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IL-1 Family Members IL-18 and IL-33 Upregulate the Inflammatory Potential of Differentiated Human Th1 and Th2 Cultures

Lars Blom and Lars K. Poulsen

The IL-1 family members IL-1β, IL-18, and IL-33 are potent cytokines in relationship to amplifying the CD4+ T cell cytokine production. To evaluate their impact on in vitro-differentiated human Th1 and Th2 cultures, such cultures were established from naive T cells, purified from healthy blood donors, and reactivated in the presence of IL-1β, IL-18, or IL-33. Interestingly, we observe modifying responses in Th1 and Th2 cultures induced by IL-18 or IL-33 but not by IL-1β, both contributing to amplifying production of IL-5, IL-13, and IFN-γ. IL-18 or IL-33 stimulation of Th1 cultures resulted in increased IFN-γ and IL-13 production concurrent with reduced IL-10 gene transcription and secretion even though Th1 cultures, in contrast to IL-18Rα, had low ST2L expression. Furthermore, adding IL-18 to Th1 cultures promoted Tbet mRNA expression and production. Th2 cultures stimulated with IL-18 or IL-33 had an increased IL-5 secretion. Interestingly, E4BP4 gene expression and the percentage of E4BP4+ cells of the recently shown IL-10 transcriptional regulator E4BP4 correlated with IL-10 gene expression and protein had low ST2L expression. Furthermore, adding IL-18 to Th1 cultures promoted Tbet mRNA expression and production. Th2 cultures stimulated with IL-18 or IL-33 had an increased IL-5 secretion. Interestingly, E4BP4 gene expression and the percentage of E4BP4+ cells of the recently shown IL-10 transcriptional regulator E4BP4 correlated with IL-10 gene expression and protein secretion in Th1 cultures. Taken together, we report that the IL-1 family “alarmins” IL-18 and IL-33 in addition to amplifying both Th1- and Th2-associated cytokines block production of the regulatory cytokine IL-10 in Th1 cultures. The Journal of Immunology, 2012, 189: 000–000.
increasing both Th1 and Th2 proinflammatory cytokine production, represses IL-10 gene transcription and secretion in differentiated Th1 cultures. IL-33 or IL-18 stimulations of Th2 cultures promote additional IL-5 secretion. Furthermore, we find that \(E4BP4\) gene expression and the percentage of \(E4BP4^+\) cells correlate with IL-18- and IL-33-mediated downregulation of IL-10 gene transcription and secretion in differentiated Th1 cultures.

Materials and Methods

Cell cultures

Cells used in this study originate from buffy coat fractions of blood portions from anonymized donors, whose identity has been anonymized for the researcher by the Blood Bank at Copenhagen University Hospital (Copenhagen, Denmark).

PBMCs were purified using Lymphoprep (Axis- Shield, Oslo, Norway) density centrifugation. The cells were washed twice with PBS buffer (PBS without Ca\(^{++}\) and Mg\(^{++}\); Invitrogen, Carlsbad, CA) and supplemented with 0.5% (v/v) EDTA (Bie & Berntsen, Rødovre, Denmark) and 0.5% (v/v) human serum albumin (ZLB Behring, Marburg, Germany).

Human naive CD\(^4^+\)CD\(^{45RA^+}\)CD\(^{56^-}\)CD\(^{25^-}\) cells were isolated using the naive CD\(^4^+\) cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. In short, PBMCs were incubated with a mixture of biotinylated Abs (Miltenyi Biotec), followed by labeling with magnetic anti–biotin-coated microbeads for magnetic depletion. The cells were depleted twice using new columns for each depletion and subsequently stained and depleted with the same mixture of biotinylated Abs and anti-biotin microbeads to ensure a purity of \(\geq99\%\) CD\(^4^+\)CD\(^{45RA^+}\)CD\(^{56^-}\)CD\(^{25^-}\) cells.

Naive CD\(^4^+\) T cells (2.5–10 \(\times\) 10\(^3\)) were cultured at 37\(^\circ\)C in 6- or 48-well flat-bottom plates (Nunc, Roskilde, Denmark) in RPMI 1640 (Sigma-Aldrich, Saint Louis, MO) supplemented with 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin, 1 mM L-glutamine (Invitrogen), 50 \(\mu\)g 2-ME (Sigma-Aldrich), and 5% (v/v) human AB serum (Copenhagen University Hospital). The naive CD\(^4^+\) T cells were cultured in media and supplemented with mouse fibroblastic L cells (CRLL-10680; American Type Culture Collection) stably expressing FcyRII/CDw32 (provided by Eva den Haan, Amsterdam, Netherlands) and staphylococcal enterotoxin B, 5 ng/ml PMA, 1 \(\mu\)g/ml mitomycin C (Sigma-Aldrich), or 1 \(\mu\)g/ml plate-bound anti-CD3 (Dako, Glostrup, Denmark).

RNA was purified using a RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. RNA concentrations were measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA synthesis was performed using SuperScript II reverse transcriptase (Invitrogen) and stored at \(-20^\circ\)C. qRT-PCR analysis of mRNA expression was performed using TaqMan Universal PCR Master mix (Applied Biosystems, Carlsbad, CA). The gene expression was determined by using the primers and probes listed in Table 1.

Bar graphs are represented as means \(\pm\) SEM. One- or two-way matched Bonferroni corrected ANOVA\(^s\) were used for statistical analysis; data from qRT-PCR and supernatant were log transformed prior to analysis. Statistical significance is designated as follows: \(*p<0.05\), **\(p<0.01\), ***\(p<0.001\).

Results

IL-18Ra and ST2L expression on differentiated CD\(^4^+\) T cells

IL-18Ra and ST2L are described to be tightly regulated, whereas their coreceptors are constitutively expressed; furthermore, IL-12–mediated expression of IL18R\(^a\) receptor expression has been shown to be Th1 and Th2 restricted, respectively (28). To confirm these findings in our system, we used naive (\(\geq 99\%)\) CD\(^4^+\) T cells differentiated under Th0, Th1, and Th2 culture conditions for a period of 5 d.

The Th1 and Th2 cultures were established at day 5 (Fig. 1A) with high expression of the master transcription factors T-bet and GATA3, respectively. As described by others (21, 25), IL-18Ra gene transcription and the percentage of IL-18Ra\(^+\) cells correlated with a Th1 subtype (Fig. 1B, 1C). Of interest, all differentiated cultures expressed the gene encoding IL-18Ra (Fig. 1C). Unlike IL-12–mediated expression of IL18Ra under Th1 culture conditions, ST2L was weakly induced by IL-4 (Fig. 1B). In contrast to flow cytometry data, ST2L mRNA expression was upregulated under Th1 and Th2 culture conditions (Fig. 1C).

IL-1 family members IL-18 or IL-33 induce an inflammatory phenotype in differentiated Th1 and Th2 cultures

To test whether Th1 and Th2 cultures respond to stimulation by the IL-1 family member IL-1\(\beta\), IL-18, or IL-33, differentiated Th1 and Th2 cultures were further stimulated with none or one of the cytokines for an additional 5 d.
FIGURE 1. Expression of IL-18Rα and ST2L on differentiated CD4+ T cell subsets. Naïve CD4+CD45RA+CD25− T cells were stimulated with fibroblast-bound anti-CD3/CD28 with IL-2 under Th0 (anti-IFN-γ), Th1 (IL-12 and anti-IL-4), and Th2 (IL-4 and anti–IFN-γ) conditions for 5 d. (A) Intracellular flow cytometry analysis of GATA3 and T-bet expression in restimulated Th0, Th1, and Th2 cultures; data are from four independent experiments using cells from two donors (n = 8). (B) Representative data are from one donor showing the geometric means of (from left) IL-18Rα and ST2L in the restimulated Th0 isotype (dotted line) and Th0 (light gray), Th1 (gray), and Th2 (black) cultures. (C) qRT-PCR analysis of ST2L and IL18Rα mRNA expression, illustrated by the cycle threshold value, of differentiated Th0, Th1, and Th2 cultures from four independent experiments using cells from two donors (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001.

Stimulation of Th1 cultures with IL-18 increased the secretion of the Th1 signature cytokine IFN-γ (Fig. 2A). Surprisingly, IFN-γ secretion was also increased in Th1 cultures stimulated with the Th2-associated IL-1 family member IL-33 (Fig. 2A). Furthermore, adding IL-18 or IL-33 to the differentiated Th1 cultures attenuated IL-10 secretion compared with the classical Th1 cultures (Fig. 2A). Overall, 5 d stimulation of the Th1 cultures with IL-18 or IL-33 but not IL-1β induced and repressed the same cytokines, indicating activation of the same signal pathway. Incubating Th2 cultured cells with IL-18 or IL-33 resulted in additional secretion of the Th2 subtype cytokine IL-5 (Fig. 2B). In contrast to the Th1 results, IL-10 secretion was not reduced in the Th2 cultures stimulated with IL-18 or IL-33, although stimulation with IL-33 showed a tendency of reduced IL-10 secretion. Collectively, these data indicate that IL-18 or IL-33 stimulation results in amplification of both Th1 and Th2 subtype cytokines concurrent with repression of IL-10 production in the Th1 cultures. In contrast, stimulation of Th1 and Th2 cultures with IL-1β did not increase production of Th1 and Th2 subtype-associated cytokines, and IL-1β was omitted in the following experiments.

To address the kinetics of the IL-18– and IL-33–mediated cytokine regulation, 5 d differentiated Th1 and Th2 cultures were incubated with none, IL-18, or IL-33 during a period of up to 5 additional days (Fig. 3).

Th1 cultures stimulated with IL-18 resulted in markedly divergent responses regarding IFN-γ and IL-10 secretion (Fig. 3A, 3C). The same pattern, even though delayed, was observed by stimulation of Th1 cultures with IL-33 (Fig. 3A, 3C). To address whether the observed delay (Fig. 3C) was a result of different biological activity of IL-18 and IL-33, Th1 cultures were stimulated with increasing concentrations of IL-18 or IL-33 (Supplemental Fig. 1). The inhibition capacity of IL-10 secretion of Th1 cultures of IL-18 or IL-33 was positively correlated with increasing protein concentrations, and IL-18 was shown to be 2- to 3-fold more biologically active than IL-33 (Supplemental Fig. 1). As for IL-18– or IL-33–mediated induction of IFN-γ in Th1 cultures, IL-5 secretion was promoted by both IL-1 family members in Th2 cultures after 24 h (Fig. 3E). In Th2 cultures IL-10 secretion was weakly repressed and seen after 120 h incubation with IL-33 or IL-18 (Fig. 3G). Analyzing IL10 mRNA expression showed IL-18– and IL-33–mediated inhibition in Th1 cultures and no change in the Th2 cultures (Fig. 3D, 3H). IL-13 secretion followed the same kinetics as IL-5 and was induced early by IL-18 stimulation in both Th1 and Th2 cultures (Fig. 3B, 3F). Representative flow cytometry plots after 24 h stimulation (Supplemental Fig. 2) showed IL-18– or IL-33–mediated induction of IL-13 mainly within the T-bet+ or GATA3+ population in the Th1 or Th2 cultures, respectively. Additionally, IL-18– or IL-33-medi-
IL-18 AND IL-33 POTENTATE Th1 AND Th2 CELL RESPONSES

FIGURE 3. IL-1 family members IL-18 and IL-33 induce an inflammatory phenotype in both differentiated Th1 and Th2 cultures. Naive CD4+ T cells were stimulated with anti-CD3/CD28 under classical Th1 (IL-12 and anti–IL-4) and Th2 (IL-4 and anti–IFN-γ) conditions for 5 d and restimulated for indicated periods with none, IL-18, or IL-33. (A–C) Relative secretion compared with Th1 of IFN-γ (A), IL-13 (B), and IL-10 (C) after stimulation of Th1 cultures with IL-18 (red) or IL-33 (blue) for indicated periods. (D and H) IL10 mRNA expression in Th1 (D) and Th2 (H) cultures with IL-18 (red) or IL-33 (blue) relative to the Th1 (D) or Th2 (H) cultures. Data are from two independent experiments using cells from two donors (n = 4). (E–G) Relative secretion of IL-5 (E), IL-13 (F), and IL-10 (G) compared with the classical Th2 culture after stimulation of Th2 cultures with IL-18 (red) or IL-33 (blue) for indicated periods. (A–C and E–G) Representative data with three (12, 24, 48, and 72 h) or five (0 and 120 h) independent experiments using cells from two donors (n = 6 or 10). *p < 0.05, **p < 0.01, ***p < 0.001.

Discussion

Recently, it has been shown that innate tissue cells can release alarmins with the ability to modulate homed effector CD4+ T cells to produce a diverse range of cytokines, all being important to eradicate encountered pathogens (30). In this study, using in vitro-differentiated human Th1 and Th2 cell cultures, we demonstrate that stimulation with the IL-1 family alarmins IL-18 or IL-33 results in amplification of powerful inflammatory cytokines as well as in Th1 cultures blocking production of the regulatory cytokine IL-10. It has been shown in vitro and in vivo that IL-10

ated induction of IL-13 in the Th1 cultures was mainly coproduced with IFN-γ (data not shown).

IL-18 increases frequency of CD4+T-bet+ and reduces the level of CD4+GATA3+ in Th1 cultures

We then asked whether induction of the master transcription factor for Th1 and Th2 differentiation of GATA3 and T-bet, respectively, was associated with the IL-18– and IL-33–dependent cytokine induction and repression. Five-day-primed Th1 and Th2 cultures were incubated with none, IL-18, or IL-33 for the indicated periods (Fig. 4). The frequency of CD4+T-bet+ cells was additionally increased in the period from 12 to 48 h simultaneously with early reduced percentage of CD4+GATA3+ cells in response to the addition of IL-18 to Th1 cultures (Fig. 4). Although the percentage of CD4+GATA3+ cells increased after restimulation of the Th2 cultures, IL-18 or IL-33 stimulation did not increase CD4+GATA3+ cells (Fig. 4B).

The positive feedback effects of IL-18 or IL-33, in relationship to inducing IL-18Rα or ST2L to the culture conditions (Supplemental Fig. 4). The stimulating capacity of IL-18 was partially blocked by Abs against IL-18Rα (Supplemental Fig. 4). However, adding ST2L Abs did not reduce the IL-33–mediated inhibition of IL-10 secretion (Supplemental Fig. 4).

IL-10 suppression correlates with E4BP4 expression in Th1

We finally wanted to examine whether transcription factor E4BP4 expression correlated with IL-10 production. Five-day-primed Th1 and Th2 cultures were incubated with none, IL-18, or IL-33 for the indicated periods (Fig. 5).

Culturing naive CD4+ T cells under Th2 culture conditions for 5 d resulted in higher E4BP4 gene expression than that seen for the Th1-cultured cells (Fig. 5A). This is in agreement with earlier studies describing IL-10 as a Th2 subtype cytokine (4). We found that IL-18– or IL-33–mediated inhibited IL-10 secretion was closely associated with E4BP4 gene transcription and the percentage of CD4+T-bet+E4BP4+ cells in Th1 cultures (Fig. 5B, 5C). In addition to being involved in IL-10 gene regulation, E4BP4 has been associated with both increased and decreased IL-13 production (8, 29). However, we observed no relationship between IL-13 production and E4BP4 expression in Th1 cultures stimulated with IL-18 or IL-33 (Figs. 2B, 5C).

To determine whether we could block the action of IL-18 or IL-33, we added commercially available blocking Abs against IL-18Rα or ST2L to the culture conditions (Supplemental Fig. 4). The stimulating capacity of IL-18 was partially blocked by Abs against IL-18Rα (Supplemental Fig. 4). However, adding ST2L Abs did not reduce the IL-33–mediated inhibition of IL-10 secretion (Supplemental Fig. 4).
can be coproduced by effector Th subsets after multiple restimulations, indicating induction of tolerance, which is exemplified by coproduction of IL-10 in specific Th1 and Th2 cells after continuous exposure of high-dose bee venom (31). If our results can be reproduced in vivo, inhibition of IL-10 production concurrently with amplification of CD4+ T cell-produced cytokines would indicate an important mechanism of the innate cells to switch a response from tolerance toward eradicating encountered pathogens. In the case of IL-33, it has been shown to be important in promoting Th2-oriented immunity, and together with it is the release by necrotic tissue cells, fitting with a mechanism to combat chronic parasite infections by modulating the local CD4+ T cells to increase cytokine production (20, 32, 33). Therefore, dysfunctional regulation of IL-18 or IL-33 could contribute to development of chronic inflammations.

We observe heterogeneity in responses of Th1 and Th2 cultures induced by IL-18 or IL-33, both contributing to amplify production of Th1- and Th2-type cytokines, in contrast to IL-1β. IL-1β has been associated with development of Th17 cells, explaining why stimulations of Th1 and Th2 cultures did not induce secretion of the investigated Th1 and Th2 cytokines (10). Interestingly, Zielinski et al. (34) recently identified IL-1β as essential in induction of human proinflammatory Th17 cells characterized by inhibited IL-10 production.

In relationship to IL-13, we found a moderate increase in its secretion in Th1 cultures stimulated with IL-18. Nakanishi et al. (35) described that stimulation of Th1 cells with IL-18 induces so-called “super” Th1 cells coproducing IFN-γ and IL-13, and these cells are involved in the development of an allergic inflammation. Moreover, other studies have shown that chronically activated Th1 cells start to coproduce IL-10 and IL-13 under the control of E4BP4, indicating that IL-10 is produced by Th1 cells as a results of negative feedback (8). Several publications support our observations of heterogenic IL-18-/IL-33-induced cytokine production of CD4+ T cell subsets; for example, in the absence of IL-12, IL-18 administration contributed to the development of a spontaneous atopic dermatitis-like phenotype, as well as increasing circulating IL-4, IL-13, and IgE, in a mouse model (36, 37). Additionally, neutralizing IL-18 signaling in an IL-18–deficient mouse model diminished allergen-induced chronic inflammation (38). Furthermore, it has been shown that Th2-skewed human CD4+ T cells produce IFN-γ in response to activation of IL-33 and Ag (9). Our Th2 cultures have added anti–IFN-γ, making us unable to detect any potentially upregulated IFN-γ production.

It has been reported that IL-18Rα and ST2L are selectively upregulated and expressed by Th1 and Th2 cells, respectively (21–25). We confirm the association of Th1 cells and high IL-18Rα expression, but we were unable to reproduce that Th2 cells have

FIGURE 4. IL-18 increases frequency of CD4+ T-bet+ and reduces the level of CD4+ GATA3+ in Th1 cultures. Naive CD4+ T cells were stimulated with anti-CD3/CD28 under classical Th1 (IL-12 and anti–IL-4) and Th2 (IL-4 and anti–IFN-γ) conditions for 5 d and restimulated for indicated periods with none, IL-18, or IL-33. (A and B) Flow cytometry analysis of frequencies of T-bet (A) and GATA3 (B) cells in Th1 or Th2 cultures for indicated periods with none, IL-18, or IL-33. Data are from three independent experiments using cells from two donors (n = 6). *p < 0.05, **p < 0.01.

FIGURE 5. IL-10 suppression correlates with E4BP4 expression. Naive CD4+ T cells were stimulated with anti-CD3/CD28 under classical Th0 (anti–IFN-γ), Th1 (IL-12 and anti–IL-4), and Th2 (IL-4 and anti–IFN-γ) conditions for 5 d and restimulated for indicated periods with none, IL-18, or IL-33. (A) E4BP4 mRNA expression at day 5 relative to Th0. (B) E4BP4 mRNA expression in Th1 cultures with IL-18 or IL-33 relative to the Th1 cultures at the indicated time point. (C) Flow cytometry analysis of frequencies E4BP4 cells in Th1 cultures with IL-18 or IL-33 relative to the Th1 cultures at the indicated time point. Data are from one (C), two (B), or five (A) independent experiments using cells from two donors (n = 2, 4, or 10). *p < 0.05, ***p < 0.001.
increased ST2L expression. This can be explained, however, by the PMA restimulation used prior to harvest, as PMA restimulation increased ST2L expression. This can be explained, however, by the PMA restimulation used prior to harvest, as PMA restimulation increased ST2L expression. This can be explained, however, by the PMA restimulation used prior to harvest, as PMA restimulation increased ST2L expression. This can be explained, however, by the PMA restimulation used prior to harvest, as PMA restimulation increased ST2L expression. This can be explained, however, by the PMA restimulation used prior to harvest, as PMA restimulation increased ST2L expression. This can be explained, however, by the PMA restimulation used prior to harvest, as PMA restimulation increased ST2L expression. This can be explained, however, by the PMA restimulation used prior to harvest, as PMA restimulation increased ST2L expression. This can be explained, however, by the PMA restimulation used prior to harvest, as PMA restimulation increased ST2L expression.


Supplementary Figure 1. The concentration of IL-18 or IL-33 was negatively associated to IL-10 secretion by the Th1 cultures. Naïve CD4+ T cells were stimulated with anti-CD3/CD28 under classical Th1 conditions, with IL-12 and anti-IL-4, for 5 days and restimulated for indicated periods with none, 0.1, 1, 3, 10, 30, and 100 ng/ml IL-18 or IL-33. (A and B) Relative secretion compared with the classical Th1 culture of IL-10, after stimulated of Th1 cultures with increasing concentrations of IL-18 (A) or IL-33 (B) for indicated periods. (A and B) Representative data of one experiment with two donors (n=2).
Supplementary Figure 2. IL-18 or IL-33 induced IL-13 production in the Th1 and Th2 cultures were mainly within cells positive for the phenotype specific transcription factor. Naïve CD4⁺CD45RA⁺CD25⁻ T cells were stimulated with fibroblast-bound anti-CD3/CD28 with IL-2 under Th1 and Th2 conditions; IL-12 and anti-IL-4 or IL-4, and anti-IFN-γ for 5 days. (A and B) Intracellular flow cytometry analysis of IL-13 and Tbet or GATA3, respectively, in the left and right column stimulated with none (top), IL-18(center), and IL-33 (bottom) in restimulated Th1 (A) and Th2 cultures (B). Data are representative of one independent experiment using cells from two donors (n=2).
Supplementary Figure 3. IL-18 and IL-33 stimulation of Th2 cultures effect regulation of \textit{ST2L} mRNA expression. Naïve CD4+ T cells were stimulated with anti-CD3/CD28 under classical Th1 and Th2 conditions, with IL-12 and anti-IL-4 or IL-4, anti-IFN-\(\gamma\), respectively, for 5 days and restimulated for indicated periods with none, IL-18, or IL-33. (A and B) Flow cytometry analysis of IL-18R\(\alpha\) (A) and ST2L (B) Geo Mean in Th1 or Th2 cultures after stimulation with none, IL-18 or IL-33 for 0, 12, 24, 48, 72, and 120 h and restimulation with PMA and ionomycin for 6 h in the presence of Bref A for the last 4 h. (C and D) qRT-PCR \textit{IL18R}\(\alpha\) mRNA (C) or \textit{ST2L} mRNA expression in Th2 (D) cultures with IL-18 or IL-33 relative to the Th2 cultures at the indicated time points. (A-D) Data are from two independent experiments each using cells from each two donors (n=4). Statistical significance is designated as follows: *\(p < 0.05\); **\(p < 0.01\).
**Supplementary Figure 4.** Partial inhibited stimulating capacity of IL-18 but not IL-33 after co-culture with control antibodies or against IL-18Rα and ST2L. Naïve CD4+ T cells were stimulated with anti-CD3/CD28 under classical Th1 conditions, with IL-12 and anti-IL-4, for 5 days and restimulated for indicated periods with 10 ng/ml IL-18 or IL-33 in combination with either none, anti-IgG1, anti-IL-18Rα or anti-ST2L. Relative secretion compared with Th1 of IL-10. Data from a single experiment using cells from two donors (n=2).