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Human Epidermal Langerhans Cells Maintain Immune Homeostasis in Skin by Activating Skin Resident Regulatory T Cells

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Abstract

Recent discoveries indicate that the skin of a normal individual contains 10-20 billion resident memory T cells (which include various T helper, T cytotoxic, and T regulatory subsets, that are poised to respond to environmental antigens. Using only autologous human tissues, we report that both *in vitro* and *in vivo*, resting epidermal Langerhan cells (LC) selectively and specifically induced the activation and proliferation of skin resident regulatory T cells (Treg), a minor subset of skin resident memory T cells. In the presence of foreign pathogen, however, the same LC activated and induced proliferation of effector memory T (Tem) cells and limited Treg cells activation. These underappreciated properties of LC: namely maintenance of tolerance in normal skin, and activation of protective skin resident memory T cells upon infectious challenge, help clarify the role of LC in skin.

INTRODUCTORY PARAGRAPH

It was recently demonstrated that there are an estimated 20 billion T cells in the skin of an adult human, with virtually all of them bearing markers that identify them as skin homing memory T cells (CD45RO/CLA/CCR4) (Clark et al., 2006a). Not only are there twice as many T cells in skin as in blood, but more than 20-fold more memory T cells with a skin homing phenotype can be found in skin rather than blood. In mouse models of skin infection (Jiang et al., 2012) and presumably in humans, these cells accumulate over time and are thought to represent a compendium of T cell memory to antigens encountered through skin (Clark, 2010). Recent studies from human CTCL patients treated with alemtuzumab demonstrate an abundant population of nonmalignant normal skin resident effector memory T cells (Tem) that do not readily recirculate, and appear to protect the host from infection locally(Clark et al., 2012). Skin dendritic cells can readily present antigen to skin resident memory T cells, a process that can occur immediately after pathogen invasion of skin and requiring no cellular recruitment or migration from blood (Heath and Carbone, 2009;

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Wakim et al., 2008). However, most studies on the role of dermal DC or LC have examined their capacity to stimulate naïve T cells (Fujita et al., 2009; Furio et al., 2010; Klechevsky et al., 2008; Mathers et al., 2009), an interaction that can occur only after DC or LC have left skin, migrated through afferent lymphatics and ultimately entered the T cell-rich area of the skin draining lymph node. Given their respective strategic locations, from a purely stochastic perspective, it is far more likely that skin resident DC's will encounter skin resident memory T cells in skin, rather than a naïve T cell in lymph node. However, because of the relatively recent discovery of skin resident memory T cells, studies on the interaction of skin resident DC and skin resident memory T cells from the same individual have not yet been published.

Human skin also contains a resident population of regulatory T cells (Treg) that represent between 5 and 10% of the total resident skin T cells (Clark et al., 2008; Clark and Kupper, 2007). To date, most human Treg cells studies analyzing their phenotype or function have been performed on rare (1-4%) blood-derived effector $CD45RO^{+}FoxP3^{hi}$ (Miyara et al., 2009; Sakaguchi et al., 2010; Wing and Sakaguchi, 2010). These effector $CD45RO^{+}$ Treg can suppress the proliferation and cytokine production of effector T cells. Although these effector Treg express CD45RO, which is the marker of conventional memory T cells, no evidence for memory and recall responses by these Treg cells has been previously demonstrated in mouse experiments until recently. Indeed, effector memory Treg cells have been shown to persist in skin and induce tolerance to a novel transgenically expressed "autoantigen" in a mouse model (Rosenblum et al., 2011). However little is known about the function of effector $CD45RO^{+}$ skin resident memory Treg cells which populate peripheral human tissues such as skin. We have previously demonstrated that human skin Treg cells can proliferate in an antigen-independent manner when cultured with IL-15 and dermal fibroblasts (Clark and Kupper, 2007), conditions that can be seen in inflamed tissue (Rappl et al., 2001). Moreover, another study showed that skin human skin-resident memory Treg cells can locally expand during cutaneous inflammation (Vukmanovic-Stejic et al., 2008) and can serve as a brake to regulate the inflammation in peripheral tissue (Dudda et al., 2008). More recently, it was shown in a human model that glucocorticosteroids can induce proliferation of Treg cells in skin(Stary et al., 2011)., Under normal conditions, however, the function and homeostasis of human skin resident memory Treg cells remain uncharacterized.

Normal human skin contains also distinct populations of dendritic cells (DC): Langerhans Cells (LC) expressing CD1a and Langerin/CD207 (Valladeau et al., 2000), and dermal resident DC (DDC) expressing CD1c, which is also known as blood dendritic cell antigen (BDCA1) (Zaba et al., 2007; Zaba et al., 2009). LC mainly populate the epidermis, and are traditionally considered as the first line of defense against exogenous pathogen invasion (Merad et al., 2008). More recently, LC function has become more controversial, with some studies suggesting their true role is not immune stimulation but rather immunoregulation (Kaplan, 2010; Lutz et al., 2010). Studies in transgenic mice have revealed a role for LC in the induction of tolerance. Data using LC-deficient mouse models indicated that LC might actually dampen inflammation and mediate tolerance, especially in contact hypersensitivity reactions (Bobr et al., 2010; Igyarto et al., 2009; Kaplan et al., 2005). At the same time, human LC have been found to be the most powerful DC subset to induce the *in vitro* differentiation and the polarization of naïve $CD4^{+}$ T cells into, respectively, either Th2 (Furio et al., 2010), Th17 (Mathers et al., 2009) or more recently Th22 (Fujita et al., 2009) effector cell phenotypes. Moreover, human LC can also prime and cross-prime naïve $CD8^{+}$ T cells (Klechevsky et al., 2008). Thus, there is substantial controversy regarding the physiologic role of LC with regard to immunoregulation versus immunostimulation (Stoitzner, 2010).

This paradox has been difficult to reconcile experimentally, in part because of certain apparent differences between human and murine skin. Langerin/CD207, an integral glycoprotein of Birbeck granules that define human LC (Valladeau et al., 2000), is expressed by a subset of dermal DC in mice that is not observed in human skin (Bursch et al., 2007). Moreover, many resident T cells in murine skin are TCR$\gamma\delta^+$ and have a limited antigenic repertoire (Nanno et al., 2007), while T cells resident to human skin are predominantly TCR$\alpha\beta$ and have great TCR diversity (Clark et al., 2006a). To further complicate matters, most human studies of LC have involved stimulation of allogeneic, rather than autologous, naïve T cells from peripheral blood, thus introducing a potential artifact into the system. These and other differences make it difficult to directly compare experimental results on human and murine LC biology, or to extrapolate experimental results involving murine LC to human skin immunity. Increasing, the trend among immunologists has been to address questions relevant to human immunology using human models(Davis, 2008, 2012), as we have chosen to do here.

In the present study, we demonstrate that LC induced the proliferation of a subset of autologous skin resident memory T cells *in vitro*. These proliferating T cells were exclusively $CD4^+CD25^+FoxP3^+CD127^-$ skin resident Treg cells, and their function was demonstrated by suppression of proliferation of autologous skin resident Tem cells. The LC mediated proliferation of these skin resident memory Treg cells could be completely blocked by antibodies to Class II MHC, as well as CD80-86, suggesting antigen presentation. Moreover, the proliferation could be blocked with antibodies to LC derived IL-15 activity or T cell derived IL-2. Immunohistochemical analysis **and FACS analysis** of normal human skin revealed that skin resident memory Treg were predominantly located within or near the epidermis or follicular epithelium, always proximity to LC. A subset of these skin resident memory Treg were positive for Ki67 *in situ*, a cell cycle antigen specific for recent or ongoing proliferation (Scholzen and Gerdes, 2000), suggesting that what we observed *in vitro* is occurring *in vivo* at steady state. Interestingly, when the skin pathogen *C. albicans* was added to the co-cultures *in vitro*, an additional subset of T cells proliferated. Further analysis showed that not only skin resident memory Treg cells, but also antigen-specific skin resident Tem cells subsets proliferated under these conditions, and that the skin resident Tem cells produced the effector cytokines IFNγ and IL-17. These data suggest that under steady state conditions in skin, LC mediate tolerance to self antigens, but can also be activated by infection to mediate protective immunity. Thus, their capacity to negatively or positively modify immune response depends upon context.

RESULTS

Langerhans cells induce proliferation of skin resident $CD4^+$ T cells in the absence of exogenous antigen

We carefully extracted and purified LC, dermal DC, and skin-resident memory T cells from the skin of multiple individual donors (Figure S1). We first isolated skin resident memory T cells from normal human skin as previously described (Clark et al., 2006a). Skin T cells are composed of $CD45RO^+$ T cells expressing skin homing addressins CLA and CCR4 and lacking co-expression of CCR7 and CD62L (Clark, 2010). In parallel, we isolated cutaneous DC from the same donor. Skin DC contained epidermal HLA-DR^+CD207^+ LC and dermal HLA-$DR^{hi}BDCA1^+$ DC (DDC) (Zaba et al., 2007; Zaba et al., 2009). Both LC and DDC expressed significant levels of CD86, CD83 and DC-LAMP (Figure S2) as described in previous studies (Bond et al., 2009; Klechevsky et al., 2008). Skin resident memory T cells were then stained with carboxyfluorescein succinimidyl ester (CFSE) and then co-cultured in presence of purified autologous skin DC. After 6 days of co-culture, T cell proliferation was assessed by flow cytometry. We observed a significant expansion of skin resident

memory T cells in the presence of LC, but no significant proliferation in the presence of DDC. Medium conditioned by LC or DDC had no effect on skin resident memory T cells proliferation (Figure 1A,B). Thus, epidermal LC were strong inducers of skin resident memory T cell proliferation whereas DDC produced negligible proliferation (Figure 1C and Figure S3). Skin resident memory T cells proliferation induced by LC became observable between day 4 and 6 (Figure 1D,E).

We next explored the phenotype of the proliferative skin resident memory T population. Skin resident memory T cells were cultured alone or in the presence of LC. After 6 days, Skin resident memory T cells were isolated and stained with antibodies directed against CD3, CD4, CD8. Strikingly, the proliferative skin resident memory T cells were overwhelmingly $CD4^+$ T cells (96.7% ± 2.7% for four donors; (Figure 2A,B), whereas the non proliferative cells were composed of a more substantial fraction of $CD8^+$ T cells. Therefore, LC but not DDC induced the proliferation of skin resident memory T cells, a process which required cell contact. CD4+ T cells were preferentially stimulated.

The proliferating skin-resident memory T cells are T_{reg} cells

In normal human skin, resident memory T cells contain a population of skin-resident memory Treg cells that are $CD4^+CD25^+FoxP3^+CD127^-$, and we then asked whether the expansion of T cells induced by LC *in vitro* might involve skin-resident memory Treg cells. To address this issue, skin isolated CFSE labeled skin resident memory T cells were again cultured with purified autologous LC. At day 6, T cells were analyzed with antibodies directed against CD4, CD25, FoxP3 and CD127 by flow cytometry. Strikingly, in the expanded proliferative T cell population, 84.8% ± 8% of the T cells were $CD4^+CD25^+FoxP3^+CD127^-$, $CTLA\text{-}4^+$, $GITR^+$ and $CD69^-$ compatible with a Treg phenotype (Figure 3A,B and Figure S4A,B), whereas the fraction of skin resident memory Treg cells in the non-proliferative, skin-resident memory T cells subset was comparable with the fraction observed in the control population. Moreover, an actual absolute expansion of Treg cells with a range of amplification from 1.5 to 3.0 fold was observed after co-culture with autologous LC (Figure 3C). Deletion of $CD25^+$ skin resident memory T cells subset prior to co-culture with autologous LC significantly reduced the expansion of skin-resident memory Treg cells (Figure 3D). Because phenotypic markers do not measure Treg function, we next assessed the ability of the expanded skin resident memory Treg population to suppress T cell proliferation in a secondary MLR. The $CD4^+CD25^+CD127^-$ skin resident memory Treg cells were harvested and isolated after 6 days of co-culture and then added to a MLR composed of CFSE labeled skin resident Tem cells and irradiated PBMC's as stimulators. The addition of this activated population of skin resident memory Treg reproducibly and significantly inhibited autologous skin resident Tem cells proliferation in this assay **(**Figure 3E **)**, and showed that there was >65% suppression of such responses at a 1:1 ratio. These results were confirmed in additional experiments in using a [^{3}H]thymidine incorporation assay (Figure 3F**)**. Again, isolated $CD4^+CD25^+CD127^-$ skin resident memory Treg cells strongly inhibited the proliferation of skin resident Tem cells. Therefore, LC specifically induced the proliferation of skin resident memory Treg cells, but not skin-resident Tem cells, under steady state conditions and these expanded skin resident memory Treg cells showed potent suppressive activity. Taken together, these results suggest that Treg cells, a minor population among skin resident memory T cells, were being specifically induced to proliferate by LC. These Treg cells demonstrated a classical phenotype ($CD4^+CD25^+FoxP3^+CD127^-$), and mediated significant suppression of proliferation of skin resident Tem cells.

LC mediated skin resident memory Treg cells proliferation is antigen-specific

We demonstrated above that LC have the unique capacity to induce proliferation of functional skin resident memory Treg cells. To further understand which signals are involved in the interaction between LC and skin resident memory T cells, we co-cultured CFSE labeled skin resident memory T cells with autologous LC in presence of different blocking antibodies: anti-MHC class I and/or II, CD80-86, CD1a, IL-2 and IL-15Rα. At day 6, expansion of skin $CD3^+$ skin resident memory T cells was assessed by dilution of CFSE using flow cytometry (Figure 4A,B). Blocking the MHC class II presentation and more specifically HLA-DR and DP, significantly reduced the proliferation of skin resident memory T cells (Figure 4C,D). Similarly, blocking the costimulatory signal CD80-86 or the addition of cytokine antibodies directed against IL-2 or IL-15Rα significantly decreased the induction of skin resident memory T cell proliferation (Figure 4B). In contrast, the proliferation of skin skin resident memory Treg cells was not dependent of CD1a or MHC Class I presentation. These data strongly suggest that proliferation of skin skin resident memory T cells by resident LC proceeds in an antigen dependent manner.

Skin resident memory Treg cells are located in the epidermis or at the papillary dermal-epidermal junction, and proliferate *in situ*

We next asked if we could detect interactions between LC and skin resident memory Treg cells *in vivo*. We examined multiple specimens of freshly obtained human skin, using immunofluoresence microscopy. As we have previously described, abundant $CD3^+$ skin resident memory T cells could be identified in uninflamed human skin, and a subset of $CD3^+$ skin resident memory T cells co-expressed nuclear FoxP3 and CD25 (Figure S5A), identifying them as skin resident memory Treg cells (Figure 5A). Interestingly, these skin resident memory Treg cells appeared to be localized in or near epidermis and follicular epithelium, in contact or close proximity to $CD1a^+$ epidermal and follicular LC **(**Figure 5B and Figure S5C). We next asked if these skin resident memory Treg cells showed evidence for proliferation by examining expression of the marker Ki67, which is expressed through all phases of the cell cycle, but is absent in cells in G_0. $CD3^+FoxP3^+$ skin resident memory T cells that expressed Ki67 could be readily observed in skin sections, while $Ki67^+$ $CD3^+FoxP3^-$ skin resident Tem cells were undetectable. The $CD3^+FoxP3^+Ki67^+$ skin resident memory T cells were predominantly located in epidermis, follicular epithelium, or upper papillary dermis, rather than in the deeper papillary or reticular dermis (Figure 5C and Figure S5B,C). Moreover we observed that $CD1c/BDCA1^+$ DDC were found close to $FoxP3^+$ skin-resident memory Treg cell in a resting state (Figure S5D). The strict association of Ki67 expression with cellular proliferation leads us to conclude that *in vivo* as well as *in vitro*, epidermal LC induce the proliferation of skin resident memory Treg cells, consistent with a tolerogenic role for LC at steady state. These results showed that Treg cells and LC co-localized in epidermis and follicular epithelium, and that a discernable population of these Treg cells appeared be entering the cell cycle, as judged by Ki67 expression. This *in vivo* data is completely consistent with the observations of LC induced Treg cells proliferation *in vitro*

Abundant skin resident memory T cells, including skin resident memory Treg cells, are present in the epidermis by FACS analysis

To confirm that skin resident memory T cells were indeed present in epidermis, we subjected skin explants to dispase treatment, which cleaves skin specifically at the dermal epidermal junction without disrupting epidermal cell-cell interactions. Dermis and epidermis were then separated, enzymatically digested, and analyzed independently. Skin resident memory T cells were readily found in the epidermis (3% all cells), and 17% of these CD3+ cells were $FoxP3^+$ skin resident memory Treg cells. LC were similarly abundant (1.2% of all

cells), and no cells with DDC markers were observed in the epidermal fraction, confirming the specificity of the separation (Figure 6A). Dermis contained a smattering of $CD1a^+$ LC (0.1%), and again skin resident memory T cells were abundant (3.6% of all cells). About 70% fewer of these skin resident memory T cells were $FoxP3^+$ skin resident memory Treg cells (6%), suggesting that skin resident memory Treg cells are enriched in the epidermal compartment (Figure 6B). Thus, our immunofluoresence histology observations were validated by FACS analysis of the dermal and epidermal compartments, that is, LC and skin resident memory Treg cells can be found together in the epidermal compartment.

During infection, LC induce proliferation of both skin resident memory Treg cells and skin pathogen-specific Tem cells

Our data strongly suggest that human LC have the ability to maintain peripheral tolerance by inducing local proliferation of skin resident memory Treg cells. However, recent studies (Fujita et al., 2009; Furio et al., 2010; Klechevsky et al., 2008; Mathers et al., 2009) on the biology and function of human LC showed their capacity to induce differentiation and expansion of naïve $CD4^+$ T cells into potent Tem cells cells rather than Treg cells, in apparent contrast with our data. To further assess the function of LC in human skin, we analyzed the proliferation of skin resident memory T cells induced by autologous LC in the presence of *Candida albicans (C.albicans).* Most immunocompetent individuals can manifest a delayed type hypersensitivity response to *C. albicans*, a ubiquitous human skin commensal which can cause skin inflammation in the setting of barrier disruption (Romani, 2000). *C. albicans* applied to the surface of intact skin provokes no response, but when injected intradermally, can induce T cell mediated inflammation which is evident by 24-48 hours (Esch and Buckley, 1988). We have previously shown that most individuals have skin resident memory T cells that can respond *in vitro* to *C. albicans*, both via proliferation and IL-17 and IFNγ production. We asked whether LC would present *C. albicans* antigens to skin resident memory T cells in an immunogenic or tolerogenic fashion. In 9 out of 10 donors, addition of *C. albicans* antigen led to substantially increased proliferation of skin-resident memory T,cells (Figure 7A,B) and proliferating skin resident memory T cells included both $FoxP3^+CD127^-$ and $FoxP3^-$ $CD127^+$ T cells, indicating that both skin resident memory Treg cells and skin resident Tem cells were proliferating (Figure 7C). Interestingly, low doses of *C. albicans* antigen favored Treg cells proliferation, while higher doses favored Tem cells proliferation (Figure 7D). This is consistent with the observation that Treg cells are most effective at lower levels of antigen. Moreover, depletion of $CD25^+$ Treg cells is associated with increased proliferation in the presence of *C. albicans* suggesting the absence of Treg cells inhibition on specific *C.albicans* Tem cells expansion (Figure 7E).

The proliferation of skin resident memory T cells to *C. albicans* could be blocked completely by antibodies to Class II MHC and CD80/86, as well as antibodies to IL-2 and IL-15Rα **(**Figure S6). Proliferating cells produced IL-17 and IFNγ (Figure S7A), and were enriched for Vβ2, Vβ3, and Vβ8 αβTcr T cells (Figure S7B). Interestingly, there was a highly reproducible >50% inhibition of proliferation with a blocking antibody to CD1a, which had no effect on skin resident memory Treg cells proliferation in the absence of *C. albicans*. Taken together, these data suggest that after pathogenic antigen exposure, LC induced proliferation of pathogen specific Tem cells as well as Treg cells. Thus, depending upon the biological context, LC can mediate tolerance or immunity.

DISCUSSION

There are several features of our study which have not been previously reported, and these are discussed below. First, we have examined the function of LC in normal human skin, and most importantly the interaction of LC with skin resident memory T cells, a population first

described in 2006 (Clark et al., 2006a). These studies are made possible by a technical advance; specifically, our ability to extract large numbers of skin resident memory T cells from human skin, as we have described previously (Clark et al., 2006b). Virtually all skin resident memory T cells have memory (CD45RO) and skin homing (CLA, CCR4) markers, most have a Tem phenotype, and all are thought to be derived from prior antigen encounters in skin draining lymph nodes (Clark, 2010). Indeed, recent mouse models have indicated that it is these skin resident memory T cells, rather than circulating T cells or antibodies, that mediate host defense upon microbial challenge (Gebhardt et al., 2009; Liu et al., 2010). More recently, this was validated in a model of human disease(Clark et al., 2012). Thus, the interaction between skin resident dendritic cells (including LC) and skin resident memory T cells is likely to be essential for rapid recall or memory responses in skin to proceed. In contrast, most recent papers describe human LC interactions with naïve T cells (Fujita et al., 2009; Furio et al., 2010; Klechevsky et al., 2008; Mathers et al., 2009), a process requiring that LC exit the epidermis and migrate down afferent lymphatics to lymph node, and most relevant to primary immune responses. We do not suggest that these studies are not important, only that they describe the biology of primary rather than memory responses.

Second, we describe the capacity of LC, but not dermal DC, to induce the proliferation of a subset of skin resident memory T cells, specifically $CD4^+CD25^+FoxP3^+CD127^-$ T cells that have the phenotype of Treg cells (Liu et al., 2006). This proliferation appears to be dependant upon antigen presentation, as it can be inhibited completely by antibodies to either Class II MHC or CD80-86. In contrast, antibodies to MHC Class I or CD1a had no affect. The proliferation could also be blocked by antibodies to IL-2 and IL-15 activity, cytokines derived from T cells (Waldmann, 2006) or LC (Munz et al., 2005; Ratzinger et al., 2004), respectively. These data are consistent with LC presenting antigen to skin resident memory Treg cells. While it is tempting to speculate that it is self-antigen that is being presented, we cannot rule out the possibility that there is some carryover of normal skin flora antigens in our culture conditions. However, a recent report in a K5-ovalbumin expressing murine model demonstrated that self-reactive skin resident memory Treg cells could be generated and persist in skin. These memory Treg cells could reduce and ultimately suppress an autoimmune T cell mediated skin disease (Rosenblum et al., 2011).

Supporting the idea that epidermal LC and skin resident memory Treg cells have ample opportunity to interact *in vivo*, our FACS analysis of dermal and epidermal compartments confirm that skin resident memory T cells in general are abundant in epidermis and dermis and that skin-resident memory Treg cells in particular are more abundant in epidermis than dermis. Immunohistochemical studies demonstrate that skin resident memory Treg cells are localized to either the epidermis or the dermal epidermal junction, and overlap with $CD1a^+$ LC. A reproducible fraction (5%) of these skin resident memory Treg cells is positive to Ki67. Ki67 is generally considered a proliferation marker, and is present during all phases of the cell cycle, but is absent in resting (G0) cells (Scholzen and Gerdes, 2000). This *in vivo* finding is completely consistent with our *in vitro* observation that LC selectively induce proliferation of the skin resident memory Treg cells subset of skin resident T cells. While the immunostaining data show that skin resident memory Treg cells are the most abundant skin resident T cells to interact with LC *in vivo*, the *in vitro* proliferation data indicate that even when skin resident memory Treg cells are a minority population, they are still preferentially induced to proliferate by LC from normal skin.

Phenotype does not prove function (Sakaguchi et al., 2010), and thus we felt it necessary to establish whether our $CD3^+CD4^+FoxP3^+CD127^-$ could suppress Tem cell immune responses. We felt that the most relevant responder Tem cells would be the autologous skin resident Tem cells derived from the same sample of skin. We were able to demonstrate quite clearly that the addition of skin resident memory Treg cells substantially blocked the

proliferation of autologous skin resident Tem cells to allogeneic antigen, using as a read-out either CFSE dilution or [^{3}H]thymidine incorporation. Thus, LC induced expansion of skin resident memory Treg cells that can suppress autologous skin resident Tem cell responses. Given the localization of these cells in the epidermis and dermis, it is tempting to speculate that LC are activating skin resident memory Treg cells to maintain tolerance to self antigens (Morelli et al., 2001; Morelli and Thomson, 2007). It is also possible that LC could present antigens from normal skin resident microbial flora to skin resident memory Treg cells (Kubo et al., 2009). Further studies will determine which of these potential roles is more important.

Most studies of human LC have focused upon their potent ability to activate and differentiate naïve T cells *in vitro*. Preferential generation of Th2 (Furio et al., 2010), Th17 (Mathers et al., 2009), and most recently Th22 (Fujita et al., 2009) cells by LC has been noted, as has the cross presentation of antigen to $CD8^+$ effector cells (Banchereau et al., 2009; Klechevsky et al., 2008). As noted above, all such interactions occur physiologically in lymph nodes, the only possible site where LC and naïve T cells will encounter each other. Given the paradox that LC are immunoregulatory within skin in the absence of exogenous antigen (our data), and the ample evidence that LC that have migrated to LN are profoundly immunostimulatory, we asked whether LC could present antigen in an immunostimulatory way to skin resident Tem cells, a population that a LC would certainly encounter during a recall immune response prior to migration to LN.

Candida antigen, via skin test, has been used clinically as a measure of immunocompetence. Typically, >75% of humans will have a positive skin test to *C. albicans* 48 hours after intradermal injection (Esch and Buckley, 1988). This time point is consistent with a memory immune response, and our unpublished data suggests that it is mediated by skin resident Tem cells specific for *C. albicans*. Thus, we reasoned that most individuals would have skin resident Tem cells that respond to *C. albicans*. We next asked whether LC could present *C. albicans* to skin skin resident Tem cells. The addition of *C. albicans* to cultures of autologous LC and skin resident memory T cells induced significantly more T cell proliferation than in its absence. Under these circumstances, two populations of T cells proliferated. First was the $CD4^+CD25^+FoxP3^+CD127^-$ population that proliferated in the absence of *C. albicans* antigen. But in the presence of *C. albicans*, a second population of T cells proliferated; these were $FoxP3^{lo}$, $CD127^+$, and produced the effector cytokines IFNγ and IL-17 (Acosta-Rodriguez et al., 2007). The cells that proliferated were also of a phenotype that suggested *C. albicans* specificity by TCR Vβ (Devore-Carter et al., 2008; Walsh et al., 1996). This latter population consists of skin resident Tem cells specific for *C. albicans*, and if their proliferation was blunted by the concurrent proliferation of skin-resident memory Treg cells, it did not occur to a substantial degree.

Given the abundance of skin resident memory T cells in normal skin, many of them specific for normal skin microbial flora (Clark, 2010; Clark et al., 2006a), it stands to reason that the activation of these cells must be tightly controlled. Our data are consistent with a model wherein LC in normal intact skin favor constitutive local proliferation and activation of skin resident memory Treg cells, thus providing a brake on inappropriate activation of skin resident Tem cells in normal skin, whether to autoantigens of foreign antigens. However, this brake appears to be readily cast aside when a pathogen is encountered, as would occur when the physical epidermal barrier is breached or disrupted and innate immune danger signals are provided. Under these conditions, LC are then capable of presenting antigen in an immunogenic fashion to skin resident Tem cells. Our data also suggest that there is a threshold of antigen below which LC induce Treg cells proliferation, but above which LC induce Tem cells activation and proliferation. Thus, depending upon the biological context, LC can function to maintain peripheral tolerance, but also have the capacity to activate

protective skin resident memory T cells that can mount an effective host response to pathogen.

MATERIALS AND METHODS

Isolation of cells from Skin

Acquisition of skin samples and all scientific studies were approved by the Institutional Review Board of the Partners Human Research Committee. Samples of normal adult human skin were obtained as discarded human tissue from cutaneous surgeries. T cells were isolated from skin using short term explant (5 days) cultures as previously described (Clark et al., 2006a). No exogenous cytokines were added. LC and dermal DC were purified from normal human skin (Zaba et al., 2007). Subcutaneous fat was excised, and remaining tissue was washed with PBS. The dermal layer was heavily scored with a scalpel and incubated with 1mg/ml type 1 collagenase (Invitrogen), 1mg/ml dispase (Invitrogen), and penicillin-streptomycin solution overnight at 37°C. Epidermal and dermal sheets were separated and placed in RPMI 1640 supplemented with 10% pooled human serum (Cellgro Mediatech), 0.1% gentamicin reagent solution (Invitrogen), and 1% 1M HEPES buffer (Sigma-Aldrich). After 48h at 37°C, cells were collected and filtered with 40μm cell strainers (BD Biosciences) and then enriched with a Ficoll-diatrizoate gradient (GE Healthcare Bio-Sciences). LC were further purified by positive selection using anti-CD1a MACS (Miltenyi-Biotec). Dermal DC were purified by positive selection using BDCA1$^+$ isolation kit (Miltenyi-Biotec).

Antibodies

All antibodies used for immunofluorescence and FACS are listed in Supplementary Table 1.

Immunofluorescence

5μm-thick sections of formalin-fixed, paraffin-embedded tissues from normal human skin, were cut, deparaffinized, and subjected to a heat-induced epitope retrieval step.. Slides were rinsed in cool running water and washed in Tris-buffered saline, pH 7.4, before incubation with primary antibodies and then incubated appropriate secondary antibodies: Alexa Fluor® 488-conjugated anti-mouse or rat or Alex-Fluor® 594-conjugated anti-mouse. Additional negative controls were performed by adding isotype mouse antibodies as primary antibodies. After subsequent washing, the sections were mounted with Prolong Gold antifade reagent with DAPI (Invitrogen) and covered with cover slip. Immunofluoresecence reactivity was viewed on an Olympus BX51/52 system microscope coupled to a Cytovision System (applied Imaging).

Candida albicans

C. albicans were seeded in RPMI medium plus 10% (vol/vol) FCS in 24-well plate. Plate was incubated for 2h at 65°C to heat inactivated yeast, and was cooled before to be used for experiments.

Analysis of T cell proliferation

For recall responses, skin isolated T cells were stained with 2 μM CFSE (Molecular Probes) at 37°C for 3 min(Parish et al., 2009). CFSE labeled T cells were washed 3 times and then were cultured (5×10^4/well) in 96-well plates in RPMI medium supplemented with 10% pooled human serum, to which autologous LC or dermal DC (2.5×10^4/well) were added. In some experiments heat-killed *C.albicans* (5×10^4) was added to the co-culture.

Blocking Experiments

Purified anti-CD80, CD86, HLA DR-DP-DQ, HLA DR, HLA DP and HLA DQ, HLA A,B,C, CD1a, IL-2 and IL-15Rα or isotypes were added to skin DC and the plate were kept for 30 min at 4°C and then washed before T cells were added.

Regulatory T Cell Assay

Regulatory T cells were isolated after 6 days of co-culture with LC, by using the $CD4^+CD25^+CD127dim/^-$ regulatory T cell isolation kit II (Miltenyi Biotec). T responders $CD4^+CD25^-$ were isolated from normal skin explant culture. CFSE labeled $CD4^+CD25^-$ (effectors) and $CD4^+$ $CD25^+$ $CD127^-$ (suppressors) T cells (2.5×10^3/well) were stimulated with plate-bound anti-CD3 (clone UCHT1, BD Pharmingen) used at a concentration of 1μg/ml, coated in PBS for 2-4 h at 37°C, in presence of irradiated T cells depleted PBMCs (2.5×10^4/well). All cells were cultured in a final volume of 200μl. Proliferation was monitored by CFSE dilution on day 5 or by pulsing (18h) each well with 1μCi of [^{3}H]thymidine (Perkin Elmer) as described previously (Baecher-Allan and Hafler, 2006).

Statistics

Statistical significance was calculated with two-tailed Student's t test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Langerhans Cell (LC) selectively and specifically interact with skin resident memory T cells.
- LC induce activation and proliferation of skin-resident memory Treg cells at steady state, and by doing so control the steady state activation of skin resident effector memory T cells (Tem) .
- In the presence of pathogen antigen, LC induce the proliferation of protective skin resident Tem cells**,** while limiting proliferation of skin-resident memory Treg cells.
- This helps resolve an ongoing controversy about whether LC are tolerogenic or immunogenic by revealing that these functions are influenced by biological context.

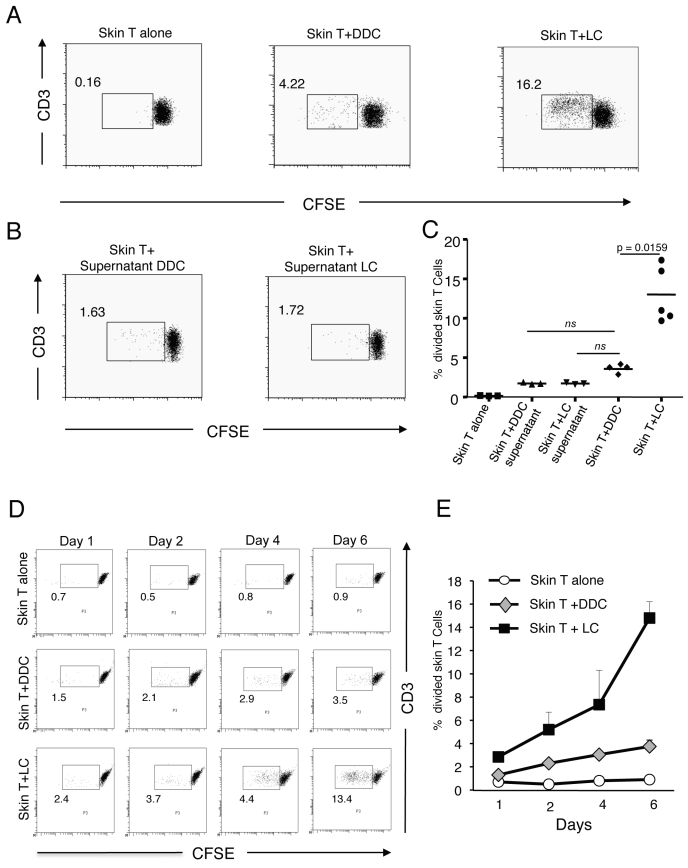

Figure 1. A subset of skin resident memory T cells proliferates in presence of autologous LC

(**A**) Proliferation of skin resident memory T cells labeled with CFSE and cultured alone, or with autologous DDC or LC at a ratio 2:1 after 6 days, or with conditioned medium from LC or DDC. Proliferation as measured by CFSE dilution, from a representative experiment. (**B**) Numbers indicate the percentage of proliferating $CD3^+$ T cells. (**C**) Percentage of proliferating $CD3^+$ skin resident memory T cells, each sign represents a single subject, two-tailed, unpaired t-test. **(D)** CFSE labeled skin resident memory T cells were co-cultured with autologous LC, DDC or alone. Proliferation as measured by CFSE dilution, from a representative experiment at different days. **(E)** Graph shows percentage of proliferating $CD3^+$ skin-resident memory T cells. Results are the mean +SD. .

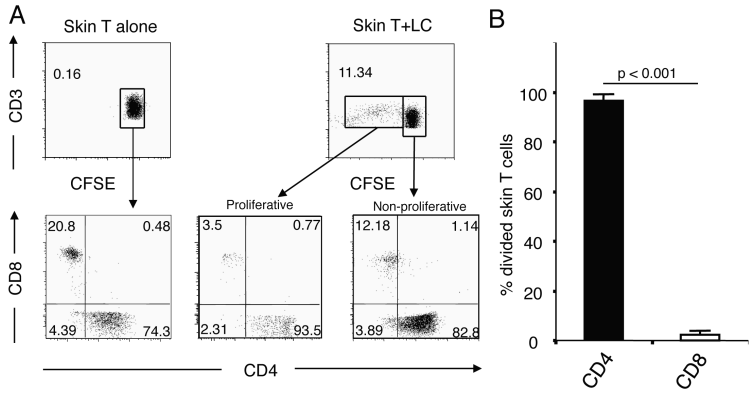

Figure 2. Proliferating skin-resident memory T cells are preferentially CD4$^+$ T lymphocytes

(**A**) Proliferation of CD3$^+$ CFSE labeled skin resident memory T cells stimulated with autologous LC. At day 6 of co-culture, skin-resident memory T cells were stained for CD4 and CD8 and proliferative and non-proliferative skin resident memory T cells subsets were analyzed. Numbers in quadrants indicate percent cells (representative experiment). (**B**) Percentage of CD4$^+$ and CD8$^+$ T cells in the proliferative population. The graph shows mean +/− SD. Data are representative of 4 independent experiments (two-tailed, unpaired t-test).

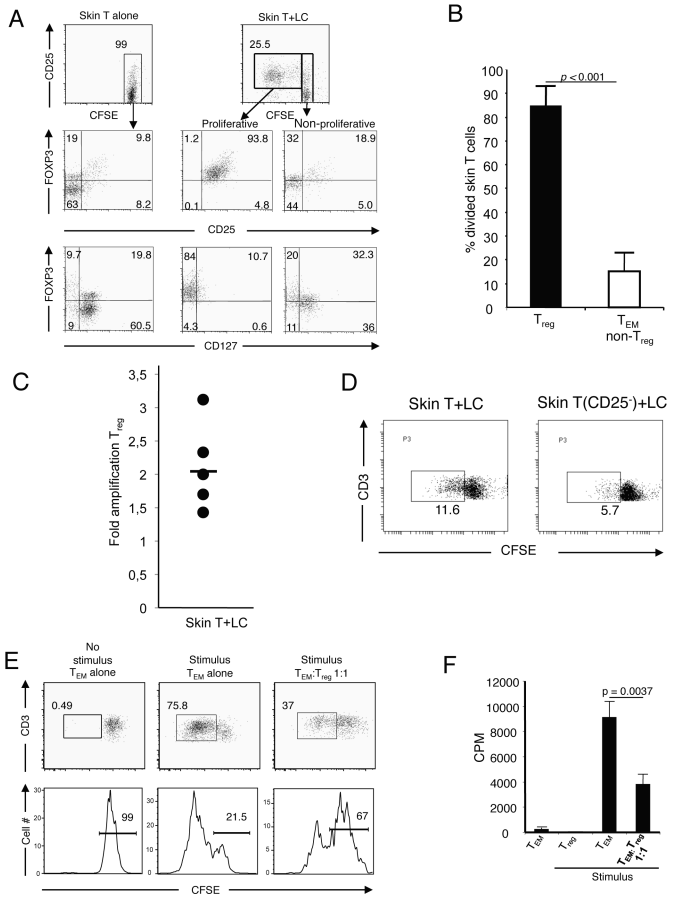

Figure 3. Proliferating CD4$^+$ skin resident memory T cells in presence of autologous LC are functional regulatory T cells

(**A**) CFSE labeled skin resident memory T cells were stimulated with autologous LC. At day 6, CD3$^+$ skin resident memory T cells were stained for CD4, CD25, CD127 and FoxP3. Numbers in quadrants represent percentage. (**B**) Percent of CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$ regulatory T cells (skin resident memory Treg cells) and skin resident memory T cells non-Treg in the proliferative population assessed for different donors. The graph shows mean +/− SD. Data are representative of 5 independent experiments (two-tailed, unpaired t-test). **(C)** Figure represents fold amplification of CD4$^+$CD25$^+$FoxP3$^+$CD127$^-$ Treg cells after 6 days co-culture with autologous LC relative to Treg cells present at day 0. Each sign represent independent experiments, bar represent mean. (**D**) The depletion of CD25$^+$ skin resident memory T cells prior to co-culture with autologous LC decreases the proliferation of skin resident memory T cells. CFSE labeled skin resident memory T cells or CFSE labeled CD25$^-$ skin resident memory **T** cells were co-cultured with autologous LC for 6 days. Proliferation was measured by the dilution of CFSE. Numbers indicate percentage of proliferative cells. (E) Expanded skin resident memory Treg cells from 6 day co-cultures were isolated and then cultured with autologous CFSE-labeled skin resident Tem cells in presence of anti-CD3 (1μg/ml) and irradiated Peripheral Blood Mononuclear Cells (iPBMCs). CFSE dilution was analyzed at day 5. These data are representative of three independent experiments with cells from different subjects. (**F**) Expanded Treg cells were cultured with skin resident Tem cells in the presence of anti-CD3 (1μg/ml) and iPBMCs. After 4 days of coculture, [^{3}H]thymidine was added to each well. The cells were harvested

after 16 hours and radioactivity was measured. The graph shows mean +/− S.D. Data are representative of two independent experiments with triplicate (two-tailed, unpaired t-test).

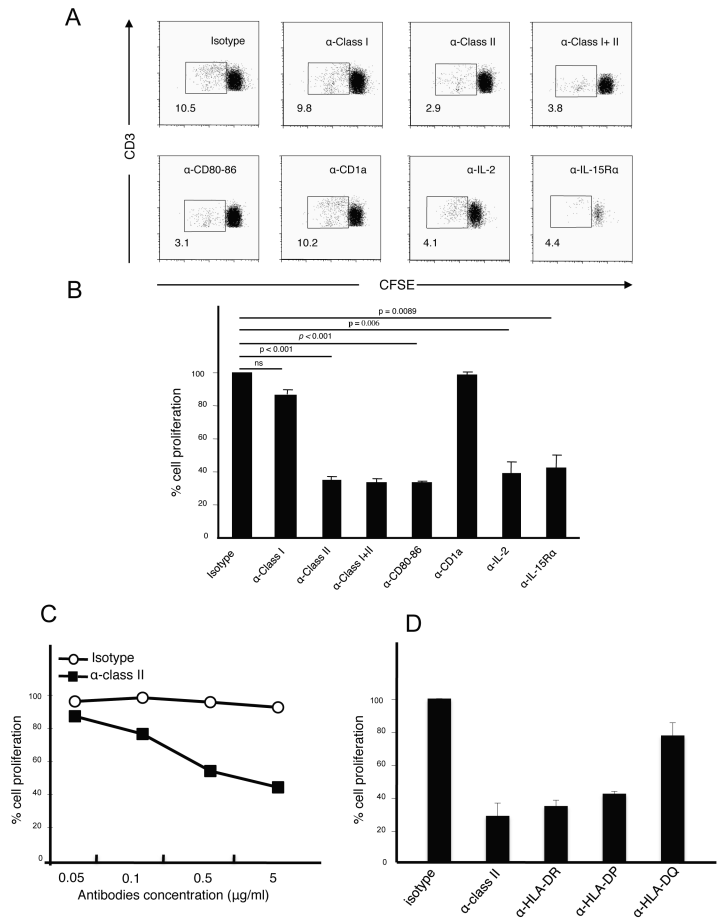

Figure 4. Expansion of skin resident memory Treg cells is dependent on MHC class II, CD80-86, and IL-2 or IL-15

(**A**) Proliferation of CFSE labeled skin resident memory T cells co-cultured with autologous LC in the presence of the indicated neutralizing antibodies or isotype-matched, non-reactive antibodies as control. Proliferation of $CD3^+$ T cells was measured by CFSE dilution. Numbers represent the percent of divided cells for a representative donor. (**B**) Percentage of skin resident memory T cells proliferating in presence of neutralizing antibodies compared to matching isotype control. The graph shows means +/− SD. Data are representative of 3 independent experiments (two-tailed, unpaired t-test). **(C)** Proliferation of $CD3^+$ T cells measured by CFSE dilution in the presence of different concentrations of pan-class II blocker. **(D)** Proliferation of CFSE labeled skin resident memory T cells co-cultured with autologous LC in the presence of the indicated neutralizing antibodies or isotype-matched control. Graph shows means +/− S.D and data are representative of 2 independent experiments with duplicate.

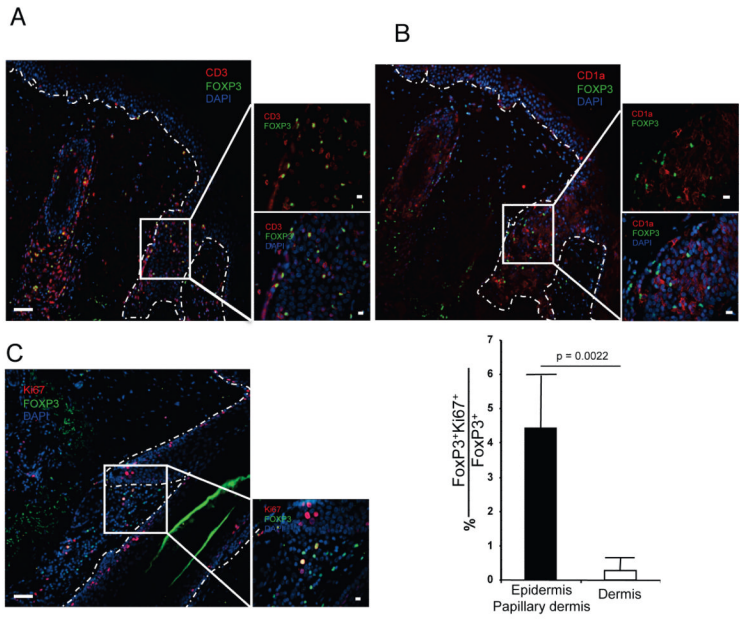

Figure 5. Skin resident memory Treg cells are found in the epidermis near Langerhans cells, and express the proliferation marker Ki67

(**A**) Immunofluorescence of skin showing expression of FoxP3 (green) and CD3 (red), FoxP3, CD3 double–positive regulatory T cells (Treg) are found mainly in the epidermis and the papillary dermis. (**B**) $FoxP3^+$ skin resident memory Treg cells are found in close proximity to $CD1a^+$ LC (red). (**C**) A proportion of $FoxP3^+$ (green) Treg cells co-express Ki67 (red), resulting in a yellow overlay. Right panel, histogram shows the percentage of FoxP3/Ki67 double positive cells in the $FoxP3^+$ population in epidermis/papillary dermis or dermis. The graph shows mean +/− S.D and data are representative of 6 independent donors (two-tailed, unpaired t-test). Scale Bar: bottom left 50μm, bottom right 10μm.

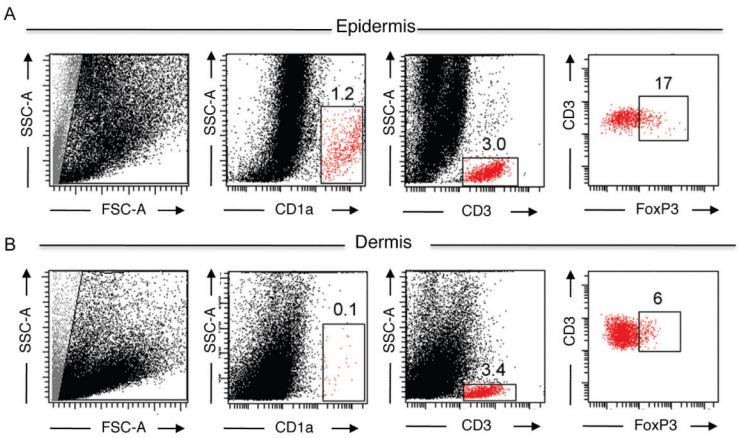

Figure 6. Skin resident memory Treg cells are abundant in epidermis

Epidermal and dermal layers from skin explants were separated using dispase treatment. (A) Epidermal cells contained abundant $CD3^+$ skin resident memory T cells and are enriched for $FoxP3^+$ skin resident memory Treg cells. $CD1a^+$ LC were also abundant in epidermis. (B) Skin resident memory T cells were abundantly present in dermis with fewer skin-resident memory Treg cells than epidermis. No substantial $CD1a^+$ LC were found in dermis. Numbers in quadrant indicate percent cells.

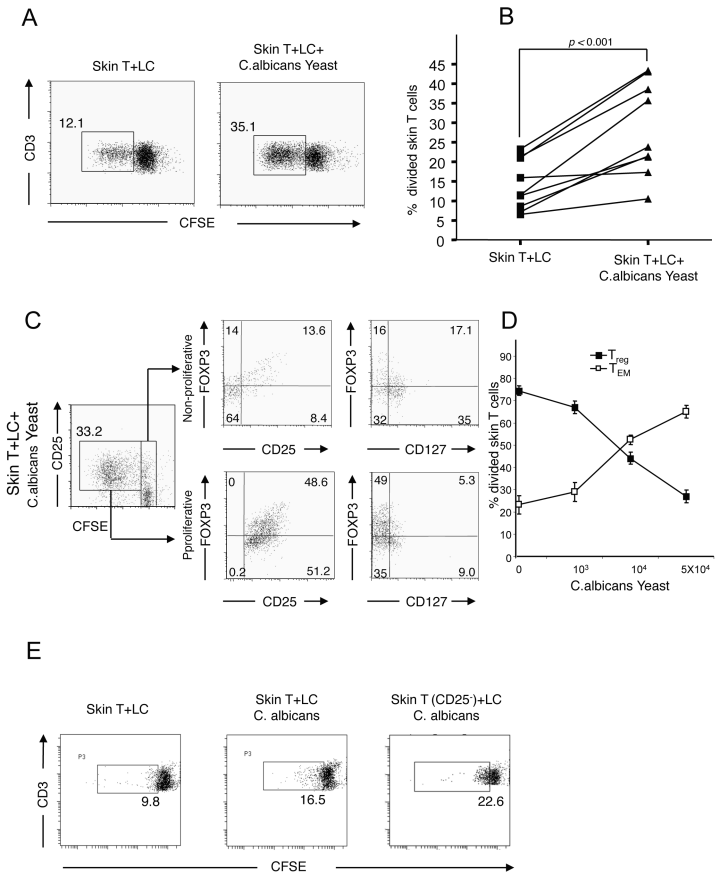

Figure 7. During infection, proliferation of skin resident memory T cells demonstrates a mixed population composed of pathogen specific skin resident Tem cells and skin resident memory Treg cells

CFSE labeled skin resident memory T cells were co-cultured with autologous LC in the presence or absence of heat inactivated *C.albicans* for 6 days. (**A**) Skin resident memory T cells were then stained for CD3. Numbers in quadrant show percentage of expanded T cells. (**B**) Data from 10 independent experiments (two tailed, unpaired t test). (**C**) CFSE labeled skin resident memory T cells stimulated with autologous LC in the presence of heat-inactivated *C.albicans*. At day 6, $CD3^+$ skin resident memory T cells were stained for CD4, CD25, CD127 and FoxP3. Numbers in quadrants represent percent cells in proliferative and non-proliferative populations. Data are representative of 3 independent experiments. (**D**) The graph shows percent skin resident memory Treg cells and skin resident Tem cells in the proliferative populations when skin resident memory T cells and LC were co-cultured with different ratio of *C.albicans*. Data are representative of 2 independent experiments with duplicate. **(E)** Depletion of $CD25^+$ increased the proliferation of skin resident Tem cells in the presence of pathogen. CFSE labeled T cells or $CD25^-$ T cells were co-cultured with autologous LC for 6 days in the presence of C.*albicans*. Proliferation was measured by the dilution of CFSE. Numbers indicate the percent of divided cells for a representative donor.