-2 was available to maintain mTregs in the skin and SDLNs. To circumvent this possibility, we used an adoptive-transfer approach with IL-2–deficient DO11 cells into T cell–deficient hosts. Because adoptive transfer of DO11/Rag\(^{-/-}\)/IL-2\(^{-/-}\) cells into K5/TGO/TCR\(^{a-a}\) hosts fails to generate Tregs upon induction of OVA expression (Fig. 2B), we provided IL-2 in trans to enable Treg generation and subsequent development of mTregs. In these experiments, CD90.2\(^{+/+}\)/DO11/Rag\(^{-/-}\)/IL-2\(^{-/-}\)/IL-7\(^{-/-}\) cells were cotransferred with WT CD90.1\(^{+/+}\)/DO11/Rag\(^{-/-}\)/IL-2\(^{-/-}\)/IL-7\(^{-/-}\) cells into K5/TGO/TCR\(^{a-a}\)/IL-2\(^{-/-}\)/IL-7\(^{-/-}\) mice, and OVA expression was induced in the skin. Approximately 30 d later, OVA expression was extinguished, and mice were maintained for \(>30\) d in the absence of Ag to generate mTregs. WT CD90.1\(^{+/+}\)/DO11/Rag\(^{-/-}\) cells were then depleted using a toxin-conjugated anti-CD90.1–depleting Ab. After deletion of IL-2–producing cells, mice were maintained for \(\sim40\) more days, after which skin and SDLNs were harvested, and mTregs were quantified (Fig. 3A). In contrast to transfer of IL-2–deficient DO11 cells alone, cotransfer with WT DO11 cells resulted in Treg generation, as Tregs derived from IL-2–deficient DO11 cells were readily detected in both the SDLNs and skin after induction of OVA expression (Fig. 3B). Depletion of CD90.1\(^{+/+}\)/IL-2–producing T cells in this adoptive-transfer model resulted in no change in the percentage or absolute number of mTregs in the skin or SDLNs (Fig. 3C). Interestingly, although WT DO11 cells were readily detected in SDLNs prior to depletion, these cells were never observed in the skin, before or after depletion (Fig. 3B). We speculate that this is secondary to a competitive disadvantage, because WT DO11 cells were cotransferred with IL-2–deficient DO11 cells at a ratio of 1:3 (i.e., WT DO11/IL-2–deficient DO11 cells). Thus, IL-2–producing T cells were most likely never present in the skin in this adoptive-transfer model. Taken together, these results indicate that IL-2 is not required to maintain mTregs in skin.

**IL-7 is required to maintain mTregs in skin**

IL-7 has long been recognized as a survival factor for memory T cells, and mTregs in the skin express high levels of IL-7R\(\alpha\) (Fig. 1). We tested whether IL-7R–mediated signaling plays...
a role in maintaining these cells in the skin and SDLNs. To do so, we used the A7R34 anti–IL-7Rα Ab clone, which does not cause Ab-dependent cell-mediated cytotoxicity and was used to mediate its effects specifically by blocking IL-7 signaling (16, 17). To generate a stable mTreg population in the skin, OVA expression was induced in K5/TGO/DO11 mice for 30 d, after which doxycycline was removed for 30 d to extinguish Ag expression. Mice were then treated for 3–4 wk with anti–IL-7Rα or control Ab, and both skin and SDLNs were harvested for assessment of DO11 T cell numbers. Consistent with previous reports, the A7R34 Ab clone stably binds to cells expressing IL-7Rα, thereby inhibiting staining of this receptor by flow cytometry (Supplemental Fig. 2C) (16). Mice treated with anti–IL-7Rα had a significant reduction in the number of DO11 mTregs and non-Treg memory T cells in the skin but not in the SDLNs (Fig. 4). There was no change in the ratio of Tregs/non-Tregs in the skin or SDLNs after anti–IL-7Rα treatment. Similar results were observed when IL-7R signaling was blocked in our adoptive-transfer model (i.e., when mTregs are generated by Ag recognition by naive CD4 T cells in the steady state), suggesting that IL-7 is required to maintain mTregs in the skin, regardless of whether they are derived from tTregs or pTregs (data not shown). Collectively, these results suggest that IL-7 plays an essential role in maintaining effector mTregs and non-Treg effector memory cells in the skin.

Fopx3-expressing Tregs are a dynamic and heterogeneous population. Different subsets of these cells have different requirements for their establishment and maintenance, which most likely depends on both their stage of differentiation and their local environment. We speculate that, at any one time, the secondary lymphoid organs of mice and humans contain a mixture of tTregs, pTregs, and mTregs in an as-yet-defined ratio.

In the skin, effector mTregs do not require IL-2 for their maintenance (Fig. 3). This may be because of an inherent difference in signaling pathways required to maintain memory versus naive T cells, or it may be specific to the tissue in which they reside. Given the abundance of effector T cells in secondary lymphoid tissues compared with the skin, it is likely that there is relatively more constitutive IL-2 production in LNs compared with the skin in the steady state. In addition, it is well known that keratinocytes are a rich source of constitutive IL-7 production (18). It is interesting to speculate that effector mTregs (and non-Treg effector memory cells) have adapted to be dependent on IL-7 in tissues in which IL-2 is less abundant. This is consistent with previous studies showing that Tregs increase expression of IL-7Rα in the absence of IL-2 (19). An alternative explanation is that mTregs in the skin are dependent on relatively low levels of IL-2 that come from a source other than T cells, such as cutaneous dendritic cells. Although possible, we feel that this explanation is less likely given that dendritic cells need to be activated to make IL-2, as they have not been shown to constitutively secrete this cytokine in the steady state (20, 21). However, even if low levels of non–T cell–derived IL-2 are capable of mediating signals to mTregs in the skin, our data suggest that this is not the dominant pathway for maintenance of these cells, because specifically blocking IL-7 signaling significantly reduces the number of cutaneous mTregs in IL-2–competent hosts (Fig. 4).

Collectively, our results provide direct evidence that signaling through the IL-7R is a major pathway required to maintain effector mTregs in the skin. We speculate that other factors in addition to IL-7 play a role in maintaining these cells. Further elucidating the fundamental biology of this unique population is required to fully understand the factors that control their differentiation and stability.
population will undoubtedly provide insight on how Tregs maintain immune homeostasis in peripheral tissues.

Acknowledgments
We thank Carlos Benetiz (Department of Pathology, University of California San Francisco) for assistance with animal husbandry; Jonathan Paw and Mike Lee (Flow Cytometry Core, University of California San Francisco) for cell sorting; and Dr. Ann Rothstein (Department of Medicine, University of Massachusetts Medical School, Worcester, MA), Dr. Katya Ravid, and Greg Martin (Transgenic/Knockout Core, Boston University School of Medicine, Boston, MA) for derivation of TRE-TGO-transgenic mice.

Disclosures
The authors have no financial conflicts of interest.

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