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Epidermal Langerhans Cell-Deficient Mice Develop Enhanced Contact Hypersensitivity

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Summary

Epidermal Langerhans cells (LCs), a distinct skin-resident dendritic cell population, acquire antigen in the skin and migrate to draining lymph nodes where they are thought to initiate adaptive immune responses. To examine the functional requirement of LCs in skin immunity, we generated BAC transgenic mice in which the regulatory elements from human Langerin were used to drive expression of diphtheria toxin. The resulting mice have a constitutive and durable absence of epidermal LCs but are otherwise intact. Unexpectedly, we found that contact hypersensitivity (CHS) was amplified rather than abrogated in the absence of LCs. Moreover, we showed that LCs act during the priming and not the effector phase. Thus, LCs not only were dispensable for CHS, but they served to regulate the response, a previously unappreciated function.

Introduction

Dendritic cells (DCs) are very efficient at activating naive T cells in vitro and in vivo (Banchereau et al., 2000). They can acquire antigen via multiple routes including endocytosis, pinocytosis, and direct infection by pathogens (Banchereau et al., 2000). DCs also have an array of receptors for pathogen-associated molecular patterns that stimulate DC activation and maturation (Iwasaki and Medzhitov, 2004). Though DCs as a class share these properties, they are nevertheless functionally and phenotypically diverse. They have been categorized based on the expression of numerous markers including CD8α, CD4, CD11b, B220, and C-type lectin receptors such as DEC205 (Henri et al., 2001; Shortman and Liu, 2002). Although DCs were initially identified because of their stimulatory capacity, a growing body of data has demonstrated that immature DCs as well as certain subsets of mature DCs promote tolerance rather than immunity (Mowat, 2003; Steinman et al., 2003; Steinman and Nussenzweig, 2002).

DCs can also be categorized by whether they are resident in secondary lymphoid tissue or in parenchymal tissues. Unlike DCs in secondary lymphoid tissue, tissue-resident DCs do not typically have direct access to naive T cells. Rather, it is thought that these cells function as sentinels that acquire Ag in the periphery and become activated locally (Hemmi et al., 2001). Once activated, they migrate to T cell zones of regional lymph nodes (LNs), where they are thought to either present tissue-acquired Ag or transfer Ag to resident secondary lymphoid tissue DCs for presentation, thereby stimulating a primary response (Carbone et al., 2004; Romani et al., 2003). Despite evidence for priming of some immune responses both directly by secondary lymphoid tissue-resident DCs and in other cases by tissue-resident DCs after migration, their relative roles remain unclear.

Langerhans cells (LC) are a subset of tissue DCs located in the epidermis and are thus the first APCs to contact pathogens at the skin surface (Romani et al., 2003). Immature LCs residing in skin collect antigen, and upon various stimuli, they mature and migrate to draining LNs (Hemmi et al., 2001; Kripke et al., 1990). Thus, they are presumed to play a key role in promoting immune responses.

LC function has been examined in a number of different systems. Contact hypersensitivity (CHS) to cutaneously applied hapten, a model for contact dermatitis, is a classical skin response in which LCs are thought to play a role (Silberberg-Sinakin and Thorbecke, 1980). Treatment with ultraviolet light prior to priming with hapten eliminates LCs from the skin and inhibits the development of CHS, thereby suggesting that LCs are required for the development of a CHS response (Schwarz, 1999; Toews et al., 1980). Supporting this notion, more recent data have demonstrated that LCs that have emigrated to the draining LN can directly present antigen secreted from keratinocytes (Mayerova et al., 2004). Moreover, LCs are sufficient for the development of cutaneous GVHD (Merad et al., 2004). Although these studies indicate that LCs can promote and are possibly necessary for T cell priming, opposite conclusions have been reached in other settings. After HSV infection of either vaginal epithelium or abraded flank skin, the DCs presenting HSV antigens to T cells in draining LNs did not appear to include LCs (Allan et al., 2003; Zhao et al., 2003). Thus, it appears that under certain circumstances, LCs may be dispensable for stimulation.

As this manuscript was being prepared, two groups reported that they had developed mice that express the diphtheria toxin (DT) receptor on LCs and have a transient depletion of LCs after injection of DT (Bennett et al., 2005; Kissenpfennig et al., 2005). Interestingly, one group observed that CHS was diminished, while the other found it was unchanged in transiently LC-depleted mice.

From these studies alone, it is clear that in spite of the likely importance of LCs in skin immune responses, their roles require more precise definition. We have addressed this by generating transgenic mice that contain an attenuated form of diphtheria toxin under control of genetic elements that restrict expression to LCs. These

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genetic elements were obtained from a bacterial artificial chromosome (BAC) containing the human Langerin gene that is specifically expressed in LCs (Giraldo and Montoliu, 2001; Valladeau et al., 2000). We selected a human BAC to allow us to test for expression of the transgene with human-specific antibodies. Once we ascertained that the human gene expressed in mice as predicted, we introduced diphtheria toxin sequence into the Langerin gene contained in the BAC and made another round of transgenic mice. Some of these founder lines constitutively lacked epidermal LCs, while other DC subsets remained intact. These mice allowed us to experimentally test the role of LCs in a prototypical CHS response. Unexpectedly, we found that CHS was amplified in the absence of LCs. Thus, LCs not only were dispensable for CHS, but they served to regulate the response.

Results

Generation of Human Langerin Transgenic Mice

To generate a mouse with a selective absence of LCs, we expressed diphtheria toxin in LCs by a genomic BAC transgenic system (Giraldo and Montoliu, 2001). We chose to use human-derived BACs to facilitate discrimination of the transgene from the orthologous engodenous mouse gene through the use human-specific reagents. This strategy has been successful in other cases; human genes on BACs are frequently (but not always) expressed in a tissue-specific manner (Attie et al., 2001; Feng et al., 2001; Miano et al., 2002).

To target LCs, we used BAC RP11-504o1, which contains the human gene for Langerin (huLangerin), which is selectively expressed by LCs (Valladeau et al., 2000). A 71 kb NotI fragment of RP11-504o1 containing Langerin and 26 kb of upstream sequence was used to generate transgenic mice. Founders were screened by PCR with primers specific for each end of the NotI fragment to ensure incorporation of the full-length DNA. A total of 8 founders from 66 live births were generated.

Transgenic huLangerin Is Expressed in Epidermal LCs

To validate LC-specific expression of the huLangerin transgene, we prepared epidermal sheets from ear and flank skin from four independent transgenic lines. Since LCs are the only epidermal cells that constitutively express MHC-II (Tsuruta et al., 1999), sheets stained with MHC-II and anti-huLangerin were examined by immunofluorescence (Figure 1A). Three out of four founder lines demonstrated strong expression of human Langerin in all LCs in every field examined. In one founder, approximately 50% of the LCs expressed detectable levels of huLangerin; this founder was not analyzed further. Cells expressing MHC-II also costained for CD11c (data not shown). There was no detectable huLangerin expression by MHC-II-negative cells.

To quantify the number of LCs expressing huLangerin, we examined epidermal single-cell suspensions by flow cytometry. As was seen by immunofluorescence, virtually all of the MHC-II$^+$ cells expressed huLangerin (Figure 1B, left). These MHC-II$^+$/huLangerin$^+$ cells also expressed murine Langerin, thereby firmly establishing them as LCs (Figure 1B, middle and right). We also examined dermal single-cell suspensions and found that transgenic huLangerin was expressed in a small subset of CD11c$^+$ cells (Figure 1C, left). The majority of these cells also expressed murine Langerin (Figure 1C, middle and right). Therefore, these are likely either LC precursors that reside in the dermis or epidermal LCs in the process of migrating though the dermis to regional LNs. The small number of dermal cells expressing murine but not human Langerin may reflect a delay in expression of the transgene compared with the engodenous murine locus. Thus, the NotI fragment of RP11-504o1 that contains the human gene for Langerin accurately expressed in all murine epidermal LCs and was a good candidate to drive expression of diphtheria toxin.

Generation of Langerin-DTA Mice

We modified RP11-504o1 to drive the expression of diphtheria toxin in LCs by using homologous recombination in *E. coli* as described by Yang et al. and modified by Misulovin et al. (Misulovin et al., 2001; Yang et al., 1997). We inserted an internal ribosome entry sequence (IRES), which allows for translation of polycistronic mRNA followed by an attenuated form of diphtheria toxin subunit A (DTA) (Maxwell et al., 1986) into the 3′ untranslated region (UTR) of the huLangerin gene (Figure 2A). Successfully recombined BAC clones were screened by PCR (data not shown) and confirmed by restriction digest with Hyp99I, which is introduced with the DTA cDNA (Figure 2B). Insertion of the IRES-DTA construct into RP11-504o1 was verified by the absence of a 71 kb band, which is seen in the unmodified DNA (left lane) and the appearance of two novel 40 kb and 31 kb bands seen in two independent clones (two right lanes). A 72 kb NotI fragment of the modified BAC was used to inject pronuclei from pure FVB embryos in order to generate a new set of transgenic mice. A total of 7 full-length founders from 28 live births were generated. All founders appeared healthy and grossly normal.

Transgenic Langerin-DTA Mice Lack Epidermal LCs

Transgenic founders were screened by immunofluorescence for the absence of LCs by staining for MHC-II in whole mounted epidermal sheets. Five of the founders showed no decrease of epidermal LCs and were not examined further. Two transgenic founders, however, showed a complete absence of MHC-II-positive cells in all fields examined (Figure 3A). Similarly, staining for MHC-II in transverse ear sections revealed an absence of epidermal LCs in transgenic mice. Importantly, MHC-II-positive APCs in the dermis were still present in Langerin-DTA mice (Figure 3B). We also did not see a reduction of $\gamma\delta^+$ (Vγ5$^+$) dendritic epidermal T cells (see Figure S1 in the Supplemental Data available with this article online) or the total number of epidermal $\gamma\delta$ T cells.

Flow cytometry of single-cell epidermal suspensions from Langerin-DTA mice revealed a virtually complete absence (30-fold reduction) of LCs in the epidermis (Figures 4A and 4D, $p < 0.0001$). In contrast, there was no significant reduction of MHC-II- or CD11c-positive cells in dermal suspensions from Langerin-DTA mice compared with littermate controls (Figures 4B–4D, $p = 0.62$). Dermal dendritic cells and macrophages are the primary MHC-II-positive cells present in noninflamed dermis.

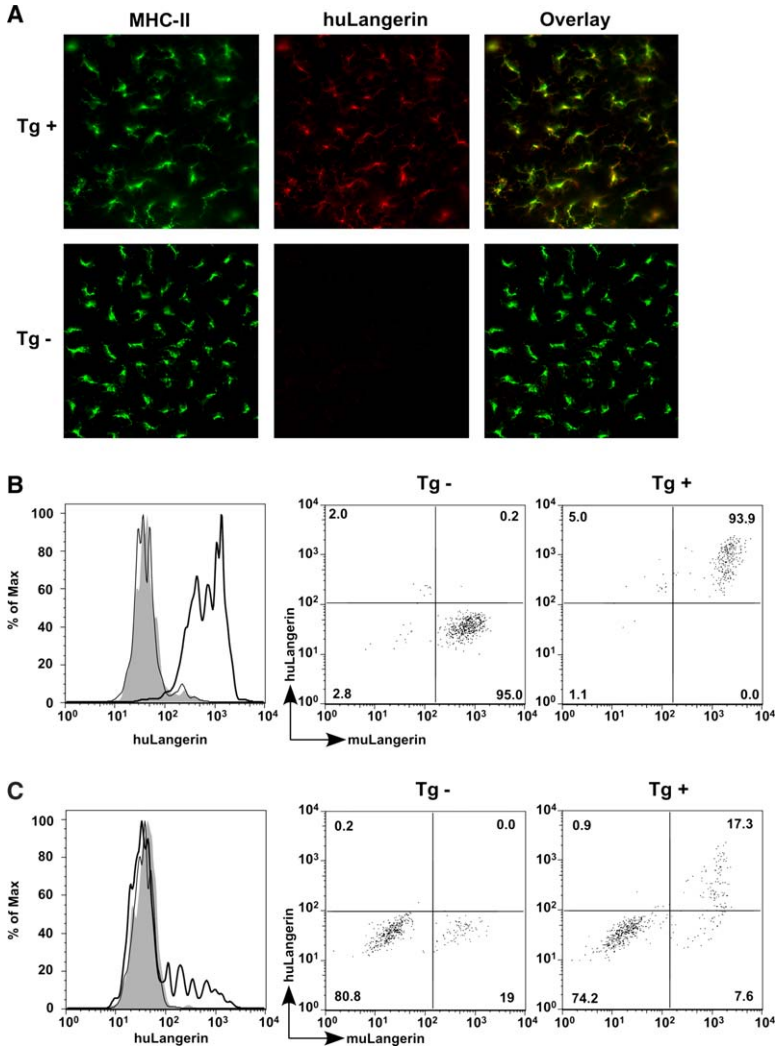

Figure 1. Transgenic huLangerin Is Expressed by All Epidermal LCs

(A) Epidermal sheets from transgene positive (top, Tg^+) and littermate controls (bottom, Tg^-) were stained for MHC-II to identify LCs (green, left) and for transgenic huLangerin (red, middle). Cells expressing both markers appear yellow when overlayed (right). Data are representative of all fields examined in three independent founders.

(B) FACS analysis of single-cell epidermal suspensions gated on live MHC-II-positive cells. Levels of huLangerin (left, Tg^+ thick line, Tg^- thin line, isotype control shaded) and comparison of huLangerin and muLangerin from Tg^- (middle) and Tg^+ (right). Numbers in quadrants indicate percentage of total cells.

(C) Analysis of dermal cells as in (B).

Thus, as expected, we did not detect any dermal $B220^+$/MHC-II^+ B cells (Figure 4C).

Flow cytometry of LN and spleen demonstrated that DC subsets were equivalent in both Langerin-DTA and control mice (Figure S2). Moreover, we also found equivalent numbers of resident murine $Langerin^+/CD11c^+$ cells in secondary lymphoid tissues and thymus from wild-type and Langerin-DTA mice (Figures 5A–5D), indicating that the deficit in these mice is restricted to epidermal LCs. The presence of murine $Langerin^+$ cells in mesenteric lymph node, thymus, and spleen has been previously reported (McLellan et al., 2002; Valladeau et al., 2002). Given the location of these cells in tissues that do not receive lymph from the skin as well as the fact that they are retained in Langerin-DTA transgenic mice that nonetheless are devoid of epidermal LCs, it is unlikely that they represent mature epidermal LCs that have migrated from skin. To directly examine the presence in cutaneous lymph node of migrating epidermal LCs, we painted FITC on the skin of both wild-type and Langerin-DTA mice. It is evident that among $CD11c^+$ LN cells 4 days after painting, the migrating (FITC+) dermal DCs that are DEC-205^{dim} are intact, whereas the migrating DEC-205^{bright} cells, which are thought to be derived from epidermal LCs, are absent (Figure 5E; Kamath et al., 2002). Taken together, these data first of all reinforce the notion that migrating LCs are DEC-205^{bright}, as this population is missing in FITC-painted Langerin-DTA mice. They also indicate that most resident $Langerin^+$ cells in cutaneous LN must not be derived from migrating LCs. Though all LCs express Langerin, not all Langerin-expressing cells are derived from epidermal LCs. This is an important point, as this substantial $Langerin^+$ population was depleted in systems that eliminate LCs though the administration of DT in mice that express the diphtheria toxin receptor under control of the native langerin locus (Bennett et al., 2005; Kissenpfennig et al., 2005). In contrast, Langerin-DTA mice have a complete absence of epidermal LCs yet retain other immunologically important cell types that normally reside in the skin and secondary lymphoid tissue.

Langerin-DTA Mice Have Enhanced Contact Sensitivity

Contact hypersensitivity (CHS) to epicutaneously applied hapten is the classic assay for examining cutaneous adaptive immunity. Importantly, CHS priming does not require prior disruption of the epidermis or stratum corneum, thus mimicking as closely as possible the manner in which the skin is normally exposed to

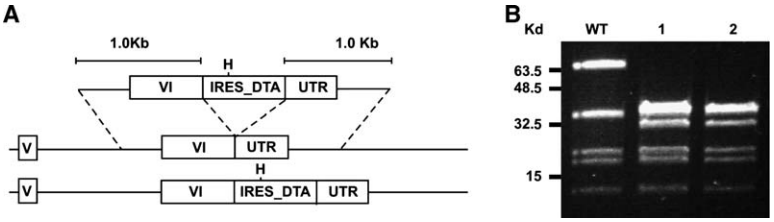

Figure 2. Generation of Langerin-DTA Mice

(A) Schematic representation of the shuttle vector used to introduce IRES-DTA into RP11-504o1 (top line). The vector contains an IRES-DTA cassette flanked by two 1.0 kb sequences homologous to the 3′ end of the Langerin gene (exon VI and 3′UTR) in RP11-504o1 (middle line). The final recombined product is pictured on the bottom line. Hyp99I sites are indicated as "H."

(B) DNA digested with Hyp99I from either the unmodified BAC (wt) or two independent recombined clones (1 and 2) run on a 1% agarose pulse field gel. Note that a 71 kb band present in the wild-type is replaced by 40 kb and 31 kb bands, indicating the successful insertion of the IRES-DTA cassette.

environmental antigens. CHS to two commonly used haptens, DNFB (2,4-dinitro-1-fluorobenzene) and oxazalone, are mediated by both CD4 and CD8 in a Th1/Tc1 response (Gocinski and Tigelaar, 1990; Wang et al., 2000). Having established that Langerin-DTA mice lack epidermal LCs, we addressed their role in the development of CHS. In the priming phase, hapten is painted on shaved but otherwise intact skin. Five days later, mice are rechallenged with the hapten on the ear and the strength of the hapten-specific response is measured by the degree of ear swelling after 24 hr.

The classical models of LC function in CHS would predict that Langerin-DTA mice would have an absent or greatly diminished CHS response (Bennett et al., 2005; Kissenpfennig et al., 2005; Silberberg-Sinakin and Thorbecke, 1980). However, we were surprised to find that ear swelling was consistently approximately 2-fold greater in Langerin-DTA mice than in age- and sex-matched littermate controls (Figure 6A). This enhanced response was seen in both founder lines (data not shown). Swelling persisted and was still evident 3 days after DNFB challenge (Figure 6C). The enhanced CHS responses were not unique to DNFB, since it was also seen with oxazalone (Figure 6B). Moreover, this effect was not due to nonspecific enhancement of inflammation since both Langerin-DTA and control mice developed a similar degree of ear swelling after application of the irritant contactants sodium dodecyl sulfate (SDS) or benzalkonium chloride (Figures 6D and 6E). Although we saw an enhanced immune response to epicutaneously applied antigen, T cells from Langerin-DTA and control animals demonstrated a similar degree of in vitro proliferation to OVA peptide when harvested 7 days after footpad immunization with OVA in CFA (data not shown).

Enhanced CHS due to Increased T Cell Priming

The mechanism responsible for the enhanced CHS response could have been operative during the priming phase, effector phase, or both. To distinguish among these possibilities, we employed a series of adoptive transfer experiments. Although, as expected in such transfer systems, the overall amount of ear swelling was less than was observed in intact mice (Wang et al., 2000), we noted an almost 2-fold increase in ear swelling in wild-type animals that received primed LN cells from Langerin DTA-mice compared with cells from littermate controls (Figure 7A). Adoptively

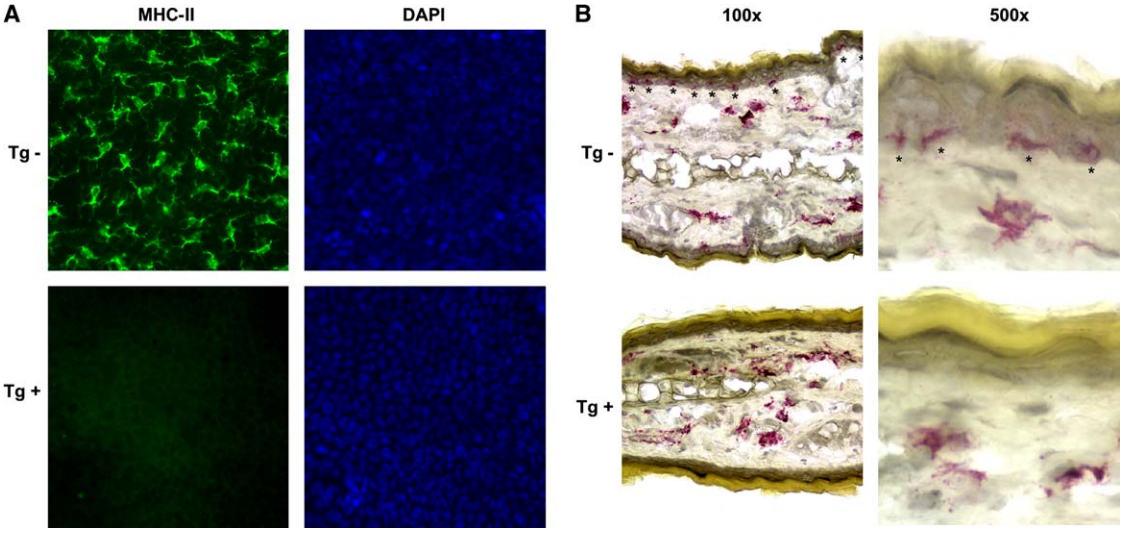

Figure 3. Langerin-DTA Mice Lack Epidermal LCs

(A) Epidermal sheets from littermate control (Tg^-, top) and Langerin-DTA (Tg^+, lower panels) mice were stained with MHC-II (green, left) to identify LCs and with DAPI (blue, right) to identify cell nuclei regardless of MHC-II expression. Data are representative of all fields examined.

(B) Transverse sections of frozen skin stained for MHC-II from littermate control (Tg^-, top) or Langerin DTA (Tg^+, bottom). LCs are indicated by asterisks. Right panels are a magnified view (500× original magnification) of the epidermis from the same sections seen on the left (100× original magnification). Data are representative of two independent founders.

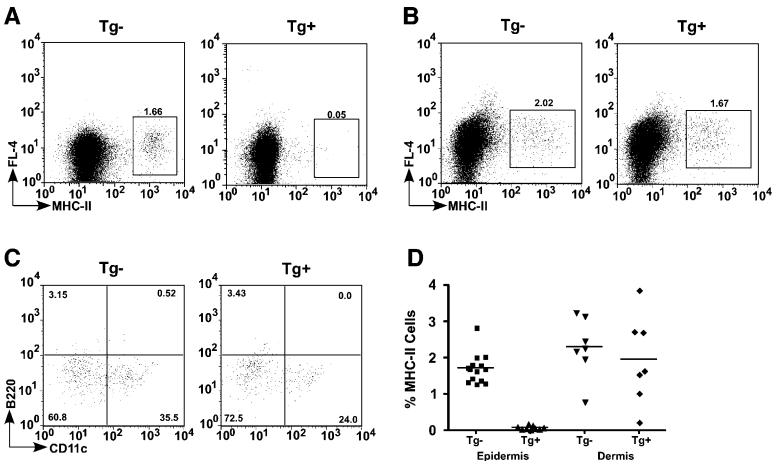

Figure 4. Langerin-DTA Mice Lack Epidermal LCs

(A and B) FACS analysis of single-cell epidermal (A) or dermal (B) suspensions gated on live cells from either Langerin-DTA (Tg^+) or littermate controls (Tg^-). Cells were stained with MHC-II to identify LCs and are plotted against an unstained channel for clarity. Numbers above gates indicate percentage of positive cells.

(C) Expression of CD11c and B220 on MHC-II^+ dermal cells. Data in (A)–(C) are representative of two independent founders.

(D) The percentage of MHC-II^+ cells found in the epidermis and dermis from both Langerin-DTA and littermate control animals are plotted from multiple experiments to better represent the range of data obtained. There is a significant reduction of MHC-II cells in epidermis ($p < 0.001$) but not in dermis ($p = 0.62$).

transferred cells from Langerin-DTA and control mice had similar numbers of B cells, CD4 T cells, and CD8 T cells (data not shown). In the reciprocal transfer experiment, Langerin-DTA and littermate controls received LN cells from primed wild-type mice. Both groups of animals developed a similar degree of ear swelling ($p = 0.42$) that was significantly greater than mice injected with PBS (Figure 7B, $p < 0.05$) or cells from naive mice (data not shown). Thus, the absence of epidermal LCs leads to enhanced CHS during the afferent phase, presumably through increased T cell priming. In contrast, the LC-deficient environment did not promote a more vigorous effector phase.

Discussion

In this paper, we have described an otherwise normal mouse that constitutively lacks a single DC subtype, epidermal LCs. We then used this model to demonstrate that the lack of LCs leads to an enhanced rather than inhibited CHS response. Finally, we went on to show via cell-transfer experiments that LCs act at the priming rather than effector phase of the response. These results alter our view of LCs by demonstrating that they are not required for the presentation of antigens acquired in skin but instead have a regulatory function. The findings highlight the importance of examining individual DC subsets in vivo and demonstrate that different DC subsets play particular roles in the generation and regulation of immune responses.

Although LCs are not required for CHS responses, they appear to have a stimulatory capacity in certain other settings. In a model of MHC-disparate allogeneic GVHD, LCs have been shown to be sufficient for the development of cutaneous disease (Merad et al., 2004). Similarly, in a separate model, ovalbumin secreted from keratinocytes appears to be presented to TCR transgenic T cells by LCs and not by other DC subsets (Mayerova et al., 2004). In addition, LCs have been presumed to play a key role in CHS based on findings that UV irradiation prior to priming with hapten leads not only to abrogated CHS responses but also to hapten-specific tolerance (Schwarz, 1999; Toews et al., 1980). An explanation for the disparity between these results and our findings could relate to the very high frequencies of antigen-specific T cells in the first two models; under such circumstances, the potential for LCs to stimulate T cells may be dominant over regulatory function. Importantly, the potential involvement of dermal DCs in these systems was not examined. This is particularly relevant for studies involving UV irradiation of the skin, since irradiation depletes both LCs and dermal DCs and has numerous other effects on the skin and immune system (Clydesdale et al., 2001; Duthie et al., 2000). Recent work with cutaneous HSV infection suggests that LCs are not responsible for presentation of antigens to T cells and raises the possibility that LCs (and/or potentially dermal DCs) could transport antigen to skin draining LNs and then transfer antigen to $CD8^+$ DCs (Allan et al., 2003; Carbone et al., 2004). Thus, although LCs may initiate certain types of responses, in many cases T cell activation can be accomplished in vivo by other APCs. In CHS, presumably dermal DCs, which are known to collect cutaneous Ags and migrate to LNs, perform this function (Itano et al., 2003).

Very recently it was reported by Kissenpfenning et al. and Bennett et al. that mice in which the gene for the diphtheria toxin (DT) receptor is "knocked-in" to the *langerin* locus have a transient depletion of $Langerin^+$ cells after injection of DT (Bennett et al., 2005; Kissenpfennig et al., 2005). Although both groups used a similar technique, Kissenpfenning et al. did not identify any specific role for LCs while Bennett et al. observed a modest decrease in CHS. Neither of these systems discerned an enhanced CHS responses as seen consistently in our mice. There are many reasons why the regulatory role of LCs could have been missed in these transient depletion studies. It is possible that the regulatory effects of LCs require their constitutive presence and short-term depletion is not sufficient to reveal this. A key difference, however, between our transgenic and the knockin systems could be the expression of the DT receptor on $Langerin^+$ cells other than LCs. DT injection eliminated epidermal LCs as well as a substantial portion of the $Langerin^+$, $CD11c^+$ $CD8^+$ DCs in skin-draining LNs in Kissenpfenning et al. (and presumably Bennett et al., though this was not as clearly documented). Langerin is also expressed by $CD8^+$ DCs in the spleen (McLellan

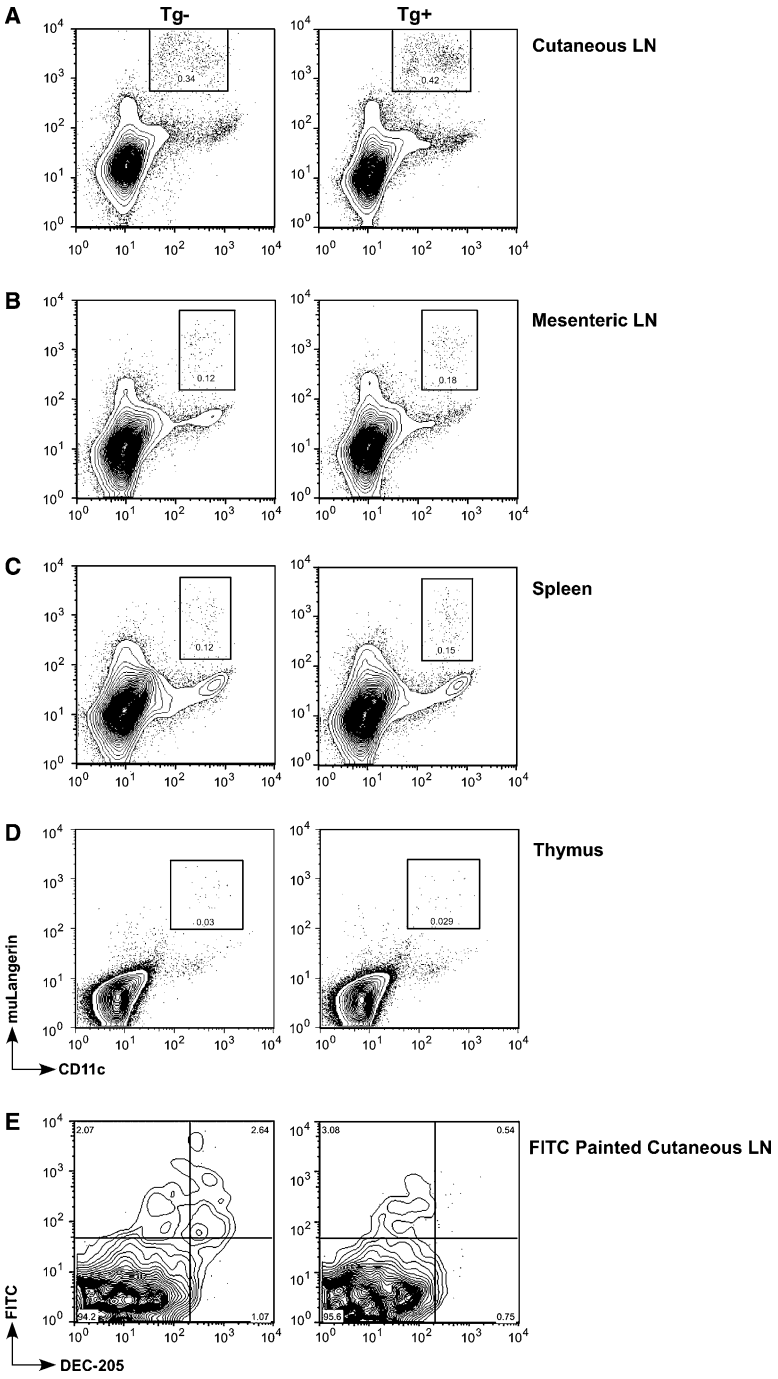

Figure 5. Resident LN and Splenic Langerin$^+$ Cells Are Retained but Migrating DEC-205^{bright} LCs Are Absent in Langerin-DTA Mice

(A–D) FACS analysis of (A) cutaneous lymph node, (B) mesenteric lymph node, (C) spleen, and (D) thymus from Langerin-DTA (Tg$^+$) or littermate controls (Tg$^-$). Live gated cells were stained with CD11c and muLangerin. Numbers in the gates indicate the percentage muLangerin cells in each tissue.

(E) Cutaneous lymph nodes were harvested 4 days after mice were painted on shaved abdomens with 100 μl of 0.5% FITC and stained with anti-CD11c and DEC-205. Live CD11c$^+$ gated cells from wild-type mice (Tg$^-$) and Langerin-DTA mice (Tg$^+$) are shown. These data are representative of three independent experiments.

et al., 2002; Valladeau et al., 2002), and many of these cells also eliminated after DT injection (Kissenpfennig et al., 2005). Since the CD8$^+$ DC subset has been shown to present antigen to T cells after cutaneous infection (Allan et al., 2003), its reduction could decrease the CHS response that otherwise would have been increased in the absence of LCs. In contrast, our Langerin-DTA mice do not have a detectable absence of Langerin$^+$ DCs (or any other DC subset) in LN or spleen (Figures 5A–5D and Figure S2). Importantly, the fact that we can observe dermal DCs but not epidermal LCs migrating into the cutaneous LN after FITC painting and still see a normal number of Langerin$^+$ cells in LN and spleen argues that not all Langerin$^+$ cells are derived from epidermal LCs (Figure 5E). Although we are not sure why expression of DTA under the control of human Langerin leads to the depletion of epidermal LCs but not other muLangerin$^+$ cells, it is clear that Langerin-DTA mice have a selective ablation of only epidermal LCs and show a clear enhancement of CHS with multiple haptens and even in cell-transfer experiments.

The enhancement of CHS that we observed in the absence of LCs indicates that LCs regulate the cutaneous immune response. Our adoptive transfer experiments showed that LCs exert their regulatory activity at the priming rather than effector phase. It is interesting to

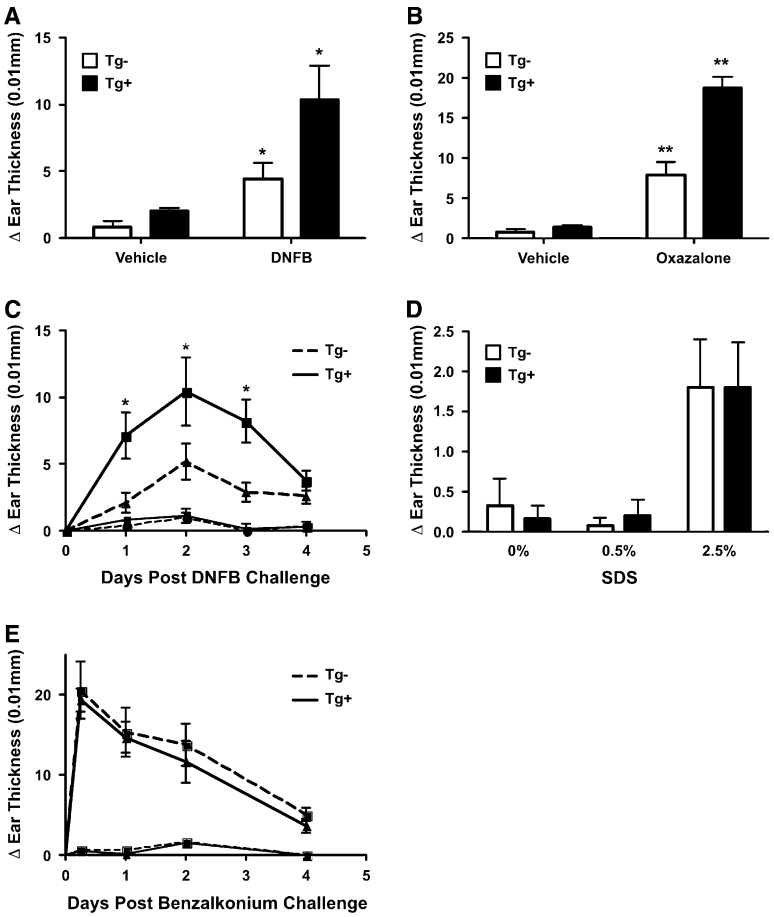

Figure 6. Langerin-DTA Mice Have Enhanced CHS but Not Irritant Contact Dermatitis

(A) Groups of 5–8 Langerin-DTA (Tg^+, black bars) and littermate controls (Tg^-, white bars) were sensitized with 0.5% DNFB or vehicle alone on shaved abdomens on day 0. All mice were then challenged with 0.2% DNFB on their ears on day 5. Data represents the change of ear thickness over baseline after 24 hr (*$p < 0.05$).

(B) As in (A) except that mice were sensitized with 3.0% oxazalone and challenged with 1.0% oxazalone. (**$p < 0.001$).

(C) Groups of 6–7 Langerin-DTA (Tg^+, solid line) and littermate controls (Tg^-, broken line) were sensitized with 0.5% DNFB (thick lines) or vehicle alone (thin lines). All mice were challenged with 0.2% DNFB 5 days later and monitored daily for change in ear thickness.

(D) Groups of 5–6 Langerin-DTA (black bars) and littermate controls (white bars) were challenged on the ear with 0, 0.5%, or 2.5% SDS. Data represent the change of ear thickness over baseline after 24 hr.

(E) Langerin-DTA (Tg^+, solid line) and littermate controls (Tg^-, broken line) were challenged with 5% Benzalkonium chloride (thick lines, n = 8) or vehicle (thin lines, n = 4) and monitored for ear swelling after 6, 24, 48, and 96 hr.

A single representative experiment from at least three independent experiments is shown. Error bars are ± standard error.

speculate on how LCs might regulate priming. CHS to DNFB and oxazalone is a CD8- and CD4-mediated Th1/Tc1 response (Gocinski and Tigelaar, 1990; Wang et al., 2000). LCs could affect T cell priming through skewing T cell polarity to a more Th-2 like response. Alternatively, LCs may favor the development or activation of regulatory T cells (Probst et al., 2005; Shevach, 2002). There are precedents for DC subsets with such functions; for example, there are specialized DC populations in the lung and gut that secrete IL-10 and preferentially lead to the production of Tregs (Akbari et al., 2001; McGuirk et al., 2002; Mowat, 2003; Rimoldi et al., 2005).

As with any knockout mouse, we cannot rule out that the enhanced CHS we observed was due to the absence of LCs during the development of the skin immune system per se. Although such a function would be quite interesting and novel, a number of lines of evidence argue against it. First, we saw no defects or alterations in other cell populations in the skin or LN that might have compensated for the loss of LCs. In addition, immune responses to Ag injected below the epidermis and to nonspecific, irritant stimuli were not exaggerated. Moreover, T cells that were primed in a wild-type environment and transferred into a LC-deficient host did not demonstrate an enhanced response in the LC-deficient

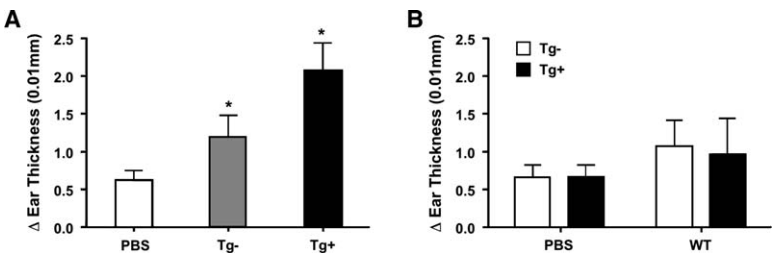

Figure 7. Enhanced CHS Can Be Adoptively Transferred to Wild-Type Mice

(A) Donor Langerin-DTA and littermate control animals were sensitized with 3.0% oxazalone on day 0. On day 5, 5×10^7 skin-draining lymph node cells from each strain were harvested and injected i.v. into groups of 6–8 unmanipulated wild-type FVB mice. Recipients were ear challenged 2 hr later and the magnitude of ear swelling was measured after 24 hr (*$p < 0.05$).

(B) In the reciprocal experiment, wild-type FVB mice were sensitized with 3.0% oxazalone and their lymph node cells were adoptively transferred on day 5 into unmanipulated Langerin-DTA (Tg^+, black bars) and littermate controls (Tg^-, white bars). There was no significant difference between Langerin-DTA and control mice that received lymph node cells from sensitized wild-type mice. Recipients of lymph node cells did show significant increase in ear thickness compared with PBS controls ($p < 0.05$). These data are representative of three independent experiments.

Error bars are ± standard error.

environment. Thus, it is very likely that the observed enhanced CHS response is due to a dynamic lack of regulation from LCs during the priming response and not from a developmental defect.

Our finding that LCs have regulatory properties in CHS has obvious implications for their role in other cutaneous immune processes such as cutaneous HSV infection, lupus, GVHD, and tumor immunosurveillance. By using the Langerin-DTA LC-deficient animals, the in vivo participation of LCs in these and other disease models can now be directly examined by us and others.

Experimental Procedures

BACs and Production of Transgenic Mice

Human BAC clone RP11-504o1 was obtained from Invitrogen (Carlsbad, CA). An IRES sequence (Clontech, Mountainview, CA) followed by an attenuated diphtheria toxin gene (tox176, kind gift of Dr. Ian Maxwell [Maxwell et al., 1986]) was inserted 83 bp 3′ of the stop codon in the 3′UTR of exon VI of Langerin by homologous recombination in *E. coli* as previously described (Misulovin et al., 2001; Yang et al., 1997). The shuttle vector for insertion of IRES-DTA into Langerin was created by PCR. First, the IRES-DTA construct was generated by overlapping PCR to contain an IRES and Kozak consensus sequences (from pIRES2EGFP) followed by tox176 attenuated DTA. Next, the shuttle vector was constructed by overlapping PCR to include 1021 bp 5′ of the insertion site in Langerin, the IRES-DTA construct, and 1066 bp 3′ of the insertion site. The construct was fully sequenced to eliminate the possibility of PCR-induced errors. Recombined BAC clones were confirmed by PCR with oligos flanking the insertion site and by Hyp99I (New England Biolabs, Beverly, MA) restriction digest. BAC DNA for microinjection was obtained with a Plasmid Maxi Kit (Qiagen, Valencia, CA) followed by NotI digestion (New England Biolabs), purification by pulse field agarose gel electrophoresis (Chef II, Biorad, Hercules, CA), and β-agarase digestion (New England Biolabs). DNA was concentrated and transferred into microinjection buffer (10 mM Tris [pH 7.4], 0.1 mM EDTA, 100 mM NaCl) by a 30 kDa molecular weight cutoff spin column (Millipore, Bedford, MA). DNA concentration was determined by Picogreen assay (Invitrogen) and injected at 1–2 ng/μl by the Yale Genomics Services Transgenic Mouse Service into pronuclei of (C57BL/6j × SJL/j) F2 embryos for huLangerin mice and into FVB embryos for Langerin-DTA mice. huLangerin mice were backcrossed with BALB/c, and the Langerin-DTA transgene was maintained on a pure FVB background. Founders were identified by PCR via STS SHGC-3151 and SHGC-143488 (see below) located on the centromeric and telomeric ends of the NotI fragment, respectively.

Oligonucleotides

STS Used to Screen for Transgene-Positive Mice

SHGC-143488: F, 5-gaggcaaatgattggcattctac-3; R, 5-ctgggaaaat tcaagaagagcct-3; SHCG-3151:F, 5-gtactaatagccagtcattgttcac-3; R, 5-ataaagagtaagatgaatggtcacag-3.

Generation of IRES-DTA

5′ IRES: 5-caagtaaagcggccgggatccgcccctctcc-3; 3′IRES: 5-catcag gatcccatggttgtggccatattatc-3; 5′ DTA: 5-gccacaaccatggatcctgatga tgttgttg-3; 3′ DTA: 5-cgtgtcaggcgatctttgtgagcggccgctaaactat-3.

Generation of Shuttle Vector

5′ A Box: 5-acgcgtcgacgcaggaagaagcttgagagg-3; 3′ A Box: 5-ggcg gaacccggcctggagtctggggaagaaag-3; 5′ Insert: 5-ccagactccaggccg ggttccgcccctctcc-3; 3′ Insert: 5-cgtgcaaagtcattcacaaagatcgcctgac acg-3; 5′ B Box: 5-gcgatctttgtgaatgactttgcacgttaatttttc-3; 3′ B Box: 5-acgcgtcgactctgtgaagagtggggaacc-3.

Mice

FVB mice were purchased from National Cancer Institute (NCI) Laboratories (Frederick, MD), Harlan (Indianapolis, IN), and Taconic (Germantown, NY). BALB/c mice were purchased from NCI. All experiments were performed with 6- to 10-week-old age- and sex-matched mice. Mice were housed in microisolator cages and fed autoclaved food and acidified water. The Yale institutional animal care and use committee approved all mouse protocols.

Antibodies

The following antibodies were used: huLangerin (DCGM4, Beckman Coulter, Fullerton, CA), muLangerin (929F3 [Valladeau et al., 2002]), CD11c (N418-FITC and APC, eBioscience, San Diego, CA), I-A/E (M5/144.15.2-FITC and biotin, Biolegend, San Diego, CA), Vγ5 (F536, Pharmingen), CD11b (M1/70-PE, Pharmingen), CD8a (Ly-2-PE, Pharmingen), mouse anti-Rat Alexa 647 (Invitrogen), and strep-avadin PE (Invitrogen). NLDC-145 (anti-DEC-205), RA3-6b2 (anti-B220), and 24G2 (anti-FcRγ) were purified from hybridoma supernatants as described (Shlomchik et al., 1993). Conjugation to biotin or Alexa-647 was performed according to manufacturer's directions (Invitrogen). huLangerin antibodies were labeled by a Zenon Bio-XX labeling kit (Invitrogen).

Flow Cytometry

Single-cell suspensions of epidermal cells were obtained from trunk or ear skin and incubated for 2 hr at 37ºC in 0.3% trypsin (Sigma-Aldridge, St. Louis, MO) in 150 mM NaCl, 0.5 mM KCl, 0.5 mM glucose. The epidermis was physically separated from the dermis and disrupted by vortexing. Dermal cells were obtained from samples from which the epidermis had been removed or from untreated whole flank skin. Samples were minced and incubated for 2 hr at 37ºC in collagenase XI (4830 U/ml, Sigma), hyaluronidase (260 U/ml, Sigma), DNase (0.1 mg/ml, ICN), 10 mM HEPES (Sigma) in RPMI (Invitrogen). The resulting cells were filtered though a 40 μM filter. Lymph node (axillary, brachial, and inguinal) and spleen cells were incubated in 400 and 150 U/ml Collagenase D (Roche Applied Science, Indianapolis, IN), respectively, for 90 min prior to erythroid cell lysis in ACK buffer (Biowhittaker, Walkersville, MD). Single-cell suspensions were pretreated for 10 min at 4ºC with 24G2 except when anti-Rat secondaries were used, in which case cells were blocked with mouse Ig (Sigma). Cells were stained for extracellular markers as described (Shlomchik et al., 1993). Propidium iodide (Invitrogen) was used for live-dead discrimination. For intracellular markers, cells were incubated in ethidium monoazide (EMA, Invitrogen) for live-dead discrimination followed by cytofix/cytoperm (Pharmingen) as per manufacturer's directions. Mice painted with FITC were shaved on their abdomens and 100 μl of 0.5% FITC (Fluorescein isothiocyanate isomer I, Sigma) in acetone: dibutylphthalate (1:1) was applied 4 days before harvest of the inguinal, axillary, and brachial lymph nodes.

Immunohistochemistry and Immunofluoresence

For immunohistochemistry, transverse tissue sections were obtained and processed as described (Hannum et al., 2000). Sections were stained with biotinylated anti-MHC-II followed by Streptavidin alkaline-phosphatase (Invitrogen) as described (Hannum et al., 2000). For immunofluorescence, epidermal sheets were prepared by treating mouse ears with Nair (Chursh and Dwight Co, Princeton, NJ) for 5 min followed by affixing them to slides (epidermis side down) with double sided adhesive (3M, St. Paul MN). Slides were incubated in 10 mM EDTA in PBS for 2 hr at 37ºC followed by physical removal of the dermis. Tissue was fixed in acetone at 4ºC for 10 min and blocked as described (Hannum et al., 2000). Tissues were stained with MHC-II FITC followed by anti-FITC Alexa 488 (Invitrogen) for signal amplification and with biotin-huLangerin followed by Streptavidin-Alexa 555 (Invitrogen).

Contact Dermatitis

Allergic contact dermatitis was induced in mice as previously described (Girardi et al., 2002). In brief, mice were sensitized on day 0 by epicutaneous application of 25 μl of 0.5% DNFB (2,4 dinitrofluorobenzene, Sigma) in acetone:olive oil (4:1), 100 μl of 3.0% Oxazalone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, Sigma) in ethanol, or vehicle alone onto dry shaven abdominal skin. On day 5, baseline ear thickness was measured with an engineer's micrometer followed by challenge with 10 μl of 0.2% DNFB or 1.0% oxazalone to both sides of one ear. Ears were measured 24 hr after challenge and data are expressed as the ear size at 24 hr minus the baseline thickness. For irritant dermatitis, mice were challenged without prior sensitization with 10 μl to both sides of one ear with the indicated

concentration SDS (sodium dodecylsulfate, Sigma) dissolved in water. Ear swelling was measured after 24 hr. Mice were also challenged with 5% Benzalkonium chloride (Sigma) in acetone:olive oil and ear swelling was measured after 6, 24, 48, and 96 hr.

Adoptive Transfer

For adoptive transfer of allergic contact dermatitis, Langerin-DTA and/or control mice were sensitized with 3.0% oxazalone to their abdomen as well as 10 μl to each paw. After 5 days, single-cell suspensions of lymph node cells (axillary, brachial, popliteal, and inguinal) were obtained by physical disruption and filtered through a 40 μM screen. Groups of unsensitized Langerin-DTA and/or control mice were injected intravenously with 5×10^7 cells. Two hours later, baseline ear thickness measurements were obtained and the mice were challenged with 10 μl of 1.0% oxazalone to both sides of one ear. Ears were measured 24 hr later and the ear thickness above baseline was calculated. All donor/recipient pairs were age-matched female mice that had been maintained on a pure FVB background.

Statistics

Statistical comparisons between groups in CHS experiments were made with a standard Student's two-tailed t test. The Mann-Whitney test was used for comparison between groups in Figure 4C.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.immunity.com/cgi/content/full/23/6/611/DC1/.

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