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CCR6 is required for epidermal trafficking of γδ T cells in an IL-23-induced model of psoriasiform dermatitis

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Abstract

A subset of CCR6$^+$, γδ-low (GDL) T cells that express Th17 cytokines in mouse skin participates in IL-23-induced psoriasisform dermatitis. We use CCR6-deficient (KO) and wildtype (WT) mice to analyze skin trafficking patterns of GDL T cells and function-blocking mAbs to determine the role of CCR6 in IL-23-mediated dermatitis. Herein, CCL20 was highly upregulated in IL-23-injected WT mouse ear skin as early as 24 hours after initial treatment, and large numbers of CCR6$^+$ cells were observed in the epidermis of IL-23-injected WT mice. Anti-CCL20 mAbs reduced psoriasiform dermatitis and blocked recruitment of GDL T cells to the epidermis. In CCR6 KO mice, GDL T cells failed to accumulate in the epidermis after IL-23 treatment, but total numbers of GDL T cells in the dermis of WT and CCR6 KO mice were equivalent. There was a ~70% reduction in the proportion of IL-22$^+$ GDL T cells in the dermis of CCR6 KO mice (vs. WT mice), suggesting that effector function as well as epidermal recruitment of GDL T cells are impaired in CCR6-deficient mice. Thus, these data show CCR6 regulates epidermal trafficking of γδ T cell subsets in skin and suggest the potential of CCR6 as a therapeutic target for psoriasis.

Introduction

The immunopathogenesis of psoriasis has been revealed in much greater depth and complexity as new data suggest that the Th17 signaling pathway plays key roles in the development of psoriasis (Fitch et al, 2007). IL-23, a key upstream player in the Th17 pathway, is an essential cytokine for the maintenance of Th17 cells, and therapeutic agents targeting the shared p40 component of IL-23 have shown remarkable clinical efficacy in psoriasis (Leonardi et al, 2008). Current models suggest that IL-23 produced by dendritic cells act to sustain dermal CC chemokine receptor-6 (CCR6)-expressing Th17 cells which

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then produce IL-22 as a major downstream effector molecule that mediates epidermal hyperplasia (Nograles, Davidovici and Krueger, 2010).

Of note, CCR6 itself is not simply a marker for Th17 cells, but several reports suggest that CCR6 has functional relevance to the trafficking and/or function of T cell subsets associated with the Th17 pathway. For example, anti-murine CCR6 antibodies have shown efficacy in ameliorating Th17-mediated autoimmune disease models of experimental autoimmune encephalomyelitis (EAE) (Liston et al, 2009) and collagen-induced arthritis (Hirota et al, 2007), but their benefit in psoriasiform skin models has never been shown. We have shown, however, that mice deficient in CCR6 fail to develop the psoriasiform dermatitis that is observed in wildtype mice after injection of IL-23 (Hedrick et al, 2009). Interestingly, there are also reports that CCR6 may be expressed by regulatory T cells and, thus, functions in some situations, including chronic EAE, to help dampen the immune response (Elhofy et al, 2009, Villares et al, 2009).

Recent data reveal that specific subsets of γδ T cells in mice are present in the dermis (Gray, Suzuki and Cyster, 2011), express CCR6 as well as the IL-23 receptor (IL-23R). and respond to IL-23 and IL-1β by secreting IL-17 and IL-22 (Sutton et al, 2009, Haas et al, 2009, Cua and Tato, 2010). In human studies, Laggner *et al*. have described a $CCR6^+$, Vγ9Vδ2 T cell subset in peripheral blood which is decreased in the blood, but increased in the skin, of psoriatic patients (Laggner et al, 2011). In addition, these authors showed by immunofluorescence microscopy that Vδ2 receptor-positive T cells are present in large numbers in psoriatic, but not healthy, skin (Laggner et al, 2011).

Drivers of Th17 inflammation, such as IL-23, and Th17 cytokines, such as IL-22, play critical roles in psoriasis through their effects in maintaining Th17 cells and stimulating epidermal hyperplasia, respectively (Fitch et al, 2007, Zheng et al, 2007). Ustekinumab, a monoclonal antibody that targets the p40 subunit of IL-23, has been shown to be very effective in ameliorating human psoriasis (Leonardi et al, 2008). We (Hedrick et al, 2009, Mabuchi, Takekoshi and Hwang, 2011) and others (Zheng et al, 2007, Chan et al, 2006) have shown that injection of IL-23 in murine skin results in a psoriasiform dermatitis with striking similarity to human psoriasis, including epidermal hyperplasia, parakeratosis, neutrophils in the cornified layer, and dense Th17-predominant inflammatory infiltrates. In the IL-23 injection model, we have previous observed that both $CD4^+$ T cells and $CD11c^+$ dendritic cells, but neither macrophages or neutrophils, are increased in the dermis of either WT or CCR6-deficient mice (Hedrick et al, 2009).

We have demonstrated that IL-23 injection in WT mouse skin results in the epidermal recruitment of $CCR6^+$, $IL\text{-}23R^+$, $V\gamma3^-$ γδ low-intermediate T cells (GDL T cells) that express high levels of IL-17 and IL-22 (Mabuchi, Takekoshi and Hwang, 2011). These γδ T cells appear to be required to maximize ear swelling in the initial response to injected IL-23 since γδ T cell-deficient mice had delayed increases in ear swelling compared to WT mice (Mabuchi, Takekoshi and Hwang, 2011). A population of γδ T cells with a similar $CCR6^+$ phenotype has also been described as highly motile and distinct in several important ways from DETC (Gray, Suzuki and Cyster, 2011), and dermal γδ T cells have now been reported to produce IL-22 and IL-17 in imiquimod-induced models of psoriasiform dermatitis (Cai et

al, 2011, Van Belle et al, 2012). It is conceivable that, under the appropriate inflammatory conditions, these dermal GDL T cells migrate in large numbers to the epidermis, where the release of IL-22 would very effectively stimulate epidermal hyperplasia.

While it was clear from our prior work that epidermal GDL T cells expressed CCR6 whereas skin resident $V\gamma3^+$ (also called $V\gamma5$, depending on nomenclature) γδ T cells known as dendritic epidermal T cells (DETC) did not (Mabuchi, Takekoshi and Hwang, 2011), the functional role of CCR6 and its ligand CCL20 in trafficking of GDL T cells to epidermis under inflammatory conditions is uncertain. Moreover, it is also clear that epidermal cells, particularly keratinocytes, can synthesize the CCR6 ligand, CCL20, when triggered by inflammatory stimuli (Schutyser, Struyf and Van Damme, 2003). CCL20 protein was immunohistologically apparent after 15 days of repeated injections of IL-23 in murine skin (Hedrick et al, 2009), but the kinetics of CCL20 production in the skin in this model has not been fully explored. Herein, to determine if CCL20 and its receptor, CCR6, were required for trafficking of GDL T cells to epidermis, we blocked CCL20 with monoclonal antibodies in our IL-23 injection model and showed that trafficking of GDL T cells to the epidermis was markedly reduced. Of therapeutic note, anti-CCL20 mAbs also reduced dermal inflammation. Intriguingly, we observed a dramatic decrease in the production of IL-22 in GDL T cells from the skin of CCR6 KO mice following IL-23 stimulation, suggesting that CCR6 may be involved in maximally triggering production of IL-22. These data reveal mechanistic insights regarding the chemokine regulation of trafficking of unconventional γδ T cell subsets in skin and suggest the potential relevance of CCR6/CCL20 as a therapeutic target for psoriasis.

RESULTS

CCL20 expression in mouse epidermis is rapidly upregulated after IL-23 injection

Harper *et al*. have shown that Th17 cytokines (IL-17A, IL-22, and TNF-α) up-regulate CCL20 in murine skin following local injection (Harper et al, 2009), and our previous study indicated that CCL20 could be detected in mouse epidermis following nearly two weeks of treatment with IL-23 (Hedrick et al, 2009), but earlier time point analysis was not performed. Since we detected CCR6 expression in GDL T cells, we asked if CCL20 was upregulated at early time points following IL-23 injection, presuming that CCL20 may be a major chemoattractant for GDL T cells migration to the epidermis. As early as 24 hr following the initiation of IL-23 treatment, CCL20 expression was highly upregulated in murine epidermis (compared to PBS-treated control skin) and was maintained at similar levels throughout the duration of the experiment (Fig. 1). Thus, CCL20 is rapidly produced by keratinocytes in vivo in response to IL-23, potentially serving as an epidermal chemoattractant for GDL T cell migration to the epidermis.

Neutralizing anti-CCL20 monoclonal antibodies reduce psoriasis-like inflammation and trafficking of IL-22-producing, CCR6-expressing GDL T cells into epidermis in response to IL-23 injection—To ascertain if CCL20 is required for the entry of GDL T cells to the epidermis during IL-23 injection-induced epidermal inflammation, we mixed IL-23 with neutralizing anti-CCL20 monoclonal antibodies (mAb)

or isotype control and injected this mixture into the skin of mice as we have previously described (Mabuchi et al, 2012). Because ear swelling is a useful measure of dermal inflammation (edema) in this model, we measured ear thickness before each injection. Whereas mice injected with the isotype control mAb and IL-23 mixture displayed the usual ear swelling over 5 days, PBS-injected ears failed to show any significant increase in ear swelling (Fig. 2a). Ears injected with neutralizing anti-CCL20 mAb plus IL-23, however, showed significant reductions in ear swelling compared to isotype control and IL-23 injected ears (Fig. 2a). A significant reduction in mean epidermal thickness was also noted in anti-CCL20 mAb plus IL-23-injected ears compared to isotype controls (Fig. 2b). Hematoxylin and eosin-stained sections from WT ears injected with isotype control mixed with IL-23 on day 5 revealed psoriasis-like changes, namely parakeratosis, epidermal hyperplasia, and mixed mononuclear inflammatory infiltrate (Fig. 2c). By contrast, mice treated with neutralizing anti-CCL20 mAb and IL-23 showed minimal parakeratosis, decreased immune cells infiltration, and reduced epidermal hyperplasia (Fig. 2c). Thus, anti-CCL20 mAb treatment markedly reduces the major epidermal and dermal inflammatory effects of IL-23 treatment.

Following treatment with isotype control mAb plus IL-23, neutralizing anti-CCL20 mAb plus IL-23, or PBS injection for 5 days, both epidermal and dermal cell suspensions were prepared for flow cytometry and RT-PCR. Pooled epidermal (Fig. 3a) and dermal (Fig. 3b) cell suspensions were stained with mAbs against γδ TCR. As expected, the number of GDL T cells was increased in the epidermis in isotype control mAbs plus IL-23 injections compared to PBS-injected ears (Fig. 3a). However, the accumulation of GDL T cells in the epidermis treated with neutralizing anti-CCL20 antibodies and IL-23 mixture was lower (76% reduction in Fig. 3a with another experiment showing 54% reduction) than that of epidermis treated with isotype control plus IL-23, approaching levels seen with PBS injection alone (Fig. 3a). On the other hand, the numbers of GDL T cells in dermis treated with isotype control mAbs plus IL-23 and neutralizing anti-CCL20 mAbs plus IL-23 were both decreased comparing to GDL T cells found in PBS-injected ears (Fig. 3b). RT-PCR analysis revealed that both epidermal (Fig. 3c) and dermal (Fig. 3d) cells from ears treated with neutralizing anti-CCL20 mAbs plus IL-23 showed significant reduction in expression of IL-22 and CCR6 mRNA when compared to isotype control plus IL-23- injected ears. Thus, anti-CCL20 mAbs block accumulation of GDL T cells in the epidermis and reduce expression of IL-22 mRNA in both the dermal and epidermal compartments.

Trafficking of GDL cells to epidermis correlates with entry of CCR6$^+$ cells into epidermis—To determine if CCR6 is required for the entry of GDL T cells to the epidermis during the IL-23 injection protocol, frozen sections of whole mouse ears were stained with mAbs against CCR6 and laminin 332 (as a marker for the basement membrane). We observed increased numbers of CCR6$^+$ cells in the epidermis of IL-23-injected, but not PBS-injected, skin localized by the 3D projections acquired using immunofluorescence confocal microscopy (Fig. 4a,b). We had previously observed CCR6$^+$ cells in IL-23-treated skin by conventional sections (Mabuchi, Takekoshi and Hwang, 2011), but the 3D projections allowed us to better appreciate the presence of large numbers of CCR6$^+$ cells that accumulated in IL-23-treated skin. Close inspection at the level of the

basement membrane (revealed by anti-laminin 332 antibodies, red) showed many $CCR6^+$ cells at (arrow head), immediately adjacent to (double arrow heads), or above (arrow) the basement membrane, suggesting that these cells may have been moving into the epidermis (Fig. 4a,b). To show $CCR6^+$ GDL T cells in relationship to γδ-high resident DETC, which express low levels of CCR6 (Mabuchi, Takekoshi and Hwang, 2011), we double-stained skin of IL-23-treated mice with differentially labeled mAbs to CCR6 (green) and the γδ TCR (red). While red, γδ-high cells were present in both PBS- and IL-23-treated skin (Fig. 4c and data not shown), green, $CCR6^+$ cells were only abundant in IL-23-treated skin (Fig. 4c), clearly showing that the $CCR6^+$ population of GDL T cells was distinct from DETC in the epidermis.

CCR6 is required for IL-22 production by GDL T cells and their trafficking to epidermis—In our prior work, we showed that CCR6 was critical for the development of a psoriasiform dermatitis following IL-23 injection since CCR6-deficient mice were unable to develop the inflammatory and hyperplastic phenotype of WT mice after exposure to IL-23 (Hedrick et al, 2009). However, in that work we were surprised to note that the numbers of Th17 and IL-22-producing cells were similar in the IL-23-treated skin of WT and CCR6 KO mice. At that time, we did not determine if unconventional T cells populations (i.e., γδ TCR T cells) were affected in numbers, particular in the epidermis, in CCR6-deficient mice. Thus, we subjected CCR6 KO mice to our IL-23 skin injection protocol. Following treatment with IL-23 or PBS injection for 5 days, both epidermal and dermal cell suspensions were stained with mAbs against CD3, γδ TCR, and IL-22 for flow cytometry. Following IL-23 injection, total numbers of GDL T cells in epidermis (as expected) were increased in the IL-23-injected WT ears compared to PBS-injected WT ears. The accumulation of GDL T cells, however, in the IL-23-treated CCR6 KO epidermis was similar to that observed in PBS-treated epidermis (Fig. 5a, left). Similarly, CCR6 KO mice failed to accumulate $CD3^+$ GDL T cells in the epidermis (Fig. 5b, left) and $IL\text{-}22^+$ GDL T cells (Fig. 5c, left) in epidermis following IL-23 injection. Whereas IL-23 injection resulted in a significant increase in the proportion of $IL\text{-}22^+$ GDL T cells in the epidermis in WT mice ($p<0.05$), it did not significantly increase this population of cells in CCR6 KO mice ($p=0.5$). This phenomenon was also observed in the dermis (Fig. 5c), suggesting the CCR6 may be necessary not only for epidermal trafficking but also for the activation of GDL T cells such that they produce IL-22 at high levels. Note that there were no significant differences between PBS- and IL-23-injected mouse ears in total numbers of GDL T cells in the dermis of either WT or CCR6 KO mice (Fig. 5a, right). Thus, CCR6 is required for both entry of GDL T cells into the epidermis following IL-23 stimulation and for maximal production of IL-22 by GDL T cells, particularly in the dermis.

DISCUSSION

Herein, we have shown in several different ways that CCR6 may be critical for the trafficking GDL T cells to the epidermis. First, anti-CCL20 mAbs block the migration of GDL T cells to the epidermis. Moreover, these neutralizing antibodies ameliorate the epidermal hyperplasia and dermal inflammation observed when IL-23 is injected into WT mouse skin. Second, GDL T cells fail to accumulate in the epidermis of IL-23-treated CCR6 KO mice, strongly suggesting that CCR6 may be required for effective trafficking to the

epidermis under these proinflammatory conditions. Confocal microscopy shows $CCR6^+$ cells clearly accumulate in murine epidermis following IL-23 injection where the proximity of these cells to the basement membrane (Fig. 4) suggest movement of $CCR6^+$ cells from the dermis into the epidermis as a result of IL-23 injection.

In other inflammatory settings, unconventional γδ T cells have been shown to be capable of producing high levels of IL-22 and other key Th17 cytokines (Sutton et al, 2009, Haas et al, 2009, Cua and Tato, 2010), but their role in skin is only just beginning to be explored. Our prior studies (Mabuchi, Takekoshi and Hwang, 2011) and those described herein revealed that a population of $CCR6^+$, $V\gamma3/V\gamma5^-$ γδ T cells (with low-intermediate expression of the γδ receptor), hence the name GDL T cells, accumulated in murine epidermis upon exposure to IL-23. GDL T cells that accumulate in inflamed skin are distinct from resident dendritic epidermal γδ T cells (DETC) that express Vγ3(5) and that are constitutively present in resting mouse skin (Gray, Suzuki and Cyster, 2011, Mabuchi, Takekoshi and Hwang, 2011). As thoroughly discussed by others, DETC serve distinct roles in immune regulation and cancer surveillance in the mouse (Girardi, 2006). In contrast to murine epidermis in which large numbers of DETC are present in the epidermis, human epidermis appears to lack an equivalent cell population (Girardi, 2006), so the immunological roles of DETC in humans are unclear. However, recent data indicate that $V\gamma9V\delta2^+$ T cells do accumulate in great numbers in the skin of patients with active psoriatic lesions (Laggner et al, 2011).

Recent human data support the potential relevance of $CCR6^+$ and γδ T cells in psoriasis. First, Kagami *et al*. have described increases (that normalized during anti-TNF-α therapy) in $IL\text{-}17A^+$, $CCR6^+$ T cells in the blood of psoriatic patients (Kagami et al, 2010). Interestingly, Laggner *et al*. found decreases in $CCR6^+$, γδ T cells in the blood of psoriatic patients while large increases in numbers of these cells were noted in the skin of patients with active disease, suggesting possible movement of γδ T cells into the skin. The large increases in $CCR6^+$ T cells in the blood may reflect the ability of conventional $CCR6^+$ αβ T cells cells to expand in number in the peripheral blood whereas $CCR6^+$ γδ T cells may alter their trafficking patterns (without necessarily expanding in numbers) during active disease.

Thus, we would propose that inflammatory triggers such as IL-23 induce sufficient CCL20 in the epidermis to begin attracting large numbers of GDL T cells that then produce IL-22, IL-17, and possibly other factors that influence epidermal hyperplasia and dermal edema. As shown in Fig. 1, CCL20 is rapidly upregulated in murine epidermis upon injection of skin with IL-23. In our prior publication (Hedrick et al, 2009), we observed that CCR6 KO (vs. WT) mice showed greatly diminished expression of IL-22 mRNA at both early (5 days) and late (15 days) time points during the IL-23 injection protocol. Of note, IL-17A and IL-17F expression was markedly reduced in CCR6 KO mice only at the early, but not at late, time points, suggesting that IL-22 expression correlated more closely with dermal edema (and psoriatic phenotype) than did IL-17A/F expression. These data substantiate recent results from Rizzo *et al*. showing that, while IL-17A has a role in the IL-23 injection model, the role of IL-22 seems to be greater (Rizzo et al, 2011).

The potential of CCR6 or its ligand, CCL20, as a therapeutic target for psoriasis has been postulated since Homey *et al*. first showed high expression of CCL20 and CCR6 in psoriatic

skin more than a decade ago (Homey et al, 2000) and has been reviewed recently at greater depth (Mabuchi et al, 2012, Hedrick et al, 2010). Indeed our current studies confirm that targeting the chemokine ligand, CCL20, with neutralizing antibodies can effectively block the development of epidermal hyperplasia and dermal inflammation although the degree of inhibition is not quite as striking as that observed when CCR6-deficient mice were treated with IL-23 (Hedrick et al, 2009). Compared to the response seen with anti-CCL20 mAb, the near complete lack of response in CCR6 KO mice might be anticipated because of the total absence of the CCR6 receptor. A CCL20-directed intervention for psoriasiform dermatitis has not to our knowledge been previously reported. Others have targeted CCR6 with monoclonal antibodies in animal models of autoimmune disease, specifically EAE (Liston et al, 2009) and collagen-induced arthritis (Hirota et al, 2007), suggesting that targeting CCL20 may also be a viable strategy for diseases other than psoriasis.

Interestingly, γδ T cells from CCR6 KO mice not only failed to accumulate in the epidermis after IL-23 treatment, but even those that did enter the epidermis expressed low amounts of IL-22 compared to WT GDL T cells (Fig. 5). This suggested that not only recruitment, but function, of γδ T cells may be impacted by the lack of CCR6. There are several possible explanations. It is well known that chemokine receptors activate PI3K and its downstream effector to impact survival and activation pathways (Murakami et al, 2003). Thus, we hypothesize that CCR6 has a role in co-activating GDL T cells. Alternatively, the lack of other cells in the dermis, possibly CD11c dendritic cells (that express IL-23 and other cytokines) that we observed to be substantially decreased in the dermis of IL-23-treated CCR6 KO mice (Hedrick et al, 2009), might have an impact on the ability of GDL T cells to become activated.

Combined with our prior study (Mabuchi, Takekoshi and Hwang, 2011), our current results now firmly implicate recruited Vγ3/Vγ5$^-$ GDL T cells as important participants in IL-23-mediated psoriasiform dermatitis. While we acknowledge that human psoriasis is likely to be driven by not just solely by IL-23, but possibly, by other cytokines such as IL-21 (Caruso et al, 2009), the therapeutic efficacy of ustekinumab (targeting the shared p40 subunit of IL-23 and IL12) (Leonardi et al, 2008) coupled with clinical data correlating the expression of Th17 cytokines with efficacy of anti-tumor necrosis factor-α antibodies (Zaba et al, 2009) suggests that the IL-23 injection model may be a useful preclinical platform for testing agents that might have efficacy in human psoriasis. Because other chemokine receptors (e.g., CXCR4 and CCR5) have been successfully targeted with small molecule inhibitors, our data suggest that a search for small molecule antagonists of CCR6 may be of value in the treatment of psoriasis and other autoimmune disorders.

MATERIALS AND METHODS

Mice

C57BL/6J WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Both C57BL/6 CCR6 KO mice and littermate control WT mice used in Fig. 5 were generated and housed at the National Institutes of Health as described (Hedrick et al, 2009). All mice were used at 8–12 weeks of age. Animal protocols were approved by the Animal

Care and Use Committees both at the Medical College of Wisconsin and the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Intradermal cytokine and neutralizing anti-CCL20 antibodies injections

We performed intradermal injection of 20 μl PBS containing 500 ng recombinant mouse IL-23 (BioLegend, San Diego, CA, USA) or 20 μl PBS alone into both ears of anesthetized mice using a 28-gauge needle every other day for 6 days as described (Mabuchi, Takekoshi and Hwang, 2011). In the same way, we also performed intradermal injection of 20 μl PBS containing 10 μg monoclonal anti-mouse CCL20/ MIP3α antibody (R&D Systems, Minneapolis, MN, USA) mixed with 500 ng recombinant mouse IL-23, 20 μl PBS containing 10 μg rat IgG_1 isotype control (R&D Systems) mixed with 500 ng recombinant mouse IL-23, or 20 μl PBS alone. Ear thickness was measured before every injection as described (Mabuchi, Takekoshi and Hwang, 2011).

Processing of epidermal and dermal cells from mouse ears

After recovery of mouse ears, skin sheets were separated from cartilage with forceps and incubated in PBS containing 0.5% trypsin (US Biochemical Corp, Cleveland, OH) for 40 min at 37°C with dermal side down to separate epidermal sheets from dermal sheets. To obtain cell suspensions, epidermal sheets were treated in DMEM (Invitrogen, Grand Island, NY, USA) containing 0.05% DNase I (Sigma-Aldrich, St. Louis, MO, USA) and 10% FBS as described (Salgado et al, 1999). Dermal sheets were incubated in 15 ml RPMI Medium 1640 (Invitrogen) containing 40 mg collagenase D (Roche, Indianapolis, IN, USA), 0.01% DNase I, and 20% FBS for 45 min at 37°C as described (Szabo et al, 1998). Cells were then filtered through a 70 μm nylon mesh and washed prior to use.

Histopathological analysis and immunofluorescence microscopy

Frozen, 5 μm sections of whole mouse ears were stained with hematoxylin and eosin. Images were acquired using an INFINITY3-1C digital camera (Lumenera, Ontario, Canada) attached to a Carl Zeiss microscope and INFINITY ANALYZE version 5.0.3 software (Lumenera). Epidermal thickness was measured at four different points on the image using INFINITY ANALYZE version 5.0.3 software.

Staining for CCR6, γδ TCR (BD Biosciences), and Laminin 332 was done using frozen, 8 μm sections of whole mouse ears. Sections were air-dried after 20 min fixation in cold acetone, blocked for 1 hr at room temperature with 5% goat serum, Fc-blocker (2.4G2, Bio X Cell, West Lebanon, NH, USA), and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS containing 3% skim milk. After washing in PBS, tissue sections were incubated with anti-mouse CCR6 monoclonal antibody or rat IgG (R&D Systems, Minneapolis, MN) overnight at 4°C. After washing in PBS, the sections were incubated with anti-laminin 332 antibody (Lazarova et al, 1996) or rabbit IgG (R&D Systems) overnight at 4°C. After washing in PBS, the sections were incubated for 30 min at room temperature with Alexa-568-conjugated goat anti-rabbit IgG and Alexa-488-conjugated goat anti-rat IgG (Invitrogen), then washed in PBS and mounted using ProLong Gold anti-fade reagent with DAPI nuclear stain (Invitrogen). Images were acquired using the

Carl Zeiss LSM510 confocal microscope with axio-observer microscope and LSM510 version 4.2 software.

Staining for CCL20 was done using frozen, 8 μm sections of whole mouse ears. Sections were air-dried after 10 min fixation in cold acetone and blocked for 1 hr at room temperature with 5% donkey serum, Fc-blocker, and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology) in PBS containing 3% skim milk. After washing in PBS, the sections were incubated with mouse CCL20/MIP-3α Affinity Purified Polyclonal antibody or normal goat IgG (R&D Systems) overnight at 4°C. After washing in PBS, tissure sections were incubated for 30 min at room temperature with Alexa-568-conjugated donkey anti-goat IgG (Invitrogen), then washed in PBS and mounted using ProLong Gold anti-fade reagent with DAPI nuclear stain. Images were acquired using an INFINITY3-1C digital camera attached to a Carl Zeiss microscope and INFINITY ANALYZE version 5.0.3 software.

Flow cytometry

Monoclonal antibodies against mouse γδ TCR (catalog # 553177), IL-22, and CCR6 were purchased from BD Biosciences (San Jose, CA, USA), BioLegend (San Diego, USA), and R&D Systems, respectively. Intracellular staining for IL-22 was done after incubating cells for 2 hr with GolgiStop™ (BD Biosciences). Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm™ Plus Kit (BD Biosciences). Flow cytometric data was analyzed using FlowJo version 7.5.5 software (Tree Star, Ashland, OR, USA).

Real-Time PCR

Extraction of RNA and subsequent analysis of mRNA expression of indicated cytokines from epidermal or dermal cell suspensions was performed as previously described (Mabuchi, Takekoshi and Hwang, 2011).

Statistics

All quantitative data was shown as the mean ± SEM unless otherwise indicated. Simple comparisons of means and SEM of data were made by using Student’s t-test (2-sided) and post hoc multiple comparisons were made by using Tukey’s test.

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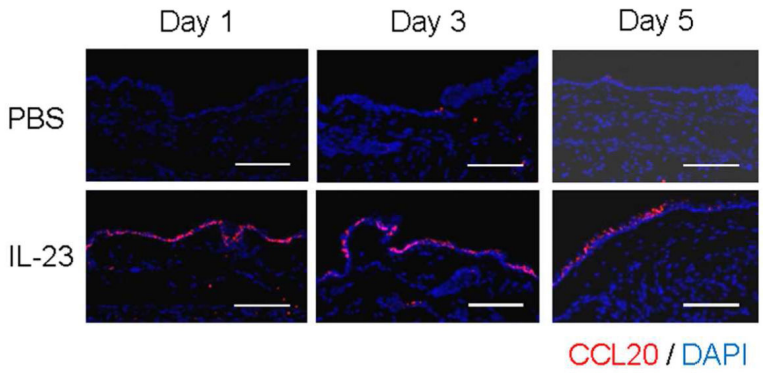

Fig. 1. CCL20 expression in mouse epidermis is upregulated within 24 hr of IL-23 injection

IL-23 was injected into ears of WT mice at day 0, 2, and 4. Mice were euthanized on the indicated days and ears were stained with anti-CCL20 antibody (red) and DAPI (nuclear counterstain in blue). Isotype control staining showed no epidermal staining with either IL-23 or PBS-injected ears (data not shown). Scale bar = 50 μm.

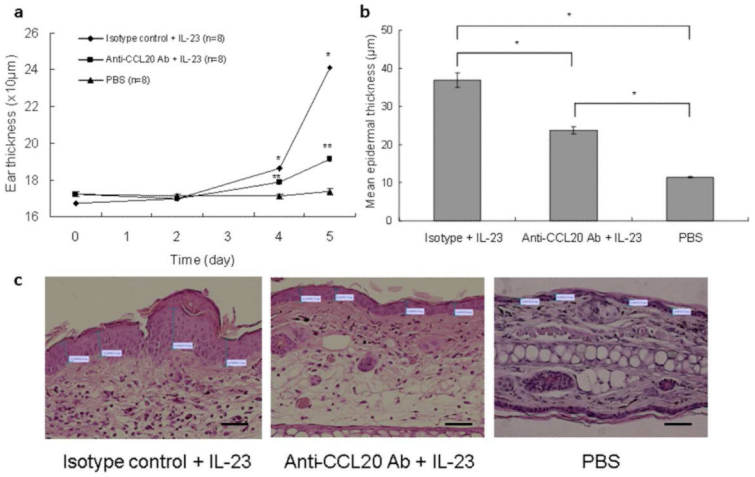

Fig. 2. Neutralizing anti-CCL20 monoclonal antibodies inhibit the ear swelling in response to IL-23 injection

(**a**) WT mice were injected with neutralizing anti-CCL20 mAb, isotype control, or PBS in ear skin with subsequent measurement of ear thickness throughout the treatment period. *P<0.01 vs. all other groups. **P<0.01 vs. PBS-injected groups. These experiments were repeated 4 times with similar results. (**b**) At day 5 following treatment with neutralizing anti-CCL20 mAb, isotype control, or PBS injection, ears were harvested and the epidermal thickness was measured (at points indicated by white rectangles) in paraffin-embedded, hematoxylin and eosin-stained tissues using Infinity Analyze analysis software (**c**). *P<0.01. Scale bar = 100 μm. Similar results were obtained in 2 independent experiments.

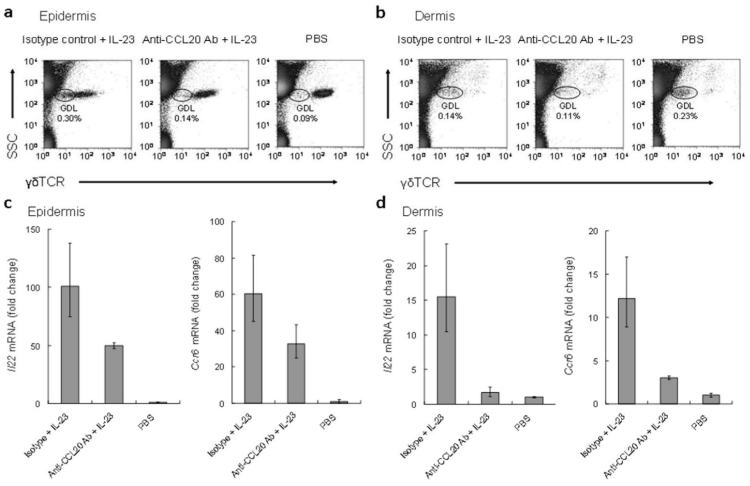

Fig. 3. Neutralizing anti-CCL20 monoclonal antibodies inhibit the trafficking of GDL T cells in response to IL-23 injection

Following treatment with neutralizing anti-CCL20 mAb, isotype control, or PBS injection for 5 days, both pooled epidermal (**a**) and dermal (**b**) cell suspensions from 4 mouse ears (per condition) were stained with mAbs against γδ TCR for flow cytometry e(**a,b**) or processed for RNA extraction for RT-PCR (**c,d**). In (**a,b**), the percentage indicated in each dot plot reflects the number of GDL cells (i.e., events) in the oval window divided by the total sample events shown in the entire dot plot. Percent reduction in epidermal GDL T cells was calculated at 76% for data shown [(% GDL cells IL23 + isotype)-(% GDL cells IL23+anti-CCL20)]/[(%GDL cells IL23+isotype)-(%GDL cells PBS)]*100. Pooled epidermal (**c**) and dermal (**d**) cell suspensions (see above) were used for quantitative RT-PCR to measure expression of IL-22 and CCR6. Fold changes were calculated for *IL-22* and *Ccr6* mRNAs normalized for *Gapdh* mRNA *vs*. the PBS treated sample. Error bars indicate SD (**c,d**) based on a single, pooled treatment group that was divided into three wells for RT-PCR measurement. Similar results were obtained in 2 independent experiments.

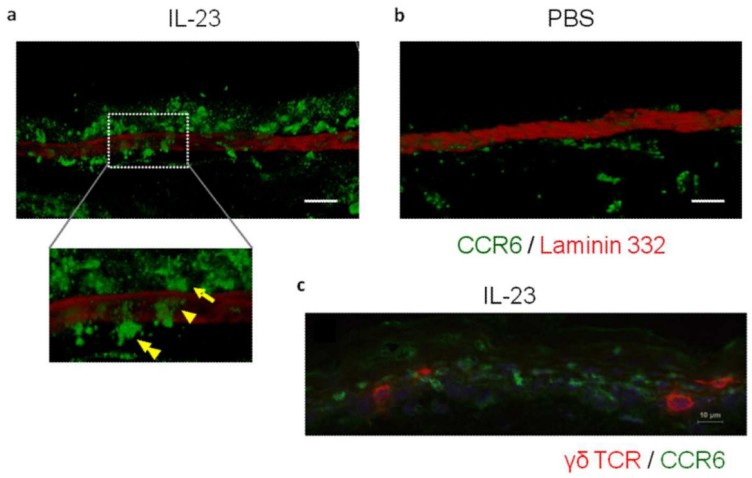

Fig. 4. Immunofluorescence staining for CCR6 expression following IL-23 injection

Following IL-23 (**a**) or PBS (**b**) injection for 6 days, mice were euthanized and ears were stained with anti-CCR6 mAbs (green) and anti-Laminin 332 mAbs (basement membrane in red), and the 3D images were acquired using a confocal microscope. The figure shows the perspective from the dermis toward the epidermis. The width of basement membrane in red shows the depth of the sections. Larger numbers of CCR6-expressing cells in the epidermis (above the basement membrane) were revealed in IL-23-, but not PBS-injected, mice. CCR6-expressing cells (**a**) at (arrow head), immediately adjacent to (double arrowheads), or above (arrow) the basement membrane in the dashed rectangular area marked in the upper figure are shown at higher magnification below. Isotype control staining showed no staining with either IL-23 or PBS-injected ears (data not shown). Scale bar = 10 μm. In (**c**), IL-23-treated skin sections were stained with labeled mAbs against CCR6 (green) and the γδ-TCR (red). Images were acquired by confocal microscopy at the level of the epidermis (x63 objective, scale bar as indicated). Similar results were obtained in 3 independent experiments.

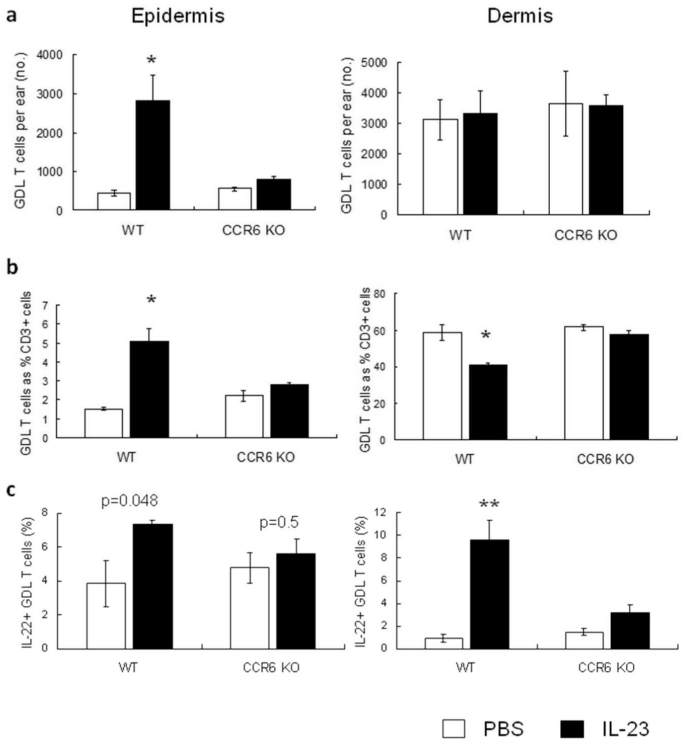

Fig. 5. CCR6 KO mice fail to accumulate IL-22$^+$, GDL T cells in response to IL-23 injection

Following IL-23 injection for 6 days, both WT and CCR6 KO mice were euthanized. Both epidermal and dermal cell suspensions were analyzed by flow cytometry with γδ TCR, CD3, and IL-22 mAbs. (**a**) Total numbers of GDL T cells per PBS- or IL-23-injected ear of WT and CCR6 KO mice were counted. The percentages of CD3$^+$ GDL T cells (**b**) and IL-22$^+$ GDL T cells (**c**) were calculated. *P<0.01 vs. all other groups. **P<0.05 vs. all other groups. Each treatment group consisted of 4 ears. All P values were calculated using Student's t-test, 2-sided. In (**c**, epidermis), p values are provided for comparisons between PBS and IL-23-treatment in WT and CCR6 KO mice. Similar results were obtained in 2 independent experiments.