

IL-18 skews the invariant NKT-cell population via autoreactive activation in atopic eczema

Sara M. Lind¹, Carlotta Kuylensstierna², Markus Moll², Emilie D. Jordö¹, Ola Winqvist¹, Lena Lundeborg³, Maria A. Karlsson³, Maria T. Linder¹, Catharina Johansson¹, Annika Scheynius¹, Johan K. Sandberg^{*2} and Mikael C. I. Karlsson^{*1}

¹ Clinical Allergy Research Unit, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden

² Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska Institutet, Stockholm, Sweden

³ Dermatology Unit, Department of Medicine and Karolinska University Hospital Solna, Stockholm, Sweden

Atopic eczema (AE) is a chronic relapsing inflammatory skin disease where the commensal yeast *Malassezia* can act as a microbial trigger factor. *Malassezia* activates human DC to produce IL-18, an innate cytokine that is elevated in serum of AE patients; however, the precise role of IL-18 in human AE etiology is unknown. Herein, we investigated the effect of IL-18 on the human invariant NKT (iNKT) cell compartment in AE. We found that IL-18 was a potent activator of human iNKT-cells and promoted a pro-inflammatory CD1d-dependent response, even in the absence of exogenous ligands. Chronic activation via IL-18 on the other hand was inhibitory and skewed the iNKT-cell pool by selectively suppressing CD4⁺ iNKT-cells. This was mimicked in AE patients where the proportion of CD4⁺ iNKT-cells was reduced in peripheral blood and coincided with elevated plasma levels of IL-18. Furthermore, a reduced CD4⁺ iNKT-cell pool was associated with elevated IgE levels in plasma, and the plasma levels of IL-18 correlated with both total IgE and disease severity in the AE patients. Based on these findings, we propose that IL-18-mediated activation and subsequent dysregulation of the CD1d-restricted iNKT-cells plays a role in the pathogenesis of human AE.

Key words: Allergy · Autoreactivity · IL-18 · Inflammation · NKT cells



Supporting Information available online

Introduction

Atopic eczema (AE) is a common chronic relapsing inflammatory skin disease affecting 15–30% of children and 2–10% of adults in

industrialized countries [1]. The disease has a complex etiology including genetic predisposition as well as environmental triggers such as the gram-positive bacteria *Staphylococcus aureus* and the commensal yeast *Malassezia sympodialis* [1, 2]. Approximately 50% of adult AE patients have serum IgE specific for *M. sympodialis*, while such reactivity is rare in other allergic

Correspondence: Dr. Mikael C. I. Karlsson
e-mail: mikael.karlsson@ki.se

*These authors contributed equally to this work.

diseases [3]. AE has been described as a biphasic disease with a Th2-like cytokine pattern (including IL-4 and IL-13) in the lesions during the initiation phase [1, 2]. The eczematous chronic stage includes production of the Th1-like cytokines IL-12 and IL-18 [1], and IL-18 has been found to be elevated in serum from patients with AE [4]. An involvement of IL-18 in AE is also suggested by the finding that single-nucleotide polymorphisms in the IL-18 gene correlate with incidence of AE [1], and that NC/Nga mice (which develop spontaneous AE-like disease) have elevated serum levels of IL-18 [4]. In addition, transgenic mice with over-expression of IL-18 in the skin develop AE-like skin lesions [5].

In a connection to AE disease, two of the dominant microbial triggers can induce IL-18 responses. DC respond with strong IL-18 production after activation with *M. sympodialis* [6], and patients suffering from sepsis caused by *S. aureus* have a stronger IL-18 response compared with patients with sepsis caused by gram-negative bacterial infection [7]. IL-18 has diverse biological actions; it enhances IL-12-driven Th1 responses but can also stimulate Th2 responses in the absence of IL-12 [8]. A wide range of cells are known to produce IL-18 and its mRNA can be found in DC, keratinocytes, macrophages, Kupffer cells and B cells [8]. The IL-18R belongs to the IL-1R/TLR superfamily and shares the MyD88-dependent signalling pathway with a number of TLR [9]. IL-18 contributes to IFN- γ (Th1-type) responses together with IL-12, whereas IL-18 alone induces a potent IgE accumulation in mice [10]. This induction of IgE is regulated by invariant NKT (iNKT)-cells, suggesting that this cell type is involved in the regulation of IgE-producing B cells in this model [11].

iNKT-cells are innate-like T cells that operate on the border between the innate and adaptive immune response. They can promote both Th1 or Th2 type cytokine responses and work as both pro-inflammatory and tolerogenic cells in the immune response [12, 13]. Human iNKT-cells express an invariant TCR carrying V α 24 and V β 11 segments, which recognizes glycolipid antigens presented by the MHC class I-like molecule CD1d. These antigens include both foreign glycolipids such as bacterial antigens or allergen-derived ligands as well as self-ligands [13, 14]. Thus, the nature of the TCR used by iNKT-cell renders them reactive to self-antigens [15]. There are CD4⁺ and CD4⁻ subtypes of iNKT-cells and they have in common that they respond rapidly to antigen stimulation with a high level of IFN- γ production [13]. Human iNKT-cell subsets, however, differ in that the CD4⁺ subset produce Th2-type cytokines such as IL-4 and IL-13 whereas the CD4⁻ subset do not [16–18]. The role of iNKT-cells in atopic diseases is controversial. The findings reported from human and mouse studies are somewhat contradictory and at the present there is no consensus on the role of iNKT-cells in, for example, asthma and atopic dermatitis/eczema [19–22].

In the present study, we investigate the role of IL-18 and iNKT-cells in AE by analysis of their interplay *in vitro*, as well as the correlation between these factors and AE disease measures. We find that IL-18 can induce potent activation of iNKT-cells to endogenous ligands and that chronic stimulation with IL-18 skews the iNKT-cell population to a CD4⁻-dominated iNKT-cell pool. Furthermore, we observe that AE patients have a decreased CD4⁺ iNKT-cell pool and that AE-plasma levels of IL-18 induce

NF- κ B signalling in iNKT-cells *in vitro*. High levels of IL-18 and total IgE also coincide with the decreased CD4⁺ iNKT-cell pool in peripheral blood of AE patients. Together, our data suggest a novel mechanism where elevated plasma levels of IL-18 in AE patients activate and skew the iNKT-cell pool in a CD1d-dependent manner and thereby contribute to the pathogenesis of AE.

Results

IL-18 is elevated in AE patients and induces a rapid NF- κ B signalling in human iNKT-cells

Studies on patients with AE have revealed elevated serum levels of IL-18 [4]. Using a larger outpatient cohort we also found a significant increase in the plasma levels of IL-18 in AE patients ($n = 78$) compared with healthy individuals ($n = 45$) (Fig. 1A and for patient information see Table 1). IL-18 can be present in serum as free cytokine and bound to the plasma protein IL-18-binding protein (IL-18 BP) [8]. We found that there was no difference in the plasma levels of IL-18 BP between AE patients and healthy controls (Supporting Information Fig. 1). Thus, we show here that AE patients have increased plasma levels of free IL-18 with the potential to contribute to AE pathogenesis. Studies in mice have shown that IL-18 can induce iNKT-cell-regulated production of IgE [10], and therefore we investigated the effects of IL-18 on activation of human iNKT-cells.

IL-18R signalling was studied using iNKT-cell lines generated from human peripheral blood from healthy individuals. The IL-18R signals through the MyD88 and NF- κ B pathway [9], and when iNKT-cell lines were stimulated with IL-18 *in vitro*, there was a fast

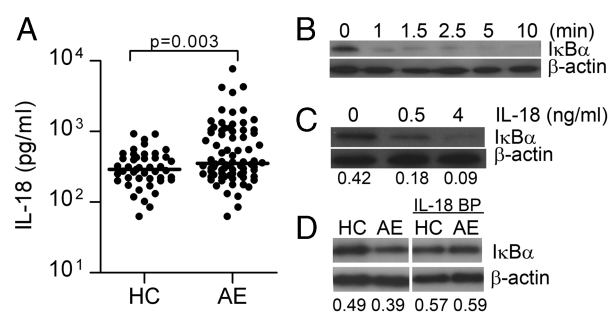


Figure 1. IL-18 is elevated in AE patients and induces rapid IκBα degradation in iNKT-cells. (A) Plasma levels of IL-18 in AE patients compared with healthy controls (HC). Each data point represents one individual patient ($n = 78$) or healthy control ($n = 45$). The horizontal line indicates the median and the groups were compared using Mann–Whitney U-test. (B–D) Western blot analysis of IL-18R signalling in iNKT-cells from healthy blood donors. (B) Stimulation using 200 ng/mL rhIL-18 for the indicated time points; one representative experiment out of three, using different healthy blood donors. (C and D) iNKT-cells were stimulated for 40 min with IL-18 (0.5 ng/mL, concentration in healthy controls; 4 ng/mL, concentration in AE patients) using either rhIL-18 (C) or healthy control/AE serum (D) as IL-18 source. The signalling induced by AE serum could be blocked by addition of IL-18-binding protein (IL-18 BP). Numbers represent the IκBα signal normalized to the β-actin signal (loading control). One representative experiment out of two using different healthy blood donors is shown in (C) and (D).

Table 1. Characterization of AE patients and healthy controls

	Healthy controls	AE patients
n	45	78
Gender (F/M) ^{a)} (n)	28/17	37/41
Age (years)	39 (20–64) ^{b)}	29 (18–65) ^{b)}
SCORAD ^{c)}	–	47 (16–81) ^{b)}
Total plasma IgE ^{d)} (kU/L)	24 (3.2–140) ^{b)}	385 (4.5–15 100) ^{b)}
M. sympodialis-specific IgE ^{e)}	0%	59%
Phadiatop [®] positive ^{f)}	0%	83%
Asthma and/or rhinitis	0%	72%

^{a)} F, female; M, male.^{b)} Median (range).^{c)} Disease severity assessed by the SCORAD Index [42].^{d)} ImmunoCAPTM (Phadia AB), reference range 1.6–122 kU/L.^{e)} m70 ImmunoCAPTM (Phadia AB), ≥ 0.35 kU/L.^{f)} Phadiatop[®] (Phadia AB) plasma IgE to any of 11 common aeroallergens, ≥ 0.35 kU/L.

and potent activation as analyzed by degradation of I κ B α (Fig. 1B). Next, to investigate whether the plasma levels of IL-18 found in AE patients were high enough to induce signalling in iNKT-cells, we stimulated cells with healthy control *versus* AE levels of IL-18. In these experiments we used either healthy control and AE patient serum or recombinant IL-18 in a concentration similar to the levels in peripheral blood of patients and controls. Interestingly, we found that both the recombinant IL-18 (4 ng/mL) as well as AE patient serum was sufficient to induce signalling in iNKT-cells (Fig. 1C and D). The enhanced signalling induced by AE patient serum was due to IL-18 since addition of a 1000-fold excess of IL-18 BP abolished the effect. There was a twofold signalling difference when using recombinant IL-18 at levels found in patients *versus* controls (calculated as the I κ B α signal normalized to the β -actin signal). Thus, IL-18 induces NF- κ B pathway signalling in human iNKT-cells and the levels found in peripheral blood of AE patients are sufficient to induce activation *in vitro*.

IL-18 activates iNKT-cells independently of exogenous ligands

To further characterize the effect of IL-18 on human iNKT-cells, we investigated the effect of IL-18 on the cytokine (IFN- γ and IL-4) response in iNKT-cells activated by the canonical ligand α -galactosyl ceramide (α -GalCer) presented by CD1d [13]. The IFN- γ response from iNKT-cells was greatly enhanced when IL-18 was added to co-cultures with iNKT-cells and DC presenting α -GalCer on CD1d and the IL-4 response also showed a similar pattern (Fig. 2A). Surprisingly, IL-18 enhanced both the IFN- γ and IL-4 response in co-cultures without addition of the exogenous ligand α -GalCer, and in the case of IFN- γ , the response was three times as high compared with that induced by α -GalCer alone (Fig. 2A). However, IL-18 did not induce release of these cytokines from iNKT-cells in the absence of monocyte-derived DC (MDDC) (Fig. 2A). Since the increased cytokine response without addition of exogenous ligand

implies that elevated levels of IL-18 potentially could shift the iNKT-cell population towards an autoreactive phenotype, we titrated the IL-18 dose to resemble the situation in AE patients. As shown in Fig. 2B, the IFN- γ response was increased by addition of 5 ng/mL of IL-18, *i.e.* similar to plasma levels in AE patients with high levels of IL-18. This was also true for IL-4, which was slightly increased by lower doses of IL-18 (Fig. 2B). In summary, the overall effect of IL-18 was greater on IFN- γ as compared with IL-4 production, both in magnitude of release and sensitivity of the response.

The CD1d molecule is known to present self-ligands that can activate iNKT-cells [13]. This activation is of low affinity compared with α -GalCer and it is thought that this autoreactive feature of iNKT-cells is important for their development as well as in regulating their response to foreign antigens [13]. To evaluate the role of CD1d in the effects of IL-18 on IFN- γ production in the absence of exogenous ligands, we stimulated iNKT-cells using CD1d-transfected 293T cells. In this experiment, we found that IL-18 induced a potent IFN- γ response in iNKT-cells only in the

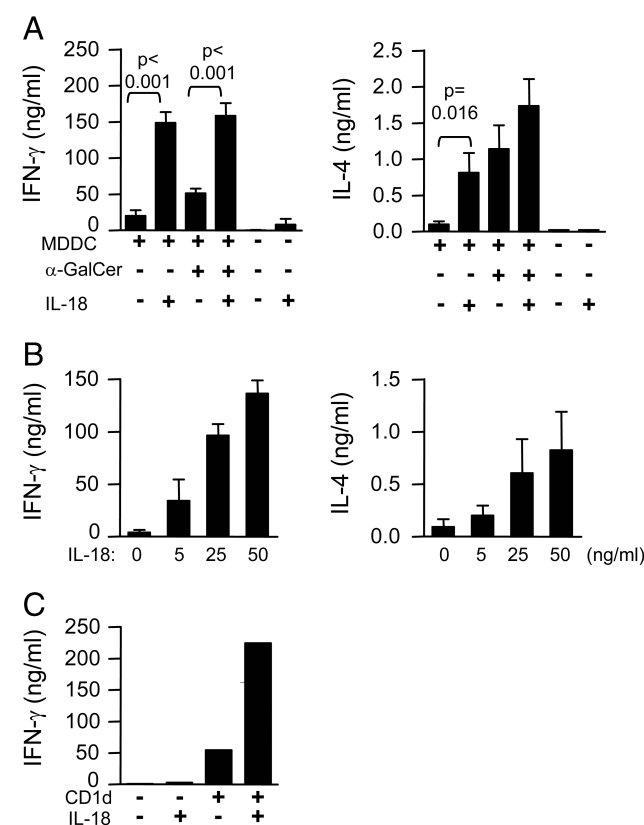


Figure 2. IL-18 activates iNKT-cells independent of exogenous ligands. (A and B) ELISA analysis of IFN- γ and IL-4 secreted by iNKT-cells co-cultured, with (A) MDDC $\pm$ α -GalCer in the presence or absence of rhIL-18 (200 ng/mL) or (B) MDDC without addition of exogenous ligand and lower doses of rhIL-18 (as indicated). (C) ELISA analysis of IFN- γ secreted by iNKT-cells co-cultured with CD1d-transfected 293T cells without addition of exogenous ligand. Results are expressed as mean $\pm$ SEM (A, $n = 5$ –7 different healthy blood donors; B, $n = 3$ different healthy blood donors), or (C) one representative experiment out of three using different healthy blood donors. All samples were collected after 72 h and analyzed in duplicates and the groups with and without IL-18 in (A) were compared using Mann-Whitney U-test.

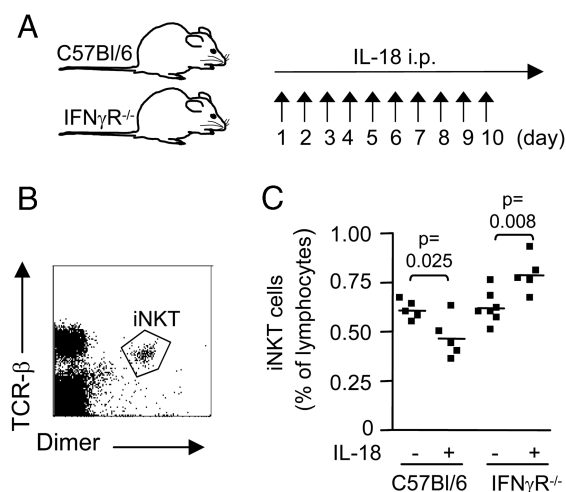


Figure 3. IL-18 decreases mouse splenic iNKT-cells *in vivo*, through an IFN- γ -dependent pathway. (A) Schematic overview of the experimental set-up, C57BL/6 (WT) and IFN γ R $^{-/-}$ mice were given daily i.p. injections of rmIL-18 for 10 days. (B and C) The iNKT-cell pool in the spleen on day 14 after the first IL-18 injection was analyzed by flow cytometry. iNKT-cells were defined as TCR- β ⁺CD1d-dimer⁺ (B) and quantified as percent of lymphocytes (C). Each data point represents one mouse and the horizontal bar indicates the mean ($n = 5$ –7). Groups with and without injections of rmIL-18 were compared using Mann–Whitney U-test.

presence of CD1d-expressing cells (Fig. 2C). Thus, these data show that CD1d, presumably presenting self-ligands, is necessary and sufficient for IL-18 to activate iNKT-cells to produce IFN- γ .

IL-18 decreases the splenic iNKT-cell pool in mice through an IFN- γ -dependent pathway

The strong activation of iNKT-cells by IL-18 *in vitro* led us to investigate the more long-term effect of IL-18 on iNKT-cells. Since IFN- γ production was greatly enhanced by IL-18 *in vitro* and injection of IL-18 in mice have been reported to result in IFN- γ production *in vivo* [23], we investigated how this cytokine contributed to the *in vivo* effect of IL-18 on iNKT-cells. C57BL/6 (WT) and IFN- γ R deficient (IFN γ R $^{-/-}$) mice were injected daily for 10 days i.p. with rmIL-18 (Fig. 3A). The splenic iNKT-cell pool in mice consists mainly of CD4⁺ cells [24] and was identified by staining with α -GalCer-loaded CD1d dimers in combination with TCR- β (Fig. 3B). The iNKT-cells decreased significantly in WT mice but not in IFN γ R $^{-/-}$ mice (Fig. 3C), where the splenic iNKT-cell pool instead was significantly increased. Thus, continuous elevated levels of IL-18 reduce the pool of iNKT-cells *in vivo* in this mouse model, and the reduction is dependent on the IFN- γ response induced by IL-18.

IL-18 induces a selective growth disadvantage for human CD4⁺ iNKT-cells in long-term cultures

In mice, the strong iNKT-cell activation mediated by α -GalCer induces a loss of iNKT-cells and renders them anergic to

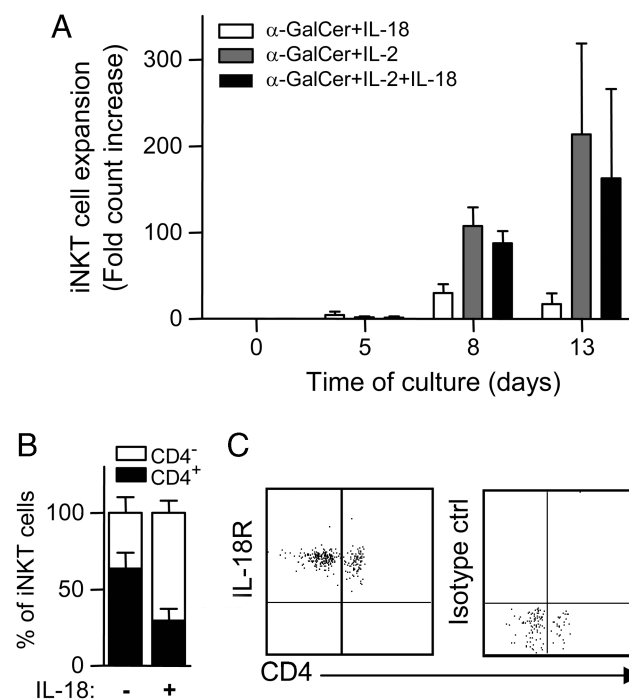


Figure 4. IL-18 induces a selective growth disadvantage for human CD4⁺ iNKT-cells in long-term culture. (A–C) Flow cytometric analysis of (A) the effect of IL-18 on the α -GalCer-mediated expansion of iNKT-cells from PBMC+IL-2. Results are expressed as fold count increase, mean $\pm$ SEM ($n = 4$ different healthy blood donors). (B) The effect of IL-18 on the proportion of CD4⁺ and CD4⁺ iNKT-cells on day 13 of the iNKT-cell expansion described in (A). Results are expressed as percent of the iNKT-cell population, mean $\pm$ SEM; ($n = 3$ different healthy blood donors). (C) IL-18R expression on the CD4⁺ and CD4⁺ subsets of iNKT-cells (gated as V α 24⁺/V β 11⁺ cells) in PBMC from a healthy donor.

subsequent stimulation [25], analogous with the negative effect on iNKT-cells by repeated injections of IL-18. As the AE patients have a chronic state of increased IL-18 levels we wanted to test the long-term effects of the IL-18-driven response on human iNKT-cells. To do this, we expanded iNKT-cells in PBMC from healthy donors using α -GalCer and IL-2 with or without addition of IL-18 for 2 wk. IL-18 was very poor at supporting α -GalCer-induced expansion without IL-2 (Fig. 4A) and we thus assessed the effects of IL-18 on the iNKT-cell expansion driven by IL-2 and α -GalCer. IL-18 had little or no effect on the expansion after 1 wk, but after 2 wk the IL-18 treated cultures showed a slight reduction in the expansion of iNKT-cells (Fig. 4A). However, when the relative proportion of CD4⁺ versus CD4⁺ iNKT-cells was analyzed, we found that addition of IL-18 for 2 wk resulted in an iNKT-cell population with fewer CD4⁺ cells (Fig. 4B). A decreased proportion of CD4⁺ iNKT-cells was also observed using 5 ng/mL of IL-18, i.e. similar to the levels in peripheral blood of AE patients (data not shown). The selective effect to reduce CD4⁺ iNKT-cells was not due to differences in IL-18R expression on the two iNKT-cell subsets (Fig. 4C). Thus, prolonged stimulation with IL-18 skews the iNKT-cell population towards a predominant CD4⁺ profile *in vitro*.

CD4⁺ iNKT-cells are decreased in patients with AE

The findings above suggest a scenario where the chronically increased IL-18 levels seen in AE patients alone could suffice to induce a chronic stimulation and subsequent decrease in iNKT-cells. In line with this reasoning, a significant decrease in peripheral blood iNKT-cells has previously been observed in AE patients [26, 27]. To follow up on our *in vitro* findings, we investigated the percentage of iNKT-cells in peripheral blood of the AE patient cohort. iNKT-cells were identified as V α 24⁺/V β 11⁺ lymphocytes (Fig. 5A). In peripheral blood from patients with elevated plasma IgE (≥ 122 kU/L) we found no difference in the total iNKT-cell population or the CD4⁺ subset, whereas the CD4⁺ iNKT-cell subset was significantly decreased ($p = 0.03$) (Fig. 5B–D). There was no difference between the proportion of total CD4⁺ T cells in peripheral blood of the AE patients and healthy controls (Fig. 5E), indicating that the difference in CD4⁺ iNKT-cells was not due to differences in CD4⁺ lymphocyte counts.

T cells are known to infiltrate lesional skin of AE patients [1] and the decreased CD4⁺ iNKT-cell population in peripheral blood could thus be due to recruitment of iNKT-cell to such inflammatory sites. To investigate this possibility, we quantified the expression of the genes encoding the TCR constant chain (*TRBC2*) and the iNKT-cell-TCR, V α 24 (*TRAV10*) and V β 11 (*TRVB25-1*), in the skin of AE patients and healthy controls using real-time PCR. As expected, the transcripts of the TCR constant chain were increased in lesional skin but we did not observe an increase in the transcripts of the iNKT-TCR (data not shown). Thus, the decrease in CD4⁺ iNKT-cells in peripheral blood is not due to recruitment to lesional skin.

To test whether the size of the CD4⁺ iNKT-cell pool in peripheral blood was indicative of elevated plasma IgE, we compared the AE patients with elevated total plasma IgE levels (≥ 122 kU/L) to the patients with plasma IgE within the reference range (< 122 kU/L). The percentage of CD4⁺ iNKT-cells was significantly lower in AE patients with elevated total plasma IgE levels (Fig. 5F). In addition, there was a tendency

towards a negative correlation between CD4⁺ iNKT-cells and IL-18, where patients with a large pool of CD4⁺ iNKT-cell had low IL-18 levels and, conversely, patients with high levels of IL-18 had a small pool of CD4⁺ iNKT-cells (data not shown). These data suggest that the increased plasma levels of IL-18, the decreased percentage of CD4⁺ iNKT-cells in peripheral blood and the increased plasma levels of IgE are connected in AE pathogenesis.

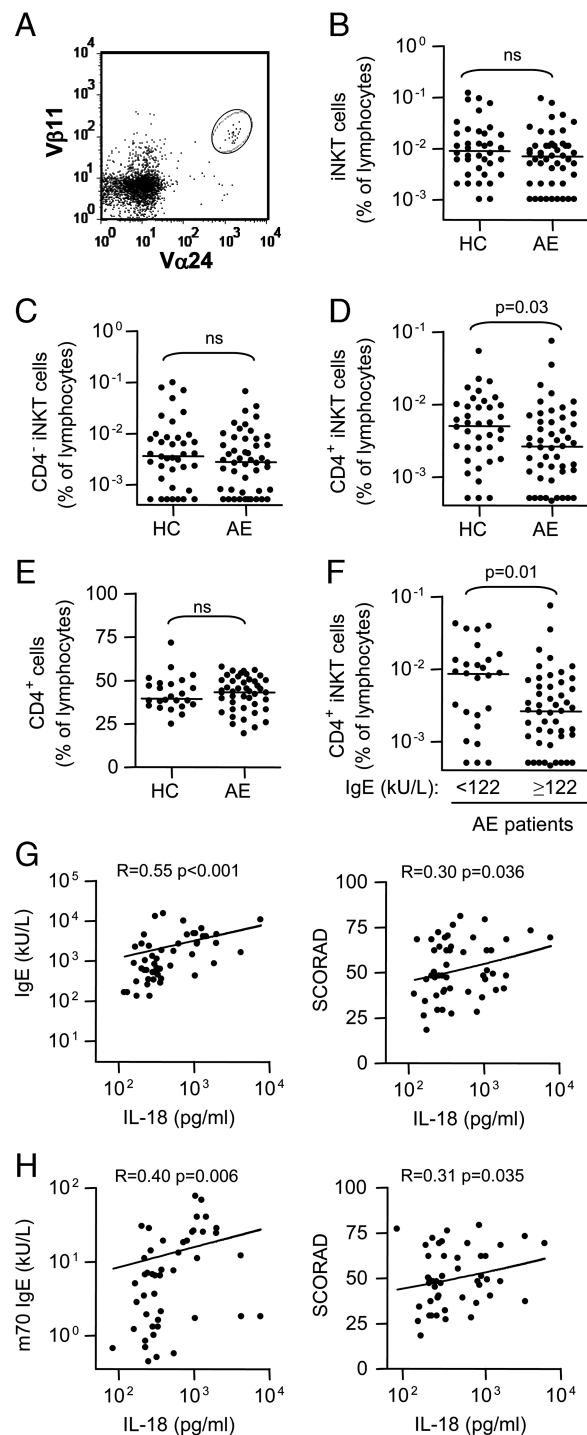


Figure 5. The CD4⁺ iNKT-cell pool is decreased in peripheral blood of AE patients and CD4⁺ iNKT-cells and IL-18 is connected to AE disease measures. (A) Representative FACS plot showing the definition of iNKT-cells as V α 24⁺/V β 11⁺ cells in peripheral blood lymphocytes of a healthy blood donor. (B–E) Flow cytometric analysis of iNKT-cells in healthy controls ($n = 38$) and AE patients with elevated total plasma IgE (≥ 122 kU/L) ($n = 49$). (B) Total iNKT-cells, (C) CD4⁺ subset of iNKT-cells, and (D) CD4⁺ subset of iNKT-cells as well as (E) CD4⁺ lymphocytes in peripheral blood. (F) Comparison of the size of the CD4⁺ iNKT-cell pool between AE patients with total plasma IgE levels < 122 kU/L ($n = 29$) and patients with elevated IgE levels, ≥ 122 kU/L ($n = 49$). Each data point represents an individual patient or healthy control and the horizontal bar indicates the median. The groups were compared using the Mann–Whitney U-test. (G) Correlation between the plasma levels of IL-18 and total IgE and disease severity, measured by the SCORAD index, in AE patients with elevated total plasma IgE (≥ 122 kU/L) ($n = 49$). (H) Correlation between plasma levels of IL-18 and M. sympodialis-specific IgE (m70) and disease severity in the AE patients with m70 IgE ≥ 0.35 kU/L ($n = 46$). The correlations were analyzed using Spearman's rank correlation.

Plasma levels of IL-18 correlate with IgE and disease severity in AE

Our data suggest that IL-18 is involved in mechanisms that can contribute to the pathogenesis of AE. IL-18 has also been suggested to correlate with plasma IgE and disease severity in adult AE patients but no significant results have been reported [4]. We therefore investigated how total and allergen-specific IgE as well as disease severity (measured by the SCORAD index) correlated with the plasma levels of IL-18 in the patient cohort. We found a correlation between plasma levels of IL-18, and total plasma IgE and SCORAD in the 49 AE patients with elevated total IgE levels (≥ 122 kU/L) (Fig. 5G). Interestingly, IL-18 also correlated with allergen-specific IgE levels and SCORAD in the 46 AE patients with *M. sympodialis* IgE ≥ 0.35 kU/L (Fig. 5H). In addition, the total IgE levels in plasma correlated with disease severity in the AE patient cohort ($r = 0.32$, $p = 0.004$). We conclude that the elevated plasma level of IL-18 in AE patients can have an impact on total IgE levels and *M. sympodialis*-specific IgE levels, as well as disease severity.

Discussion

AE is different from many other inflammatory disorders in that lesions go through a distinct initiation stage, with a cytokine profile predominantly including IL-4, IL-5 and IL-13, and then shifts into a chronic stage dominated by IFN- γ and IL-12 [1]. We have with the present study aimed at introducing IL-18 into the picture and especially its effect on iNKT-cells. The most striking novel findings are that IL-18 induces CD1d-dependent activation of iNKT-cells to endogenous ligands and that prolonged IL-18 stimulation skews the iNKT-cell compartment. In addition, we find that low levels of CD4⁺ iNKT-cells are associated with elevated plasma levels of IgE in the AE patients.

The size of the “natural” pool of iNKT-cells vary to a large extent among individuals and these cells typically make up a small population of lymphocytes [13]. However, a reduced proportion of iNKT-cells in peripheral blood has been associated with development of disease, for example in patients with systemic lupus erythematosus [28]. In a previous study on AE by Takahashi *et al.*, data show a reduced iNKT-cell population in peripheral blood [26]. In contrast to our findings, they report that the CD4⁺ subset had a tendency to be more reduced than the CD4⁺ subset. The reason for these discrepancies is unknown, but a difference in patient cohorts is plausible. The decreased pool of CD4⁺ iNKT-cells in peripheral blood of AE patients could be due to that individuals with a smaller CD4⁺ iNKT-cell pool develop the disease or, alternatively, that the decreased pool of CD4⁺ iNKT-cells is part of the pathogenic process, or a consequence, of the disease. Our results point towards the latter possibility and we believe this iNKT-cell phenotype to be IL-18 driven because (i) prolonged exposure to IL-18 *in vitro* selectively reduces the expansion of CD4⁺ iNKT-cells and (ii) administration of IL-18 alone is sufficient to reduce the splenic iNKT-cell pool (in mice

dominated by the CD4⁺ subtype [24]). This is most likely through autoreactive CD1d-dependent activation of iNKT-cells since only endogenous ligands are present. In humans, CD4⁺ iNKT-cells produce a mixture of Th1 and Th2 cytokines, including both IFN- γ and IL-4, whereas the CD4⁺ subset produces mainly Th1 cytokines such as IFN- γ and TNF- α [16–18]. Selective reduction of CD4⁺ iNKT-cells could thus reduce the anti-inflammatory, Th2-like, effects of iNKT-cells [29] and thereby add to the chronic Th1-type inflammation described in AE patients [1, 2]. Activated iNKT-cells have also been reported to inhibit Th2-type responses, for example by releasing IL-21, which induces apoptosis in IgE⁺ B cells [30]. We suggest that a skewed iNKT-cell pool could disturb such inhibitory actions by iNKT-cells and possibly also contribute to elevated IgE levels in AE patients. This is in concordance with the finding that AE patients with elevated IgE levels have a significantly smaller CD4⁺ iNKT-cell pool in peripheral blood compared with the patients with total plasma IgE levels within the reference range (< 122 kU/L).

iNKT-cells are prominent producers of both IFN- γ and IL-4 and we show here that IL-18 increases the production of both cytokines from human iNKT-cells activated by antigen presented on CD1d. Our results are in line with studies in mice showing that IL-18 can increase the cytokine response by iNKT-cells activated with α -GalCer [31]. A striking novel finding is that IL-18 alone can increase the production of cytokines from human iNKT-cells activated by endogenous ligands presented in CD1d context, both on MDDC and CD1d-transfected cells, a feature previously only described for IL-18 together with IL-12 [32]. The magnitude of the response was greater for IFN- γ compared with IL-4 and we conclude that IL-18 converts human iNKT-cells to an autoreactive phenotype by inducing a strong pro-inflammatory response to self-antigens presented through CD1d. This is similar to the recently described activation of iNKT-cells by IL-12 (produced by DC in response to TLR signalling) in synergy with self-glycolipid-CD1d complexes [33].

IL-18 could induce pro-inflammatory autoreactive activation of iNKT-cells at concentrations found in peripheral blood of AE patients, suggesting that this mechanism could be at work in AE patients. Activation of iNKT-cells by ligands presented by CD1d can occur on various locations such as in the secondary lymphoid organs and in the skin [34]. In relation to antibody responses, B cells that express high levels of CD1d are important for iNKT-cell-enhanced antibody responses in mice [35]. Also, keratinocytes express CD1d in the skin [36] where iNKT-cells also have been found in elicitation sites of allergic contact dermatitis [37] and keratinocytes are also known to produce IL-18 [8]. Thus, iNKT-cells can infiltrate inflamed skin and it is likely that there will be systemic as well as tissue-specific effects that may differ depending on the context of iNKT-cell activation.

In many autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, increased levels of IL-18 correlate with severity of the autoimmune pathologies [38]. Interestingly, about 25% of AE patients have IgE antibodies against self-proteins and some allergens in AE have human homologues [1]. IgE reactivity against manganese superoxide dismutase, a human homologue of

the Mala s 11 allergen [39], correlates strongly with activity of AE [40]. Here, we find that the plasma levels of IL-18 correlate with disease severity in patients with IgE reactivity against *M. sympodialis*. The plasma level of IL-18 also correlates with total IgE and disease severity in the patients with elevated IgE levels (≥ 122 kU/L), supporting that IL-18 could potentially drive the disease in these patients.

The data we describe in this study suggest a novel model where elevated levels of IL-18 contribute to AE pathogenesis by a dual effect on human iNKT-cells. Microbes, such as *Malassezia*, can induce production of IL-18, by for example DC or keratinocytes, in the initial stage of an AE lesion. The high levels of IL-18 activate iNKT-cells to become pro-inflammatory (release IFN- γ) in response to both self and foreign ligands presented by CD1d. Later, in the chronic stage, the continuous stimulation via IL-18 skews the iNKT-cell repertoire with selective suppression of the tolerogenic CD4⁺ iNKT-cells. We speculate that these events may open up for a pro-inflammatory chronic immune response, possibly also including deregulated IgE-producing B cells, and contribute to the subsequent development and maintenance of the chronic stage of AE.

Materials and methods

Subjects

Clinical outpatients with AE and healthy controls were recruited in the Stockholm area (see Table 1). Inclusion criteria for the AE patients were diagnosis according to the UK working party criteria [41], moderate to severe eczema (as assessed by the SCORAD system [42]) and skin lesions not only restricted to the hands. The healthy controls had no clinical symptoms or history of allergy or skin diseases. Exclusion criteria for the participants in the study were other skin diseases than AE, autoimmune diseases, immune deficiencies, malignant diseases, pregnancy or lactation, immunosuppressive treatment and age below 18 or above 65 years. Use of systemic glucocorticoids or systemic antifungal treatment was not allowed for 2 months before the investigation. The subjects were interviewed about their medical history and blood samples were drawn for analysis. All participants gave their informed consent. The study was approved by the Swedish Regional Ethics Committee.

IgE analysis and ELISA

Total IgE, *M. sympodialis*-specific IgE (m70) and specific IgE to any of 11 common aeroallergens (Phadiatop[®]) were analyzed in blood plasma using ImmunoCAP[™] (Phadia AB, Uppsala, Sweden). Total IgE above 122 kU/L was considered as elevated. Human IL-18, IFN- γ and IL-4 were measured using specific ELISA kits from Invitrogen (Carlsbad, CA), R&D systems (Minneapolis, MN) and Mabtech AB (Stockholm, Sweden), respectively. Mouse

IgE was measured using a matched antibody pair (BD Pharmin-gen, San Jose, CA) and streptavidin AP (DAKO, Glostrup, Denmark). All ELISA samples were assayed in duplicate.

Western blot analysis

iNKT-cell lines were stimulated with rhIL-18 as follows: (i) with 200 ng/mL rhIL-18 for the indicated time points, (ii) for 40 min with the indicated concentrations of rhIL-18 or healthy controls or AE patient serum pre-incubated at 37°C for 10 min $\pm$ 2 μ g/mL IL-18 BP (R&D Systems). IL-18R signalling was detected using a rabbit anti-I κ B α antibody (Cell Signaling, Danvers, MA) followed by an HRP-conjugated antibody to rabbit IgG (Cell Signaling). β -actin was detected with a HRP-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were visualized with a commercial ECL kit (Cell Signaling).

Cell culture

CD1d-restricted iNKT-cell lines and MDDC were generated from healthy blood donors as described previously [43]. Briefly, iNKT-cell lines were generated by expanding iNKT-cells from PBMC, using IL-2 (Peprotech, Rocky Hill, NJ) α -GalCer (Kirin Brewery, Tokyo, Japan) and 10% FCS (Invitrogen, Carlsbad, CA), and monocytes were differentiated to MDDC using IL-4 (R&D systems, Minneapolis, MN), GM-CSF (Peprotech) and heat-inactivated human AB serum (Cambrex, East Rutherford, NJ).

To obtain stable CD1d transfectants, the CD1d gene was cloned into the pcDNA3.1/Zeo(+) expression vector (Invitrogen), and transfected into 293T cells using Lipofectamine 2000 (Invitrogen). Stable 293T cells expressing CD1d were selected using 50 μ g/mL Zeocin (Invitrogen) and CD1d expression was confirmed by flow cytometry.

Short-term effects of IL-18 on iNKT-cells were investigated by co-culture of antigen-presenting cells (MDDC, 293T cells or CD1d-transfectants), loaded with 100 ng/mL α -GalCer, with iNKT-cells $\pm$ 5–200 ng/mL rhIL-18 (MBL, Woburn, MA) for 72 h. Long term effects of IL-18 on iNKT-cells were evaluated by expanding iNKT-cells from PBMC (as described in [43]) $\pm$ 200 ng/mL rhIL-18 for 2 wk. The expansion of iNKT-cells was analyzed by flow cytometry.

Mouse experiments

IFN γ R^{-/-} mice [44] were backcrossed to the C57BL/6 strain for > 10 generations. IFN γ R^{-/-} and C57BL/6 mice were treated with rmIL-18 (MBL) 2 μ g/mouse/day i.p. for 10 days. The serum IgE levels were monitored throughout the experiment and the mice were sacrificed at day 14. The rmIL-18 contained < 0.005 ng/mL LPS as measured by the Limulus Amebocyte Lysate endochrome method (Charles River Endosafe, Wilmington, MA). Mice were maintained at the animal facility at the Department of Microbiology Tumor and Cell Biology, Karolinska Institutet, and used

at the age of 8–12 wk. The work was approved by the Swedish Regional Ethics Committee for animal welfare and conducted under federal guidelines.

Flow cytometry

The following anti-human antibodies were used to stain PBMC and iNKT-cell lines; V β 11-FITC, V α 24-PE, V α 24-biotin (Immunotech, Miami, FL), CD1d-PE, CD4-PerCP and streptavidin-Allophycocyanin (BD Pharmingen), IL-18R α -PE and mouse IgG1k-PE (R&D Systems).

Single-cell suspensions were prepared from mouse spleens and strained through a 70- μ m nylon screen. Erythrocytes were lysed with ACK lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA at pH 7.2) and the cells were stained with anti-mouse TCR- β -Allophycocyanin, and CD1d dimers (BD Pharmingen). Fluorescently labeled CD1d dimers loaded with α -GalCer were prepared according to the manufacturer's protocol (BD Pharmingen). Prior to CD1d dimer staining, Fc-receptors were blocked with purified anti-CD16/32 (BD Pharmingen).

Samples were analyzed on a FACSCalibur (Becton Dickinson, Lincoln Park, NJ) using FlowJo software (ThreeStar Ashland, OR).

Statistical analysis

Mann–Whitney *U*-test was used to test the difference between two groups. Correlations were analyzed using Spearman's rank correlation. *p* < 0.05 was considered significant.

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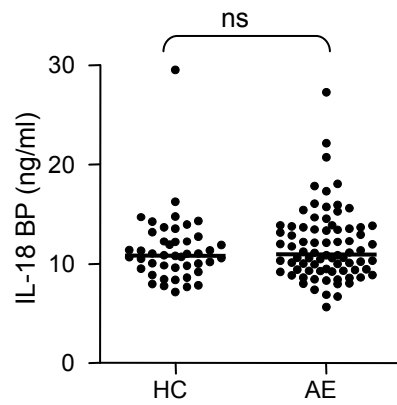
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- Abbreviations:** AE: atopic eczema · α -GalCer: α -galactosyl ceramide · HC: healthy controls · IL-18 BP: IL-18-binding protein · iNKT: invariant NKT · MDDC: monocyte-derived DC
- Full correspondence:** Dr. Mikael C. I. Karlsson, Karolinska Institutet, Department of Medicine Solna, Clinical Allergy Research Unit L2:04, Karolinska University Hospital, 171 76 Stockholm, Sweden
Fax: +46-8-335724
e-mail: mikael.karlsson@ki.se
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**IL-18 skews the invariant NKT-cell population *via* autoreactive activation in
atopic eczema**

Sara M. Lind, Carlotta Kuylenstierna, Markus Moll, Emilie Domange Jordö,
Ola Winqvist, Lena Lundeberg, Maria A. Karlsson, Maria Tengvall Linder,
Catharina Johansson, Annika Scheynius, Johan K. Sandberg and
Mikael C. I. Karlsson



Supplementary figure 1

No difference in the plasma levels of IL-18 binding protein (BP) between AE patients and healthy controls. Each data point is an individual patient (n=78) or healthy control (n=45), the horizontal line indicates the median.

Supplemental materials and methods

ELISA

Plasma levels of IL-18 binding protein were measured using a matched antibody pair from R&D systems (Minneapolis, MN) and streptavidin AP (DAKO, Glostrup, Denmark). The ELISA samples were assayed in duplicate.