IL-18 together with anti-CD3 antibody induces human Th1 cells to produce Th1- and Th2-cytokines and IL-8

Hitomi Hata1, Tomohiro Yoshimoto2,3, Nobuki Hayashi2,3, Toshikazu Hada1 and Kenji Nakanishi2,3

1Department of Internal Medicine and 2Department of Immunology and Medical Zoology, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan
3Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan

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Abstract

Although IL-18 was initially regarded as a factor that enhances IFN-γ production from Th1 cells, later studies revealed its potential to induce Th2 cytokine production from T cells, NK cells and basophils/mast cells. Very recently, we demonstrated that passively transferred memory phenotype Th1 cells induce airway inflammation and hyperresponsiveness in a host mouse by production of Th1-, Th2-cytokines, GM-CSF and chemokines, when the transferred cells are stimulated in the host mice with nasally administered Ag and IL-18. Moreover, IL-18 is suggested to contribute to asthma exacerbation in human patients. Therefore, it is important to determine whether human Th1 cells also have the potential to produce these soluble factors when stimulated with anti-CD3 and IL-18 in vitro. Here we demonstrated that only Th1 cells, but not Th2 cells, produce IFN-γ, IL-13, GM-CSF and IL-8 after stimulation with anti-CD3 and IL-18. Furthermore, highly purified IFN-γ-producing Th1 cells have the same potential. Thus, human Th1 cells may become very harmful cells, when stimulated with Ag and IL-18 in vivo, and produce IFN-γ, IL-13, GM-CSF and IL-8, which in combination might induce severe inflammation such as airway inflammation.

Introduction

Th1-dominant immune responses have been generally considered pathological in autoimmune disease but protective against Th2-related diseases (1,2). However, recent studies revealed that Th1 cells rather augment Th2 cell-mediated airway hyperresponsiveness (AHR) (3–7). Indeed, nasal administration of IFN-γ and IL-13 induces severe bronchial asthma in mice (8). Furthermore, activated Th1 cells by themselves induce AHR and airway inflammation by recruitment and activation of neutrophils (9). In fact, airways of asthmatic patients, particularly those with long history of bronchial asthma, often show co-presence of Th1 and Th2 cells and increased neutrophil numbers and IFN-γ, TNFα and IL-8 levels (10–12). Nevertheless, we still do not understand the mechanism of how Th1 cell express pathological effect on the lung. Therefore, it is important to determine the pathological relevance of Th1 cells in bronchial asthma.

IL-18 was originally identified as a factor that enhances IFN-γ production by Th1 cells in the presence of anti-CD3 antibody and IL-12 (13–15). Therefore, injection of a mixture of IL-12 and IL-18 inhibits Th2-induced IgE response by induction of IFN-γ-producing cells in vivo (16). However, our very recent study has demonstrated that passively transferred freshly prepared Ag-specific Th1 cells or Th2 cells become adapted as a resting memory phenotype in naive host mice and induce airway inflammation and AHR, when they are stimulated with nasally administered Ag and IL-18 in vitro. In contrast, Ag-challenged Th1 cell-transferred mice lack in AHR, although they show moderate peribronchial inflammation mainly consisting of neutrophils and lymphocytes. Thus, Ag challenge only induces delayed-type hypersensitivity (DTH) reaction in the lung, while Ag plus IL-18 challenge stimulates Th1 cells to produce cytokines and chemokines that induce dense airway inflammation and AHR. It is widely accepted that IL-9, IL-13 and GM-CSF are principally responsible for inducing bronchial asthma (18–22). Indeed, Ag plus IL-18-stimulated Th1 cells, capable of producing Th1 cytokine (IFN-γ), Th2 cytokine (IL-9, IL-13), chemokine (RANTES, macrophage inflammatory protein-1α) and GM-CSF (17), induce bronchial asthma by production of both Th1- and Th2-cytokines. Local neutralization of IL-13 inhibits
eosinophilic infiltration and AHR in Th2 cell-transferred mice (17). In contrast, same treatment cannot inhibit AHR in Th1 cell-transferred mice, even though this treatment markedly reduces eosinophilic infiltration in the airway (17). These results suggest that Th1 cells induce AHR in an entirely different manner from Th2 cells.

Serum IL-18 levels were reported to be increased in patients with acute bronchial asthma (23). Furthermore, IL-18 levels during acute asthma exacerbation were significantly higher than on remission days. Therefore, it is highly probable that Th1 cells which when stimulated with Ag and IL-18 produce Th1- and Th2-cytokines, GM-CSF and chemokines, which in combination induce AHR and airway inflammation in the patients. This type of asthma might be classified as Th1 asthma. Thus, it is very important to determine whether human Th1 cells, like mouse Th1 cells, also have the potential to become 'highly pathological cells' under Ag plus IL-18 stimulation condition.

Here, we have demonstrated that newly polarized human Th1 cells and highly purified IFN-γ-producing Th1 cells, both of which strongly express IL-18Rα chain, markedly increase their production of IFN-γ, IL-13, GM-CSF and IL-8 upon stimulation with anti-CD3 and IL-18. These results suggest that Th1 cell might have the potential to induce tissue injuries by production of both Th1- and Th2-cytokines as well as GM-CSF and IL-8, when they encounter Ag plus IL-18 in the organs, such as lung.

Methods

Reagents

Recombinant human IL-2, IL-4, IL-12 and IFN-γ were purchased from R&D (Minneapolis, MN). Recombinant human IL-18 was purchased from MBL Co. (Nagoya, Japan). FITC- or CyChrome–anti-human CD4 mAb, FITC–anti-human CD45RA mAb, FITC–anti-human IFN-γ mAb, PE–anti-human IL-13 mAb and anti-human IL-12 mAb were obtained from Pharmingen (San Diego, CA). PE–anti-human IL-18Rα mAb (Clone 70625), anti-human CD3c mAb, anti-human IL-4 mAb and anti-human IFN-γ mAb were purchased from R&D.

Generation of Th1 or Th2 cells in vitro

Naive CD4+CD45RA+ T cells from peripheral blood of healthy donors were isolated as described in our previous report (24). Th1 and Th2 cells were generated by culturing CD4+CD45RA+ T cells (1 × 10^6/ml) with PHA (1 μg/ml), IL-12 (50 ng/ml) and neutralizing anti-IL-4 mAb (500 ng/ml) or PHA (1 μg/ml), IL-4 (200 ng/ml) and neutralizing anti-IL-12 mAb (10 μg/ml) in 24-well plate in a total 1 ml volume of RPMI 1640 supplemented with 10% FCS, 2-mercaptopethanol (50 μM), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (50 μM), respectively. These stimulated T cells were washed on day 3 and expanded in the culture medium containing 100 U/ml of IL-2 for an additional 4 days.

Isolation of IFN-γ+ Th1 cell

For isolation of IFN-γ+ Th1 cells, polarized Th1 cells were recultured with immobilized anti-CD3 (5 μg/ml for coating) and IL-2 (100 U/ml) in 24-well plate. We followed the manufacturer’s recommended procedure to enrich IFN-γ-expressing living Th1 cells. After 3 h, only adherent cells were collected and incubated with the anti-CD45/anti-IFN-γ bispecific antibody-capture matrix (Miltenyi Biotec) for 5 min on ice. The treated cells were transferred to 50-ml conical tubes and cultured at a concentration of 5 × 10^4 cells/ml in 20 ml of warmed medium and placed in a 37°C water bath. After 30 min, cells were washed with cold 0.5% bovine serum albumin (BSA) in PBS. Matrix-captured IFN-γ was detected with PE–anti-human IFN-γ and was positively isolated with anti-PE microbeads by using auto MACS (Miltenyi Biotec).

In vitro culture

Freshly polarized Th1 and Th2 cells, and sorted IFN-γ+ cells from newly polarized Th1 cells were recultured at 1 × 10^5/0.2 ml/well with immobilized anti-CD3 (5 μg/ml) in the presence of various concentrations of IL-18 (~500 ng/ml). After 6–72 h of culture, supernatants were harvested and tested for IL-4, IL-5, IL-8, IL-13, IFN-γ and GM-CSF contents by ELISA (R&D). In some experiments, Th1 cells were stimulated with immobilized anti-CD3 and IL-18 in the presence or absence of anti-human IFN-γ.

Flow cytometry

Polarized Th1 cells (1 × 10^6/ml) were restimulated in 24-well plates with immobilized anti-CD3 with or without IL-18 (100 ng/ml) for 72 h with a pulse of 2 μM monensin during the final 3 h to inhibit cytokine secretion. The analysis of intracellular IFN-γ+ and/or IL-13+ staining was performed as described in our previous reports (17). For determination of IL-18Rα chain expression on Th1 and Th2 cells, after FcγR blocking with human IgG, cells were incubated with FITC–anti-human CD4 and PE–anti-human IL-18Rα chain mAb or control PE-mouse IgG1 mAb for 30 min at 4°C in PBS containing 1% FCS. Samples were analyzed on a FACS Calibur (BD Bioscience, San Jose, CA).

Results

IL-18 induced IFN-γ, IL-8, IL-13 and GM-CSF production from polarized human Th1 cells

Naive human CD4+CD45RA+ T cells isolated from peripheral blood of healthy donors were primed in vitro under Th1- or Th2-inducing conditions for consecutive 7 days stimulation. Upon challenge with immobilized anti-CD3, Th2 cells produced significant amounts of IL-4, IL-5 and IL-13 but not IFN-γ, and Th1 cells predominantly produced IFN-γ, IL-8 and GM-CSF (Fig. 1A). Additional IL-18 stimulation failed to enhance Th2 cytokine production from Th2 cells. In contrast, this treatment markedly enhanced production of IFN-γ, IL-8, IL-13 and GM-CSF from Th1 cells (Fig. 1A). The mechanism underlying this difference in IL-18 responsiveness between Th1 and Th2 cells is principally explained by preferential expression of IL-18Rα chain on Th1 cells (24,25). As illustrated in Fig. 1(B), human Th1 cells express high level of IL-18Rα chain, while naive human CD4+ T cell and Th2 cells express this Ag meagerly. Thus, IL-18R expressing human Th1 cells showed the
capacity to produce Th1- and Th2-cytokines, IL-8 and GM-CSF in response to anti-CD3 plus IL-18.

Because IL-12 is known to induce IFN-γ production from Th1 cells but inhibit IL-4 production from Th2 cells in vivo and in vitro (25,26), it is important to test the effect of IL-12 on IL-13 production from Th1 cells. We found that IL-12 augments IFN-γ production from anti-CD3-stimulated Th1 cells but decreases their IL-13 production (data not shown). We also found IL-12 diminishes the production of IL-13 from anti-CD3 plus IL-18-stimulated Th1 cells (data not shown). Thus, IL-12 as well as IL-18 enhances IFN-γ production from anti-CD3-stimulated Th1 cells; however, only IL-18 stimulation induces IL-13 production from Th1 cells.

We simultaneously examined IL-18-dose responsiveness of anti-CD3-stimulated Th1 cells in terms of their production of IFN-γ, IL-8 and IL-13. Thus, we stimulated Th1 cells with various doses of IL-18 (~500 ng/ml) in the presence of immobilized anti-CD3 for 3 days. As illustrated in Fig. 2, Th1 cells dose-dependently increased IFN-γ, IL-8 and IL-13 production in response to IL-18. Again, additional stimulation of anti-CD3-stimulated Th2 cells with high doses of IL-18 failed to enhance their cytokine production (data not shown). Therefore, only human Th1 cells have unique potential to produce both Th1- and Th2-cytokines as well as IL-8 in response to Ag plus IL-18 (Fig. 2).

**Kinetics of IL-18 induced IFN-γ, IL-8 and IL-13 production from Th1 cells**

We next performed kinetic study of the production of these cytokines after stimulation with immobilized anti-CD3 alone or after co-stimulation with anti-CD3 plus IL-18. As illustrated in

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**Fig. 1.** Anti-CD3 plus IL-18-stimulated human Th1 cells but not Th2 cells produced IFN-γ, IL-13, GM-CSF and IL-8. (A) CD4⁺CD45RA⁺ human T cells were cultured under Th1-inducing condition, PHA (1 μg/ml), IL-12 (50 ng/ml) and anti-IL-4 mAb (500 ng/ml) or Th2-inducing condition, PHA (1 μg/ml), IL-4 (200 ng/ml) and anti-IL-12 mAb (10 μg/ml). Cells were washed on day 3 and expanded in the culture medium containing 100 U/ml of IL-2 for an additional 4 days. These primed T cells (1 × 10⁶/0.2 ml/well) were further stimulated with medium alone or IL-18 (100 ng/ml) in the presence of immobilized anti-CD3 in 96-well plate for 72h. Supernatants were harvested and measured for IL-4, IL-5, IL-8, IL-13, IFN-γ and GM-CSF contents by ELISA. Data presented are means ± SD of triplicate cultures. Data presented are representative of four independent experiments with similar results. (B) Surface expression of IL-18Rα chain on human naive CD4⁺ T cells Th1 and Th2 cells by flowcytometry. The percentages shown represent the proportion of IL-18Rα chain positive cells among CD4⁺ cells.
Fig. 3. Human Th1 cells produced IFN-γ first and IL-8 as well as IL-13 later in response to anti-CD3 plus IL-18. Polarized human Th1 cells (1 × 10^5/0.2 ml/well), as described in the legend to Fig. 1, were further stimulated with medium alone or IL-18 (100 ng/ml) in the presence of immobilized anti-CD3 for 6–72h. Supernatants were harvested and measured for IL-8, IL-13 and IFN-γ contents by ELISA. Data presented are means ± SD of triplicate cultures. Data presented are representative of four independent experiments with similar results.
in Fig. 5(A), 23.1% of Th1 cells expressed IFN-γ on their cell surfaces, when Th1 cells stimulated with anti-CD3 for 3 h were examined by the affinity matrix technology, which allows the detection of IFN-γ production from living cells. We then positively purified IFN-γ-producing living IFN-γ+CD4+ Th1 cells (98.9%) by auto MACS (Fig. 5A). Culturing of resultant IFN-γ+ Th1 cells with anti-CD3 and IL-18 substantially increased their production of IFN-γ, IL-8 and IL-13 but not IL-4 (Fig. 5B). Thus, we could formally conclude that highly purified IFN-γ-expressing living human Th1 cells, which are strongly positive for IL-18Rα chain, produced IFN-γ, IL-8 and IL-13 upon stimulation with anti-CD3 and IL-18. These results taken together indicate that human Th1 cells produce both Th1 cytokines (e.g. IFN-γ) and Th2 cytokines (e.g. IL-13) as well as GM-CSF and chemokines (e.g. IL-8) in response to Ag plus IL-18.

Discussion

Th1 cells are originally regarded to produce only Th1 cytokines such as IFN-γ when stimulated with anti-CD3 or TCR engagement. Since they promptly produce IFN-γ following stimulation, their property to produce IL-13 at later time might have been ignored. However, murine Th1 cells have the unique potential to produce both Th1- and Th2-cytokines as well as GM-CSF and chemokines when they are stimulated with Ag plus IL-18 (17). Furthermore, passively transferred freshly prepared Th1 cells become memory phenotype and, upon in vivo challenge with Ag plus IL-18, become very harmful cells that induce airway inflammation and AHR by production of both Th1- and Th2-cytokines as well as GM-CSF and chemokines in the host mouse (17). This IL-18/Ag-challenged-Th1 cell-induced AHR is resistant to the treatment with soluble IL-13Rα2-human Fc fusion protein (IL-13Rα2-Fc), which selectively binds to and neutralizes IL-13 in vivo (17). In contrast, Ag-challenged-Th2 cell-induced AHR is completely blocked by the treatment with IL-13Rα2-Fc. Based on these observations, we concluded that IL-18/Ag-induced bronchial asthma is a kind of Th1 asthma, in which both IFN-γ and IL-13 are actively produced (17). Indeed, administration of a mixture of IFN-γ and IL-13 was shown to induce most severe bronchial asthma (8). Therefore, it is very important to show that human Th1 cells, like mouse Th1 cells, have the capacity to produce both IFN-γ and IL-13. Here we showed that Th1 cells produce IFN-γ first and IL-13 and IL-8 later in response to stimulation with anti-CD3 in vitro (Fig. 3). We also showed that additional IL-18 stimulation not only shortened the time required for induction of IL-8 and IL-13 production but also markedly augmented production of these cytokines (Fig. 3). Furthermore, we demonstrated these IL-18-induced IL-8 and IL-13...
IL-18 production from IL-18-stimulated human Th1 cells

productions are IFN-γ-independent, because we could exclude the possibility that IL-18 indirectly induces the expression of IL-8 and IL-13 via induction of endogenous IFN-γ from Th1 cells (data not shown). This is the first report demonstrating that human Th1 cells can produce both Th1- and Th2-cytokines as well as IL-8 and GM-CSF upon stimulation with anti-CD3 and IL-18.

Although Th2 cells are often found in the lungs of asthmatic patients, concomitant presence of Th1 cells has also been reported (10,11). These results have been interpreted to indicate that Th1 cells protect against Th2 cells (1,2). Later studies, however, indicated that Th1 cells rather augment bronchial asthma instead of antagonizing (3–9). Therefore, it is eagerly needed to elucidate the relevant role of Th1 cells in regulation of airway inflammation and AHR. Our very recent study clearly revealed that IL-18 acts on memory Th1 cells to induce airway inflammation and AHR in a naive host mouse (17). Ag-stimulated Th1 cells only produce Th1 cytokines and induce DTH in the lung, while Ag plus IL-18-stimulated Th1 cells produce both Th1 and Th2 cytokines and induce very severe and pathological DTH, leading to induction of bronchial asthma characterized by airway inflammation associated with dense neutrophil infiltration and substantial eosinophilic infiltration (17). In contrast, Th2 cells produce only Th2 cytokines and never produce IFN-γ and never increase Th1 cytokine when stimulated with Ag plus IL-18 (17). Thus, only Ag plus IL-18-stimulated human Th1 cells produce both Th1 and Th2 cytokines, suggesting their causative role in severe tissue injuries. As patients with acute bronchial asthma increased IL-18 levels during acute asthma exacerbation, it is intriguing to speculate that human Th1 cells may develop into harmful cells that induce airway inflammation and AHR when they are stimulated in the lungs with Ag and IL-18.

We showed that human Th2 cells produced IL-13 (7 ng/ml) upon challenge with anti-CD3 stimulation in vitro (Fig. 1). We also demonstrated that human Th1 cells produced a comparable level of IL-13 (4.5 ng/ml) in response to anti-CD3 plus IL-18 (Fig. 1). However, compared to the level of IL-13 (70 ng/ml) produced by Ag-specific mouse cloned Th1 or Th2 cells upon challenge with Ag plus IL-18 or with Ag alone in vitro, respectively (17), human Th1 or Th2 cells produced a significantly lower level of IL-13. There are several possibilities that account for this discrepancy. First, we used polyclonal activators (PHA, anti-CD3) to stimulate human T cells, while Ag-specific mouse T cells form OVA-TCR transgenic mice are stimulated with same Ag. Second, perhaps most importantly, in our previous report (17) we induced memory response of mouse cloned Th1 cell, while in this report we used anti-CD3 antibody instead of Ag to induce human T cell response. Thus, we suspect that Ag-specific human Th1 cells might have the potential to produce larger amounts of IL-13 when stimulated with same Ag and IL-18 and that such T cells might induce bronchial asthma in vivo.

Ag plus IL-18-stimulated Th1 cells also produced MIP-α, which is regarded as chemokine for neutrophils (27). Taking the advantage of human immune system in which IL-8 is an authentic chemotactrant for neutrophils (27,28), we measured IL-8 levels in the culture supernatants of Th1 cells stimulated with Ag and/or IL-18. Here, we could reveal that human Th1 cells also have the potential to produce IL-8 in response to Ag plus IL-18 (Figs 1–3). Thus, Th1 cells induce recruitment and activation not only of neutrophils by production of IL-8, GM-CSF and IFN-γ, but also of eosinophils by production of IL-13. In line with this, the increased neutrophil numbers and IL-8 levels in airway secretions is linked to the course of severe asthma (12). IL-18-stimulated Th1 cells also produce IL-13, which has been shown to induce smooth muscle constriction of bronchi (29). Therefore, Ag plus IL-18-stimulated human Th1 cells might be highly pathological cells that might worsen airway inflammation and AHR by production of both Th1- and Th2-cytokines.

Bronchial asthma is often triggered by viral or bacterial airway infection (30). As IL-18 is stored in airway epithelial cells (31) and is released from them upon stimulation of the toll like receptor (TLR)-mediated pathways (15,32), we could assume the possibility that some types of infectious agents might stimulate bronchial epithelial cells to produce IL-18, which may stimulate Th1 cells to induce severe airway inflammation and AHR. Indeed, both mouse and human bronchial epithelial cells stimulated with bacterial products, such as Staphylococcus aureus peptidoglycan (PGN) or Salmonella minnesota lipopolysaccharide (LPS), produce IL-18 (unpublished observation). Furthermore, patients with acute bronchial asthma express high levels of IL-18 in their serum (23). It was recently demonstrated that human airway epithelial cells constitutively express TLR4 complex and transduce signal after stimulation with LPS (33). Therefore, it is important to consider the possibility that IL-18 released from airway epithelial cells following infections might be a therapeutic target for the treatment of Th1 cell-induced airway inflammation and AHR. Furthermore, IL-18 induction of Th2 cytokines in Th1 cells might provide new insights into the establishment of treatment for bronchial asthma, particularly in severe asthma cases that usually contain both Th1 and Th2 cells in the lungs.

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>AHR</td>
<td>airway hyperresponsiveness</td>
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<tr>
<td>DTH</td>
<td>delayed-type hypersensitivity</td>
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<tr>
<td>IL-13Rα2-Fc</td>
<td>IL-13Rα2-human Fc fusion protein</td>
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<tr>
<td>TLR</td>
<td>toll like receptor</td>
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References


