

IL-18 is a key proximal mediator of contact hypersensitivity and allergen-induced Langerhans cell migration in murine epidermis

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Abstract: Langerhans cells (LC) migrate rapidly from epidermis to lymph node following epicutaneous application of antigen. In this study, we have explored the role of IL-18, a cytokine with structural similarities to IL-1 β , in murine LC migration and contact hypersensitivity (CHS), which to oxazolone (OX) and 2,4-dinitrofluorobenzene (DNFB) was suppressed significantly in IL-18 knockout (IL-18 $^{-/-}$) mice and could be rescued by local intradermal administration of IL-18 prior to sensitization, suggesting that the defect in these mice was in the afferent phase of CHS. To determine the effect of IL-18 on LC migration, mice were treated topically with OX or DNFB, and remaining LC numbers were assessed. A significant decline in remaining epidermal LC occurred in wild-type (WT) mice but did not occur in IL-18 $^{-/-}$ mice. Sodium lauryl sulfate, a nonantigenic LC migratory stimulus, induced equivalent LC migration in IL-18 $^{-/-}$ and WT mice. In IL-18 $^{-/-}$ mice, IL-1 β and TNF- α were equally able to mobilize LC from epidermis, indicating that migration in response to these cytokines is not dependent on IL-18 and suggesting that IL-18 acts upstream of these cytokines in the initiation of antigen-induced LC migration. Moreover, IL-1 β but not IL-18 was able to rescue the defective CHS response observed in caspase-1 $^{-/-}$ mice, which have no functional IL-1 β or IL-18. These data indicate that IL-18 is a key proximal mediator of LC migration and CHS, acting upstream of IL-1 β and TNF- α , and may play a central role in regulation of cutaneous immune responses. *J. Leukoc. Biol.* 83: 000–000; 2008.

Key Words: oxazolone · lymph nodes · caspase-1

INTRODUCTION

Langerhans cells (LC) are bone marrow-derived APCs that reside in the epidermis [1, 2]. They play a central role in the initiation and regulation of immunological responses in skin, typified by contact sensitization and the contact hypersensitivity

response (CHS) to epicutaneously applied chemical antigens. LC are required for the induction of antigen-specific responses in the skin, most notably, initiation of host defense against invading microorganisms and the induction of anti-tumor immunity.

To present antigen encountered in the skin to the immune system, LC must migrate from epidermis to draining lymph nodes [3]. A considerable body of data indicates that this migratory response is under the control of at least two cytokines derived from epidermal cells, IL-1 α and TNF- α . Intradermal injection of TNF- α or IL-1 α into murine skin results in migration of LC from epidermis and their subsequent accumulation in draining lymphoid tissue [4, 5]. Similar experiments in human skin confirm that TNF- α is also a potent initiator of LC migration from epidermis in man [6]. Moreover, neutralizing antibodies against either cytokine are capable of blocking migration of LC following topical application of antigen [5]. That this inhibition is of functional relevance is exemplified by the observation that intradermal injection of anti-IL-1 β antibody prior to sensitization can suppress the murine CHS response effectively [7].

We have shown previously that the cysteine protease caspase-1 plays an important regulatory role governing murine LC migration and CHS [8]. This enzyme is responsible for processing IL-1 β and the structurally related cytokine IL-18 from their inactive precursor forms into mature bioactive cytokines capable of receptor binding and initiation of their respective signaling pathways. We have begun to investigate the role of IL-18 in LC migration and have shown that like IL-1 α and TNF- α , intradermal injection of IL-18 is capable of initiating LC migration from epidermis in mice, with subsequent accumulation of mature dendritic cells (DC) in draining lymph nodes [9]. Moreover, migratory LC can release functional IL-18 [10], and antibodies against IL-18 are able to inhibit antigen-induced LC migration [9]. Taken together,

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these data strongly suggest that like IL-1 α and TNF- α , IL-18 is involved in the regulation of LC migration from epidermis to lymph node. Compatible with this is the observation that human DC express functional IL-18R [11].

Current data suggest that IL-1 β and TNF- α are required for LC migration, and their interdependence is complex. Thus, antibodies to TNF- α are able to inhibit IL-1 β -induced LC migration, and anti-IL-1 β antibodies are able to inhibit TNF- α -induced migration [5]. To date, a more proximal cytokine signal has not been identified, and in the current study, we sought to determine whether IL-18 might provide such a signal. We were also interested in defining the role played by IL-18 in cutaneous inflammatory responses typified by CHS and have compared this with nonantigen-specific inflammation induced by skin irritants.

MATERIALS AND METHODS

Cytokines, antibodies, other reagents

Recombinant murine (rMu)IL-1 β (specific activity $1\text{--}2\times 10^8$ U/mg), IL-18 (specific activity $1\text{--}2\times 10^8$ U/mg), and TNF- α (specific activity 2×10^8 U/mg by L929 cytotoxicity assay) were purchased from R&D Systems (Oxon, UK), PeproTech EC Ltd. (London, UK), and Genzyme Diagnostics (West Malling, Kent, UK), respectively. Cytokines were diluted in sterile PBS containing 0.1% BSA as carrier and were administered locally by intradermal injection into ear pinnae (30 μ l). Biotin-conjugated rat anti-mouse I-A^d/I-E^d mAb (clone 2G9) and streptavidin-FITC conjugates were purchased from PharMingen (San Diego, CA, USA). All other reagents, including contact sensitizers 2,4-dinitrofluorobenzene (DNFB) and oxazolone (OX) and skin irritant sodium lauryl sulfate (SLS), were purchased from Sigma Chemicals Ltd. (Poole, Dorset, UK) unless otherwise indicated.

Animals

IL-18 knockout (IL-18^{−/−}) and wild-type (WT) mice have been described previously in detail [12]. Caspase-1^{−/−} and WT control mice were the kind gift of Dr. W. Wong (BASF Corp., Worcester, MA, USA) and have also been described in detail [13, 14]. All animals were housed in a conventional animal facility with a 12-h light/dark cycle. Mice were used between 6 and 12 weeks of age and in individual experiments, were age-matched to within 2 weeks. All experiments were carried out under the provisions of the Animals (Scientific Procedures) Act 1986, UK.

CHS

Groups of at least three mice were sensitized by application of 150 μ l 0.5% DNFB in 4:1 acetone/olive oil (AOO) to abdominal skin. Five days later, 25 μ l 0.25% DNFB was applied to dorsal and ventral surfaces of the right ear and AOO alone to the left ear. At intervals thereafter (24, 48, and 72 h), challenge-induced ear swelling (relative to the AOO-treated ear) was measured using a modified, spring-loaded micrometer (Mitutoya Inc., Japan). In parallel experiments, OX was used in the same volumes and diluent at concentrations of 1% (sensitization) and 0.5% (elicitation). In cytokine rescue experiments, the dorsal surface of the right ear was pretreated by intradermal injection of 30 μ l IL-18 (50 ng), IL-1 β (50 ng), or diluent alone 30 min prior to sensitization with allergen (30 μ l 1% OX or 0.5% DNFB). Five days later, the left ear of the mice was challenged with 30 μ l 0.5% OX or 0.25% DNFB, respectively, and ear swelling (relative to prechallenge) was measured as described above. All experiments were repeated at least three times, and representative data are shown.

Irritant dermatitis

Groups of at least three mice received 25 μ l of the skin irritant SLS (10%), dissolved in dimethylformamide (DMF) on the dorsal and ventral surfaces of the right ear and DMF alone to the left ear. At intervals thereafter (2, 6, and

24 h), ear swelling (relative to the DMF-treated ear) was measured using a modified, spring-loaded micrometer (Mitutoya Inc.). All experiments were repeated at least three times, and representative data are shown.

Immunohistochemical staining of LC

LC numbers and morphology were evaluated en face in epidermal sheets prepared as described previously [8]. Briefly, ears were harvested and split into dorsal and ventral halves using fine forceps. Dorsal ear halves were then incubated in 0.02 M EDTA at 37°C for 90 min to allow separation of epidermis and dermis. Epidermal sheets were peeled away carefully from dermis, washed twice in PBS, fixed in acetone at -20°C for 20 min, and washed again. Epidermal sheets were then incubated with biotinylated rat anti-mouse I-A^d/I-E^d antibody diluted to 5 $\mu\text{g}/\text{ml}$ in PBS with 0.1% BSA for 45 min at room temperature, washed, and subsequently incubated for a further 45 min with FITC-labeled streptavidin diluted 1:100 in PBS with 0.1% BSA. Following further washing, epidermal sheets were mounted whole in glycerol, slides were coded, and LC were counted in 10 high-power fields in the central portion of the ear using an eye-piece with a calibrated grid (0.25 mm $\times$ 0.25 mm at $\times$ 40 magnification) on a fluorescence microscope (Axiophot, Zeiss, New York, NY, USA). The evaluator was blinded to the nature of slides examined. Results are expressed as mean $\pm$ SEM numbers of LC/mm² epidermis.

Statistical analyses

The statistical significance of differences of means of experimental groups was calculated using a two-tailed Student's *t*-test. Mean differences were considered to be significantly different when $P < 0.05$. All data are presented as mean $\pm$ SEM, and error bars are indicated on figures where SEM $> 5\%$ of the mean.

RESULTS

IL-18 is required for an optimal CHS in murine skin

To explore the role of IL-18 in murine CHS, IL-18^{−/−} and WT mice were sensitized on abdominal skin with 1% OX and challenged on one ear 5 days later with 0.5% OX. Ear swelling was measured over the following 72 h. Although a vigorous swelling response occurred in WT mice, maximal ear swelling in IL-18^{−/−} animals was less than 65% that observed in WT mice (**Fig. 1**). A similar reduction of CHS was seen when DNFB, an alternate contact sensitizer, was used. These data strongly suggest that IL-18 is required for optimal contact sensitization in murine skin.

IL-18 is not required for irritant contact dermatitis (ICD) in murine skin

Having shown a role for IL-18 in allergen-mediated murine skin inflammation, we were next interested in determining whether cutaneous inflammatory responses were generally suppressed in the absence of IL-18. We therefore studied the ear swelling induced by the skin irritant SLS, where 10% in DMF was painted onto ear skin of IL-18^{−/−} and WT mice, and ear thickness was measured over the following 48 h. Ear swelling occurred in both groups, maximal at 24 h, and no significant difference was evident between IL-18^{−/−} and WT mice (**Fig. 2**). Consistent with this is that the irritant response to contact allergens observed in unsensitized IL-18^{−/−} mice shown in Figure 1 did not differ from that observed in unsensitized WT mice.

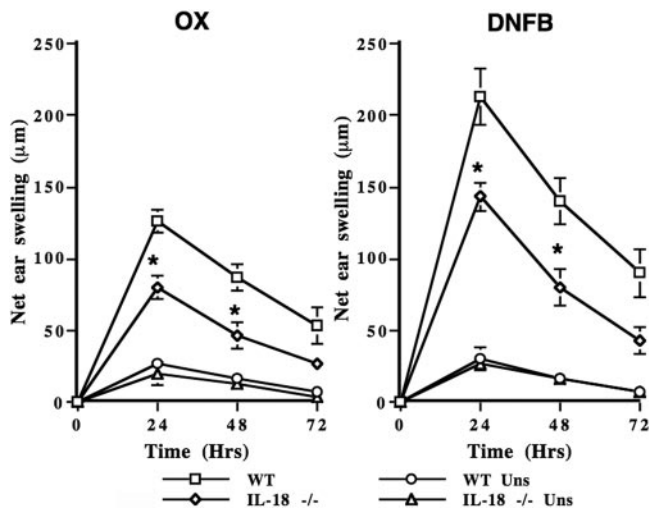


Fig. 1. The CHS is suppressed in IL-18^{-/-} mice, which were sensitized with OX or DNFB and challenged 5 days later on one ear as described. Marked ear swelling developed in WT mice (□) but was suppressed significantly in IL-18^{-/-} mice (◇); *, $P < 0.05$. Challenged but unsensitized control animals (○, WT Uns; △, IL-18^{-/-} Uns) were used as controls; $n = 3$ mice/group. Results are representative of three independent experiments.

IL-18 is required for normal epidermal LC migration and the afferent phase of CHS

Because of our previous data suggesting that IL-18 is capable of initiating LC migration from skin [9] and the observation that migrating LC release biologically active IL-18 [10], we hypothesized that LC migration might be deficient in IL-18^{-/-} mice and that this might account for

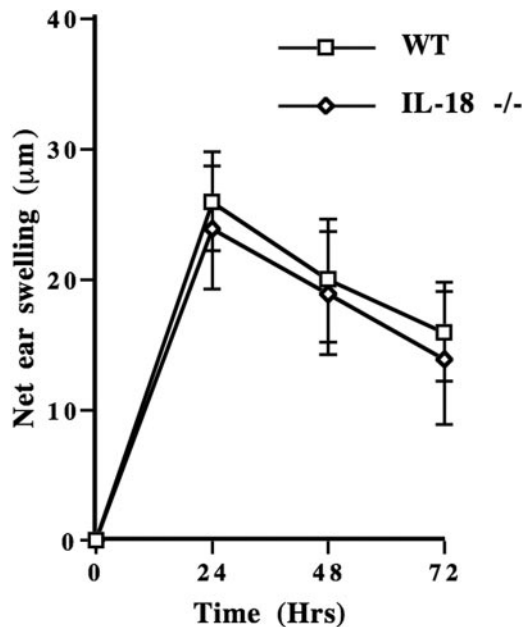


Fig. 2. ICD is normal in IL-18^{-/-} mice. Ten percent of SLS (25 μ l) in DMF was applied to ear skin of WT and IL-18^{-/-} mice as described. Ear swelling developed in both groups, maximal at 24 h, and no significant difference could be identified between WT and IL-18^{-/-} mice; $n = 3$ mice/group. Results are representative of three independent experiments.

the decreased CHS response shown in Figure 1. Therefore, LC were enumerated in epidermal sheet preparations derived from IL-18^{-/-} and WT mice before and 4 h following local epicutaneous application of 1% OX or 0.5% DNFB in AOO (**Fig. 3**). In WT mice, both allergens induced a significant reduction (OX, 25%; DNFB, 29%; both, $P < 0.05$, compared with vehicle control) in epidermal LC numbers, as has been reported in other mouse strains, suggestive of LC migration away from epidermis. Resting LC numbers and morphology in IL-18^{-/-} mice were equivalent to those observed in WT animals, but no significant change in epidermal LC numbers was seen following allergen application. It is interesting, however, that an increase in the intensity of MHC class II expression by LC in IL-18^{-/-} mice after stimulation was detected, suggestive of LC activation (data

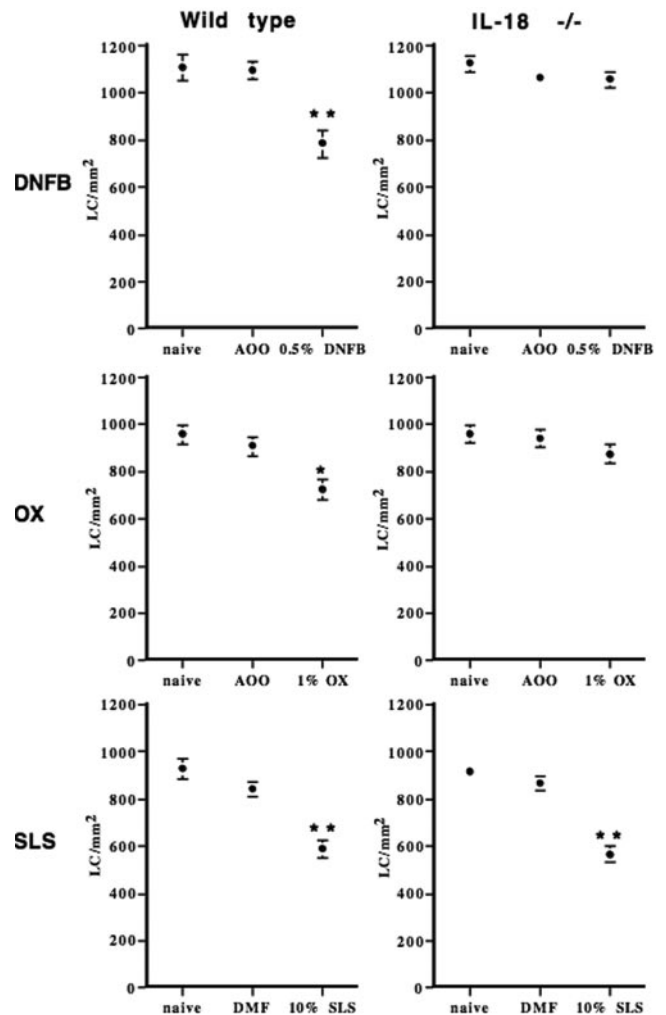


Fig. 3. LC migration in response to allergen stimulation is impaired in IL-18^{-/-} mice. LC numbers in epidermal sheets prepared following topical application of skin allergens DNFB or OX and the skin irritant SLS were enumerated by immunohistochemical staining for MHC class II antigens. Application of DNFB or OX resulted in a significant fall in epidermal LC numbers in WT mice, but this decline was absent in IL-18^{-/-} mice. In contrast, application of SLS induced a marked fall in epidermal LC density in WT and IL-18^{-/-} mice. Results are expressed as the mean number of LC/mm² ($\pm$ SEM); $n = 4$ ears/group; *, $P < 0.05$; **, $P < 0.01$, compared with vehicle control. Results are representative of three independent experiments.

not shown). To explore the responsiveness of LC in IL-18^{-/-} mice to a nonantigenic migratory stimulus, we also examined the reduction in epidermal numbers induced by the skin irritant SLS. WT and IL-18^{-/-} mice displayed an equivalent reduction (WT, 36%; IL-18^{-/-}, 38%; both, $P < 0.01$, compared with vehicle controls) in remaining epidermal LC 4 h following skin painting with SLS, indicating that IL-18^{-/-} LC can, given an appropriate signal, be induced to migrate. As with allergen application, an increase in the intensity of MHC class II expression by LC in both groups of mice was observed after SLS stimulation, suggestive of LC activation.

If the role played by IL-18 in CHS was predominantly in the regulation of LC migration, we reasoned that injection of IL-18 into skin at the time of sensitization would restore the CHS ear-swelling response. Thus, IL-18^{-/-} mice were sensitized on the skin of one ear with 1% OX 30 min after intradermal injection of 50 ng rMuIL-18 or diluent alone. Five days later, mice were challenged with 0.5% OX on the contralateral ear pinna, and swelling of the challenged ear was monitored over the following 72 h. As with abdominal sensitization (Fig. 1), IL-18^{-/-} mice showed a suppressed ear-swelling response in this model, but this could be rescued completely by preinjection of IL-18 (Fig. 4). IL-1 β was able to substitute for IL-18 in this model, whereas diluent alone was without effect. Neither IL-18 nor IL-1 β preinjection enhanced the swelling response observed in WT mice.

These data confirm that IL-18 has an obligatory role in the murine CHS response and strongly suggest that it is required

primarily in the sensitization rather than the elicitation phase of CHS.

IL-18 acts proximally to IL-1 β and TNF- α in initiation of LC migration and murine CHS

We were next interested in determining the relationship between IL-18 and the other cytokines (notably, IL-1 β and TNF- α), implicated previously in induction of LC migration. Thus, 50 ng IL-1 β , IL-18, or TNF- α was injected into IL-18^{-/-} or WT ear skin as described previously, and remaining epidermal LC at 4 h (IL-1 β and IL-18) or 30 min (TNF- α) were enumerated by immunostaining of epidermal sheet preparations. All three cytokines induced an equivalent reduction of epidermal LC numbers in IL-18^{-/-} and WT mice (Fig. 5), indicating that the migratory response to these cytokines is not dependent on secondary secretion of IL-18.

We have shown previously that the CHS response is absent in caspase-1^{-/-} mice that lack functional IL-1 β and IL-18, the principal cytokines processed by this enzyme [8]. To explore the interdependency of these two cytokines further in the initiation of CHS, we examined the ability of each to rescue the defective CHS response in caspase-1^{-/-} mice. In the absence of cytokine pretreatment caspase-1^{-/-} mice displayed a suppressed CHS response compared with WT controls, confirming our previous data [8]. Intradermal injection of 50 ng IL-18 immediately prior to sensitization was without effect, whereas injection of 50 ng IL-1 β fully restored the CHS in these mice (Fig. 6). Similar experiments performed in IL-18^{-/-} mice demonstrated that both cytokines were equally capable of restoring the defective CHS response (Fig. 4).

Taken together, these data indicate that IL-18 acts proximally to IL-1 β in the induction of CHS and that its effect is mediated, at least in part, by control of LC migration from the epidermis.

DISCUSSION

The experiments described in this paper indicate that IL-18 plays a key proximal role in the induction of allergic CHS in murine skin. It is required for normal LC migration in response to allergen and appears to act upstream of IL-1 β and TNF- α , two cytokines that have previously been shown to govern LC migration from epidermis to lymph node.

IL-18 bears strong structural relationships to IL-1 β [15]. Both molecules require proteolytic cleavage by caspase-1 to generate the mature functional cytokine from an inactive precursor form [16]. We have demonstrated previously that caspase-1 plays a key regulatory role in LC migration [8], presumably by modulation of processing and release of IL-1 β and IL-18. Within the epidermal microenvironment, keratinocytes [17] and LC [18, 19] express IL-18 and caspase-1, and thus, either cell may be the source of this signal. IL-18 may also be released in a noncaspase-1-dependent manner following the ligation of Fas ligand (FasL) [20]. Keratinocytes have been shown to express Fas and FasL in a number of inflammatory states [21, 22], and thus, this mechanism may also be

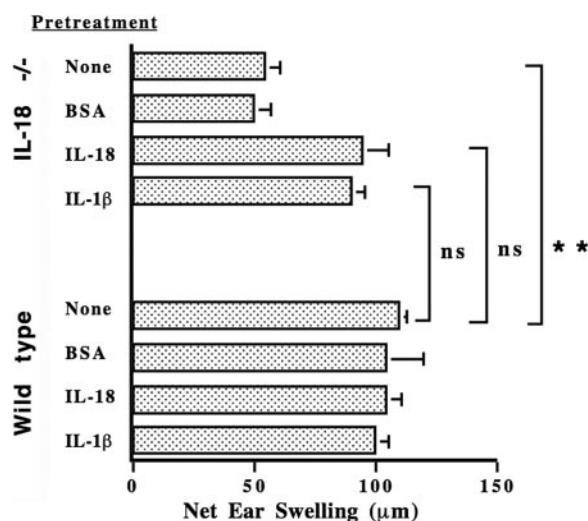


Fig. 4. Intradermal administration of rMuIL-18 prior to sensitization with oxazolone fully restores CHS in IL-18^{-/-} mice. Ear skin of WT and IL-18^{-/-} mice was pretreated locally by intradermal administration of 30 μ l IL-18 (50 ng), IL-1 β (50 ng), or diluent alone, 30 min prior to sensitization with 0.5% OX, and 5 days later, ears were challenged as described. IL-18^{-/-} mice pretreated by intradermal injection of diluent alone or untreated showed suppressed ear swelling as expected, but this attenuated response was restored to levels seen in WT mice following pretreatment with IL-18 or IL-1 β . Note that neither IL-18 nor IL-1 β pretreatment enhanced ear swelling in WT mice; $n = 5$ mice/group. ns, Not significant; **, $P < 0.01$, compared with vehicle control. Results are representative of three independent experiments.

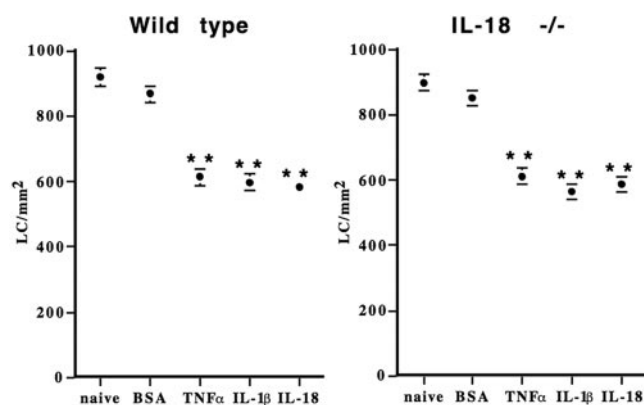


Fig. 5. Intradermal injection of TNF- α , IL-1 β , or IL-18 induces LC migration in WT and IL-18 $^{-/-}$ mice, which received 50 ng MuTNF- α , IL-1 β , or IL-18 by intradermal injection into ear pinnae. Control mice were untreated or received diluent alone. Epidermal sheets were prepared 4 h (IL-1 β and IL-18) or 30 min (TNF- α) after treatment, and LC numbers were determined following immunofluorescent labeling of MHC class II-positive cells. Administration of all three cytokines resulted in a similar decline in LC numbers in WT and IL-18 $^{-/-}$ mice. BSA alone was without effect. Results are expressed as the mean number of LC/mm² ($\pm$ SEM). **, $P < 0.01$, compared with vehicle control; $n = 4$ ears/group. Results are representative of three independent experiments.

of importance in the generation of mature IL-18 in the context of human inflammatory skin disease.

Our data indicate that IL-18 has a critical role in the regulation of inflammation within the skin. Thus, it is unsurprising that unregulated, transgenic overexpression of IL-18 in skin leads to uncontrolled inflammation [23]. Interfering with IL-18 activity is therefore an attractive therapeutic option in inflammatory skin disease, and pharmacologic use of IL-18-binding protein has been shown to inhibit contact sensitization in mice [24]. The data presented in this paper would suggest that because of the proximal signaling role played by IL-18 in LC mobilization, this approach would have significant advantages over other targets for cytokine blockade.

It has been well-established that IL-1 β and TNF- α are required for the induction of LC migration in response to allergen [4, 5, 25, 26]. The current hypothesis is that following allergen contact with epidermis, LC release IL-1 β , which stimulates production of TNF- α by neighboring keratinocytes that express the signal-transducing type-1 IL-1R. LC, which express IL-1R [27] and TNFR- α [28], are stimulated by the presence of both cytokines to enter the migration pathway, with loss of E-cadherin expression and induction of matrix metalloproteinase-9 and other markers associated with maturation and antigen presentation [3]. Both of these cytokines can induce the expression of the other under appropriate conditions [29], and it is therefore difficult to determine their relative importance to LC migration. However, the data presented herein, taken together with antibody inhibition studies published previously [9], indicate clearly that IL-18 acts upstream of TNF- α and IL-1 β .

Our current data do not define the source of IL-18 release, although keratinocytes or LC may be responsible. Keratinocytes [30, 31] and DC [11] have been shown to express and signal through the IL-18R, and DC are responsive to this

cytokine in other ways [32]; thus, either may be the responder cell potentially within the epidermis. Alternatively, IL-18 may stimulate the release of preformed TNF- α from mast cells, which could promote LC migration subsequently [33]. That IL-18 should be a proximal mediator in LC migration is in accord with previous data indicating that IL-18 can induce synthesis and release of a number of other cytokines, including TNF- α , IL-6, IL-8, and IL-1 β . In some cells, this is a direct effect [34]; in others, it requires the presence of TNF- α [35]. Although formal proof from cellular studies is awaited, it is therefore highly likely that IL-18 could induce production of both downstream cytokines involved in regulation of LC migration within the epidermis.

Our finding that IL-18 has no apparent role in ICD is of interest for a number of reasons. First, it indicates that the role for IL-18 in CHS is not simply part of a general requirement for IL-18 in skin inflammation. Second, it implies that IL-18, which is stored preformed in considerable quantities in murine and human keratinocytes [17], is not immediately bioactive. Were IL-18 directly proinflammatory in skin, we would have predicted that at least part of the ICD ear-swelling response, which occurs as a result of release of preformed, proinflammatory cytokines such as IL-1 α from damaged keratinocytes [36], would have been compromised in IL-18 $^{-/-}$ mice. This was not the case. An alternative explanation is that bioactive IL-18 does not have a direct, proinflammatory effect in skin, although we consider this unlikely, as IL-18 is capable of inducing expression of endothelial adhesion molecules [37], as well as

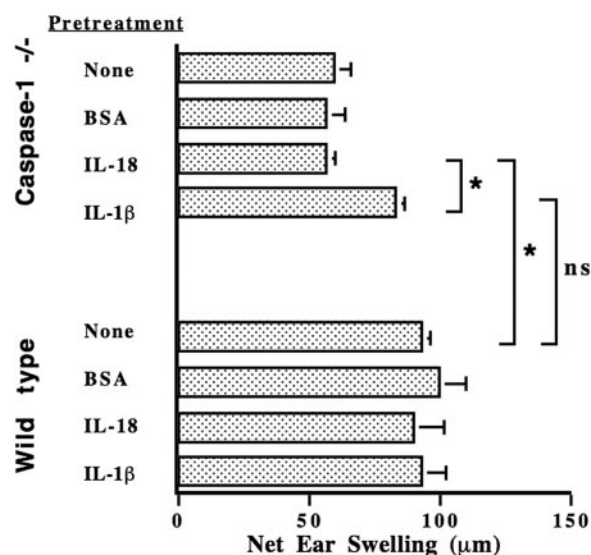


Fig. 6. IL-1 β but not IL-18 is able to restore the normal CHS in caspase-1 $^{-/-}$ mice. MuIL-1 β or IL-18 (50 ng) in 0.1% BSA was administered by intradermal injection locally to ear pinna skin of WT and caspase-1 $^{-/-}$ mice 30 min prior to sensitization with 1% OX on the same ear. Five days later, the contralateral ear pinna was challenged with 0.5% OX, and swelling was measured 24 h after challenge. WT mice exhibited marked ear swelling, whereas caspase-1 $^{-/-}$ mice without cytokine pretreatment showed significant suppression of this response. Preinjection of caspase-1 $^{-/-}$ ear skin with IL-1 β fully restored normal ear swelling, but IL-18 and BSA were without effect. Neither cytokine affected ear swelling significantly in WT mice. *, $P < 0.05$; $n = 5$ mice/group. Data are representative of three independent experiments.

promoting synthesis and release of other cytokines including TNF- α , which is known to be proinflammatory when released in the cutaneous microenvironment [34]. Finally, and perhaps most important, the data suggest that the presence of IL-18 is not obligatory for LC migration in all circumstances. Although the mechanisms and biological role of LC migration in irritant dermatitis are unclear, there is increasing evidence that preformed IL-1 α release is important in initiating this response [38]. It therefore seems likely that given such a signal, LC are able to avoid the requirement for IL-18 in migration in some situations.

A considerable body of evidence is now accumulating that indicates that IL-18 plays a key role in epithelial defense against microorganisms. Several skin-specific viruses (e.g., human papilloma virus [39, 40] and pox viruses [41, 42]) have evolved means of suppressing IL-18 in the skin by production of inhibitory binding proteins analogous to the endogenously produced IL-18-binding protein. Moreover, mice deficient in IL-18 are prone to experimental infection by a number of organisms including *Leishmania* and *Staphylococcus aureus* [12], both of which are major skin pathogens. A central role for IL-18 in the initiation of LC migration following antigen penetration of epidermis fits in well with this role and underscores the key role played by IL-18 in epithelial defense.

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