Th1 Responses Are More Susceptible to Infliximab-mediated Immunosuppression than Th17 Responses

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Th17 Responses

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Abstract

Background Treatment with infliximab, a chimeric anti-tumor necrosis factor (TNF)- α antibody, is highly efficient in patients with inflammatory bowel disease (IBD). It neutralizes soluble TNF- α and induces apoptosis of transmembrane TNF- α -positive macrophages and T cells in the gut. Recently, T helper (Th) 17, as well as Th1, responses have been implicated in the pathogenesis of IBD.

Aims To clarify the effects of infliximab on Th1 and Th17 responses *in vitro*.

Methods Naive CD4⁺ T cells isolated from peripheral blood of healthy volunteers were stimulated under Th1- or Th17-inducing conditions in the presence of 10 μ g/ml of infliximab or control immunoglobulin G1 (IgG1). The concentrations of interferon (IFN)- γ , interleukin (IL)-17 and TNF- α in the culture supernatants were determined by enzyme-linked immunosorbent assays. Th1 and Th17 cells were immunostained with infliximab or control IgG1 and transmembrane TNF- α -positive cells were analyzed by flow cytometry. Annexin V staining and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays were conducted to analyze the percentages of apoptotic cells.

Results Both Th1 and Th17 cells expressed soluble and transmembrane TNF- α at comparable levels. Although infliximab suppressed both IFN- γ secretion by Th1 cells and IL-17 secretion by Th17 cells, the level of suppression of IFN- γ production was more profound than that of IL-17 production. Infliximab increased annexin V- and TUNEL-positive apoptotic cells under

Th1-inducing conditions but not under Th17-inducing conditions.

Conclusions Infliximab suppressed Th1 and Th17 differentiation *in vitro*, probably through different mechanisms. Th1 responses are more susceptible to infliximab-mediated immunosuppression than Th17 responses.

Keywords: Infliximab, Tumor necrosis factor, T helper 1, T helper 17, Apoptosis

Introduction

Human inflammatory bowel diseases (IBDs), which include Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders of the intestinal tract with unknown etiologies, for which no current cures exist. Over the past few years, it has become evident that both CD and UC are caused by excessive immune reactivity in the gut [1]. Classically, T helper (Th) cell responses have been divided into two distinct categories according to their characteristic cytokine profiles, namely Th1 responses with increased interferon (IFN)- γ production and Th2 responses with increased expressions of interleukin (IL)-4 and IL-13. In the era of the Th1/Th2 paradigm, CD was considered to be a Th1-dominant disorder, while UC was thought to be associated with augmented Th2 responses [1]. However, recent studies have revealed a novel class of CD4⁺ effector T cells with a distinct cytokine expression profile that does not fall into either category. These cells are named Th17 cells [2, 3] because of their characteristic production of the pro-inflammatory cytokine IL-17. IL-12, which induces Th1 differentiation, and IL-23, which is important for the maintenance of Th17 cells, are both heterodimeric molecules and share a common component, the p40 subunit [4, 5]. Neutralizing antibodies (Abs) against p40 or genetic deletion of p40 in knockout mice were originally investigated to block Th1 responses, but also affected Th17 responses. As a consequence, some mouse disease models of autoimmune inflammation, such as experimental autoimmune encephalomyelitis and collagen-induced

1 arthritis, which were originally considered to be Th1-dominant disorders, have turned out to
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4 be Th17-mediated disorders [6–8]. Th17 responses are now considered to play major roles in
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7 certain human diseases such as rheumatoid arthritis and multiple sclerosis [9]. Recent studies
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10 have indicated a link between IBD and Th17 responses. Hölttä et al. [10] reported that the
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13 mRNA expressions of the Th17 cytokines IL-17 and IL-23 are increased in CD. Kobayashi et
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16 al. [11] reported that the mRNA expressions of IL-17 and IFN- γ are upregulated in both CD
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19 and UC, although IL-17 upregulation is more prominent in UC while IFN- γ upregulation is
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22 more marked in CD [11]. In some experimental murine models of IBD, Th17 cells rather than
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25 Th1 cells have been shown to be the major effector cells [12, 13]. Accumulating evidence
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28 suggests that Th17 responses are involved in the pathogenesis of IBD.
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32 Infliximab is a chimeric monoclonal antibody (mAb) against tumor necrosis factor
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35 (TNF)- α and one of the major therapeutic agents for CD [14–16]. It has also been proven to
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38 show efficacy as a treatment for UC [17–19]. Infliximab acts not only by neutralizing TNF- α
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41 but also by inducing apoptosis of TNF- α -producing cells that express transmembrane TNF- α
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44 on their surface through complement-dependent cytotoxicity (CDC) or antibody-dependent
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47 cell-mediated cytotoxicity (ADCC) [20, 21]. The production of TNF- α by intestinal mucosal
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50 mononuclear cells is increased in CD [22], and treatment with infliximab induces apoptosis of
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53 both macrophages and T cells [23–26]. Th17 cells have been reported to produce TNF- α [8,
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56 27]. Given the recent implication of Th17 responses in the pathogenesis of IBD, it is of
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1 importance to establish whether Th17 cells are susceptible to infliximab-mediated apoptosis.

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4 To the best of our knowledge, the effects of infliximab on Th17 cells as well as Th1 cells have
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7 not been clarified. Therefore, we carried out this study to examine the effects of infliximab
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10 treatment on human Th1 and Th17 responses *in vitro*.
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16 **Methods**

17 **Abs and Reagents**

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23 Infliximab was a kind gift from Tanabe-Mitsubishi Pharmaceutical (Tokyo, Japan).
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26 Human immunoglobulin G1 (IgG1) as a relevant isotype control was obtained from Ikwan
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29 (Bangkok, Thailand). Recombinant human IL-2, IL-12, IL-1 β , IL-6 and IL-23 and mAbs
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32 against human IL-12 and IL-4 were purchased from R&D Systems (Minneapolis, MN).
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36 Anti-human CD3 and CD28 mAbs were purchased from BD PharMingen (San Diego, CA).
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42 **Samples and Isolation of Naive CD4⁺ T cells**

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45 Heparinized peripheral blood was drawn from healthy volunteers. Peripheral blood
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48 mononuclear cells (PBMCs) were collected by density-gradient centrifugation using
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51 Lymphocyte Separation Media (MP Biomedicals, Santa Ana, CA). The cells were separated
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54 magnetically using a Naive CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach,
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57 Germany) to isolate naive CD4⁺ T cells.
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Cell Culture

Naive CD4⁺ T cells were stimulated with beads bearing anti-CD2, anti-CD3 and anti-CD28 Abs (T Cell Activation/Expansion Kit; Miltenyi Biotec) at a ratio of 1 bead/cell, according to the manufacturer's instructions, at a density of 1×10^6 cells/ml in RPMI 1640 medium (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (FBS) (Cell Culture Technologies, Gravesano, Switzerland), 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA), 1% HEPES buffer (Sigma) and 1% non-essential amino acids (Invitrogen). To induce Th1 differentiation, recombinant human IL-2 (20 ng/ml), recombinant human IL-12 (10 ng/ml) and anti-human IL-4 mAb (5 µg/ml) were added to the culture medium. To induce Th17 differentiation, recombinant human IL-1β (10 ng/ml), recombinant human IL-6 (20 ng/ml), recombinant human IL-23 (100 ng/ml), anti-human IL-12 mAb (5 µg/ml) and anti-human IL-4 mAb (5 µg/ml) were added to the culture medium as previously described [28]. Cells were cultured in the presence of infliximab (10 µg/ml) or control human IgG1 (10 µg/ml), or without an immunoglobulin.

To examine infliximab-induced apoptosis of TNF-α-bearing cells, cells were cultured under ADCC conditions. CD56⁺ natural killer cells were enriched from PBMCs using MACS CD56 microbeads and MACS positive selection columns (Miltenyi Biotec) in accordance with the manufacturer's protocol. Naive CD4⁺ T cells and CD56⁺ cells were incubated at a

ratio of 5:2 in the presence of 10% heat-inactivated FBS.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IFN- γ , IL-17 and TNF- α in the culture supernatants were measured by a sandwich ELISA technique using a Human IFN- γ ELISA Ready-SET-Go Reagent Set (eBioscience, San Diego, CA), Human IL-17 DuoSet (R&D Systems) and Human TNF- α /TNFSF1A DuoSet (R&D Systems), respectively. IFN- γ and IL-17 secretions were assessed after stimulation of cells with T-cell-activating beads as described above for 3 days in the presence or absence of infliximab. For measurements of TNF- α concentrations, the cells were stimulated with T-cell-activating beads for 7 days in the presence or absence of infliximab. The cells were then washed extensively and restimulated with 10 μ g/ml of immobilized anti-CD3 mAb and 3 μ g/ml of soluble anti-CD28 mAb for 48 h without addition of infliximab, because the presence of infliximab interfered with the ELISA reaction for TNF- α .

Detection of Cell Surface TNF- α

Naive CD4⁺ T cells were cultured under Th1- or Th17-inducing conditions with anti-CD3 and anti-CD28 mAbs for 0, 48, 60, 72 and 96 h. Activated T cells were washed and incubated with infliximab or control human IgG1 as a primary Ab for 30 min. The cells were

then washed and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG F(ab')₂ fragment (Jackson ImmunoResearch, West Grove, PA) as a secondary Ab. The cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA) and the CellQuest software (BD Biosciences).

Apoptosis Assay

To measure the effects of infliximab on the apoptosis of Th1 and Th17 cells, two different methods, namely annexin V staining and terminal deoxynucleotidyl transferase (TdT)-deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assays, were performed. For annexin V assays, the cells were stained with annexin V and 7-amino-actinomycin D (7-AAD) using an Annexin V-Phycoerythrin Apoptosis Detection Kit (BD PharMingen) according to the manufacturer's protocol. The cells were washed in phosphate-buffered saline (PBS) and labeled for 15 min at room temperature in the dark with 5 µl of staining solution per 100 µl of cell suspension. After washing, the percentages of annexin V-positive 7-AAD-negative cells were determined by flow cytometry within 1 h of staining.

TUNEL assays were performed using an Apo-Direct kit (BD PharMingen) according to the manufacturer's instructions. At the end of the culture period, the cells were fixed with 1% paraformaldehyde for 1 h on ice and stored in 70% (v/v) ethanol at -20°C for 12–18 h. The fixed cells were washed with PBS and incubated in a staining solution containing TdT

enzyme and dUTP-FITC for 1 h at 37°C. After washing, the percentages of dUTP-FITC-positive cells were determined by flow cytometry.

Statistical Analysis

The Mann-Whitney U-test was applied for comparisons of the levels of suppression by infliximab of Th1 and Th17 cytokine secretion. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Both Th1 and Th17 Cells Produce Soluble and Membrane-bound TNF- α

First, we analyzed the expressions of soluble and membrane-bound TNF- α in Th1 and Th17 cells. As shown in Fig. 1A, the cells cultured under Th1-inducing conditions produced abundant IFN- γ . In contrast, the cells cultured under Th17-inducing conditions did not secrete detectable levels of IFN- γ . On the other hand, the cells cultured under Th17-inducing conditions produced high levels of IL-17 (Fig. 1B). The cells cultured under Th1-inducing conditions also expressed IL-17, but the levels were much lower than those in cells cultured under Th17-inducing conditions (Fig. 1B). These results confirmed that Th1 and Th17 cells were successfully induced under the Th1- and Th17-inducing conditions, respectively. Importantly, both Th1 and Th17 cells secreted abundant soluble TNF- α at

comparable levels (Fig. 1C). Fig. 2 shows the data for transmembrane TNF- α expression on the surface of Th1 and Th17 cells stained by infliximab. Transmembrane TNF- α was expressed at comparable levels on cells cultured under both Th1- and Th17-inducing conditions (11.4% vs. 14.8%) at 60 h post-stimulation (Fig. 2A). The kinetics of the transmembrane TNF- α expressions were similar between the Th1- and Th17-inducing conditions, with peaks at 60 h (Fig. 2B).

Infliximab Suppresses Th1 and Th17 Cytokine Secretion

Next, we investigated the effects of infliximab on cytokine secretion by Th1 and Th17 cells. Infliximab blocks the actions of TNF- α not only by neutralizing soluble TNF- α but also by inducing apoptosis of immune cells that express transmembrane TNF- α on their surface through CDC and ADCC mechanisms. In our pilot studies, similar results were obtained for both CDC and ADCC experimental settings. Therefore, we conducted the following experiments using the ADCC experimental settings. Under Th1-inducing conditions, cultured naive CD4⁺ T cells produced abundant IFN- γ . Infliximab profoundly suppressed this IFN- γ expression, while control IgG1 had no effect (Fig. 3A). Only small amounts of IFN- γ were produced under Th17-inducing conditions (Fig. 3B). CD4⁺ T cells cultured under Th1-inducing conditions produced only trace amounts of IL-17 (Fig. 3C). Under Th17-inducing conditions, high levels of IL-17 were produced by CD4⁺ T cells. Infliximab

only partially suppressed this IL-17 production, while control IgG1 had no effect (Fig. 3D). CD4⁺ T cells under Th1- and Th17-inducing conditions produced high levels of TNF- α upon restimulation. Treatment with infliximab completely abolished the TNF- α expression under both conditions (Fig. 3E and F). The levels of suppression were more profound for IFN- γ production than for IL-17 production in three independent experiments ($P<0.05$) (Fig. 3G).

These results indicate that infliximab suppressed both Th1 and Th17 differentiation. However, the levels of suppression were more profound for Th1 induction than for Th17 induction. Although the suppressions of Th1 and Th17 differentiation were partial, treatment with infliximab completely blocked the generation of TNF- α -producing cells during the induction of Th1 and Th17 differentiation.

Infliximab Induces Apoptosis of Th1 Cells but not Th17 Cells

Next, we determined whether infliximab induced apoptosis of Th1 and Th17 cells. To detect apoptotic cells, we conducted annexin V staining and TUNEL assays. Fig. 4A shows representative results of annexin V staining assays. In these assays, annexin V-positive 7-AAD-negative cells (lower right areas of the dot plots) represented apoptotic cells. Activated T cells treated with infliximab showed a higher degree of apoptosis than cells treated with control IgG1 (11.6% vs. 5.5%) under Th1-inducing conditions at 48 h after the addition of infliximab. In contrast, there was no obvious difference in the percentages of

apoptotic cells between infliximab- and control IgG1-treated cells (7.1% vs. 6.8%) under Th17-inducing conditions. The kinetics of the percentages of annexin V-positive 7-AAD-negative apoptotic cells are shown in Fig. 4B. Under Th1-inducing conditions, the percentages of apoptotic cells were higher among infliximab-treated cells than among control IgG1-treated cells at 12, 24 and 48 h. In contrast, under Th17-inducing conditions, the percentage of apoptotic cells was slightly higher in infliximab-treated cells than in control IgG1-treated cells at 12 h, but no obvious differences were observed at 24 and 48 h.

In the TUNEL assays, infliximab-treated cells exhibited more TUNEL-positive apoptotic cells than control IgG1-treated cells (24.4% vs. 2.1%) at 24 h after the addition of infliximab under Th1-inducing conditions (Fig. 5A). In contrast, no obvious difference was observed in the percentages of TUNEL-positive apoptotic cells between infliximab- and control IgG1-treated cells (3.8% vs. 1.7%) under Th17-inducing conditions (Fig. 5A). Fig. 5B shows the kinetics of the TUNEL-positive apoptotic cells. Under Th1-inducing conditions, the percentage of apoptotic cells was higher in infliximab-treated cells at 12 h, reached a peak at 24 h and then returned to the background level at 48 h. In contrast, no obvious difference was observed between infliximab- and control IgG1-treated cells at any of the time points under Th1-inducing conditions.

Discussion

IL-12 and IL-23 are key cytokines for Th1 and Th17 responses, respectively, and are both heterodimeric molecules that share a common p40 subunit [4, 5]. An anti-IL-12 Ab that recognizes the p40 subunit neutralizes both IL-12 and IL-23, and IL-12 p40 gene-targeted mice are deficient in both IL-12 and IL-23. Consequently, some Th17-mediated diseases were previously considered to be Th1-induced disorders, until the recent discovery of the Th17 cell subset. Notably, rheumatoid arthritis and multiple sclerosis were originally considered to be Th1-mediated diseases, but are now considered to be induced by Th17 [9]. CD is considered to be a Th1-mediated disorder [1]. However, evidence for a connection between IBD and Th17 has recently been accumulating. Hölttä et al. [10] reported that IL-17 and IL-23 are increased in CD, suggesting that Th17 responses are involved in its pathogenesis. Kobayashi et al. [11] reported that the mRNA expressions for IL-17 and IFN- γ are upregulated in CD and UC, although IL-17 upregulation is more prominent in UC and IFN- γ upregulation is more marked in CD [11]. It is therefore probable that Th1 and Th17 responses are both augmented in IBD, although the Th1/Th17 balances may differ between CD and UC. Based on these findings, it is likely that Th17 responses play important roles in the development of inflammation in IBD. To develop efficacious therapies for IBD, it is important to clarify how Th17 responses can be suppressed in the gut of patients with IBD.

TNF- α is a pivotal inflammatory cytokine in the pathogenesis of IBD [1, 29]. Infliximab, a chimeric mAb against TNF- α , is highly efficacious against the induction and

1 maintenance of moderate to severely active CD [14–16]. It has also been proven to be
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4 effective in the treatment of refractory UC [17–19]. It blocks the actions of TNF- α not only by
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7 neutralizing TNF- α but also by inducing apoptosis of TNF- α -producing cells that bear
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10 transmembrane TNF- α on their surface [20, 21]. In the gut of patients with IBD, TNF- α is
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13 prominently produced by immune cells [22, 29]. It is known that treatment with infliximab
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16 induces apoptosis of macrophages and T cells in CD [23–26]. Generally, macrophages
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19 produce much more TNF- α than T cells. However, animal models have suggested the
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22 importance of TNF- α produced by lymphocytes in the pathogenesis of IBD. Specifically, mice
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25 lacking TNF- α AU-rich elements, in which immune cells produce abundant TNF- α , develop
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28 spontaneous colitis and arthritis [30]. Double-mutant mice with a TNF- α AU-rich element
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31 mutation introduced into a recombination-activating gene-1-deficient background develop
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34 arthritis but not colitis [30], suggesting that lymphocyte-derived TNF- α is essential for the
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37 development of IBD. Consequently, it is of interest to establish whether Th1 and Th17 cells
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40 produce TNF- α , and whether infliximab is capable of blocking the actions of TNF- α produced
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45 by Th1 or Th17 cells.

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48 Although it has been reported that Th17 cells produce TNF- α [8, 27], data regarding
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51 TNF- α production by Th1 cells are relatively sparse. In this study, we have shown that human
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54 Th1 and Th17 cells produce soluble and transmembrane TNF- α at comparable levels.
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57 Treatment with infliximab strongly reduced the IFN- γ production by Th1 cells, but only
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1 partially and less markedly blocked the IL-17 production by Th17 cells. These results suggest
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4 that infliximab suppresses both Th1 and Th17 differentiation. However, Th17 responses are
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7 less sensitive to infliximab-mediated suppression than Th1 responses. Th1 and Th17 cells
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10 produced abundant TNF- α upon restimulation. Although the inhibition of Th1 and Th17
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13 differentiation by infliximab was partial, treatment with infliximab during Th1 and Th17
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16 differentiation completely abolished the TNF- α production, suggesting that infliximab either
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19 inhibits the development of or eliminates TNF- α -producing T cells.
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23 As mentioned above, infliximab induces apoptosis of TNF- α -producing cells through
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26 CDC or ADCC mechanisms [21] as well as neutralizing soluble TNF- α . Treatment of Th1
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29 cells with infliximab increased the percentages of annexin V-positive and TUNEL-positive
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32 apoptotic cells. Surprisingly, although some Th17 cells expressed transmembrane TNF- α
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35 similarly to Th1 cells, infliximab did not increase the percentages of annexin V-positive and
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38 TUNEL-positive cells among Th17 cells. These results indicate that infliximab inhibits Th1
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41 responses at least partially by inducing apoptosis of Th1 cells. In contrast, Th17 cells appear
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44 to be relatively resistant to infliximab-mediated cytotoxicity, and neutralization of TNF- α by
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47 infliximab suppressed the differentiation of Th17 cells without apoptosis. These findings
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50 suggest that TNF- α is important for Th17 differentiation, in accordance with a previous study
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53 in which TNF- α was used to induce Th17 differentiation [33]. These differences in the
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56 susceptibilities to infliximab-mediated apoptosis may explain the differences in the
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1 sensitivities of Th1 and Th17 differentiation to infliximab-mediated suppression. Although
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4 infliximab failed to induce apoptosis of Th17 cells bearing TNF- α on their membranes, it was
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7 able to completely block TNF- α production by Th17 cells as well as that by Th1 cells *in vitro*.
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10 However, *in vivo*, the ability of infliximab to elicit apoptosis in target cells may be important
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13 for its efficacy in the treatment of CD. Among several anti-TNF- α agents that are efficacious
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16 for rheumatoid arthritis, some have failed to show efficacy for treatment of CD [31]. The
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19 superiority of infliximab for CD treatment may be explained by the difference in its abilities
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22 to exert cytotoxicity toward transmembrane TNF- α -positive cells [25, 32]. Indeed, it has been
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25 reported that infliximab treatment increases the number of apoptotic T cells in the lamina
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28 propria of CD patients [24–26]. Further studies are awaited to establish whether apoptotic Th1
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31 or Th17 cells are increased after infliximab therapy in the gut of patients with IBD.
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36 In this study, we have shown that Th1 and Th17 cells produce abundant TNF- α in
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39 soluble and transmembrane forms. Although treatment with infliximab suppressed both Th1
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42 and Th17 differentiation *in vitro*, IFN- γ production by Th1 cells was more profoundly
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45 suppressed than IL-17 secretion by Th17 cells. Infliximab induced apoptosis of Th1 cells but
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48 not Th17 cells. These results indicate that infliximab suppresses Th1 and Th17 differentiation
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51 through different mechanisms and that Th17 responses are more resistant to
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54 infliximab-mediated immunosuppression than Th1 responses.
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FIGURE LEGENDS

Figure 1. Secretion of IFN- γ , IL-17 and TNF- α by cells cultured under Th1- and Th17-inducing conditions. Naive CD4⁺ T cells isolated from peripheral blood samples from healthy volunteers were stimulated with T-cell-activating beads in the presence of IL-2, IL-12 and anti-IL-4 mAb for Th1-inducing conditions or IL-1 β , IL-6, IL-23, anti-IL-12 mAb and anti-IL-4 mAb for Th17-inducing conditions. For analyses of IFN- γ and IL-17 secretion, the cells were cultured for 3 days. For measurements of TNF- α secretion, the cells were cultured under the above conditions for 7 days, washed with PBS and restimulated with anti-CD3 and anti-CD28 mAbs for 48 h. The supernatants were collected and the concentrations of the cytokines were determined by ELISA. Values are means $\pm$ SD. ND, not detected.

Figure 2. Expression of transmembrane TNF- α on the cell surface. Naive CD4⁺ T cells were stimulated under Th1- or Th17-inducing conditions. After 0, 48, 60, 72 or 96 h, the cells were washed with PBS and incubated with infliximab or control human IgG1 as a primary Ab. Subsequently, the cells were stained with FITC-conjugated goat F(ab')₂ anti-human IgG as a secondary Ab and analyzed by flow cytometry. (A) Histograms showing the binding of infliximab (filled histograms) and control human IgG1 (open histograms) on the cell surface at 60 h. The percentages of the gated areas (M1) are shown for infliximab vs. control IgG1. (B) Kinetic analysis of the percentages of transmembrane TNF- α -positive cells under Th1-

and Th17-inducing conditions. Representative results of two independent experiments are shown.

Figure 3. Suppression of IFN- γ , IL-17 and TNF- α production by infliximab. Naive CD4⁺ T cells were stimulated under Th1- or Th17-inducing conditions. Infliximab, control IgG1 or no Ab was added to the culture medium. Naive CD4⁺ T cells were cocultured with CD56⁺ cells at a ratio of 5:2. For analyses of IFN- γ and IL-17 secretion, the cells were cultured for 3 days. For measurements of TNF- α secretion, the cells were cultured under the above conditions for 7 days, washed with PBS and restimulated with anti-CD3 and anti-CD28 mAbs for 48 h. Infliximab or control human IgG1 was not added to the culture medium of the second stimulation. The supernatants were collected and the concentrations of the cytokines were determined by ELISA. Values are means $\pm$ SD. ND, not detected. Representative results of three independent experiments are shown. Panel G shows the percent suppressions of IFN- γ secretion from Th1 cells and IL-17 secretion from Th17 cells induced by infliximab, which were calculated from the results of three independent experiments. * P <0.05.

Figure 4. Annexin V assays for cell apoptosis induced by infliximab. Naive CD4⁺ T cells were stimulated under Th1- or Th17-inducing conditions. After 48 h of culture, CD56⁺ cells and infliximab (10 μ g/ml) or human IgG1 (10 μ g/ml) were added to the culture medium and

incubated for the indicated times. The cells were stained with annexin V and 7-AAD and analyzed by flow cytometry. (A) Dot plots of the annexin V and 7-AAD staining at 48 h after the addition of infliximab or control IgG1. The percentages of annexin V-positive 7-AAD-negative cells, which represent apoptotic cells, are shown. (B) Kinetic analysis of the percentages of annexin V-positive 7-AAD-negative cells. The indicated times refer to those after the addition of infliximab or control IgG1 to the culture medium.

Figure 5. TUNEL assays for cell apoptosis induced by infliximab. Naive CD4⁺ T cells were stimulated under Th1- or Th17-inducing conditions. After 48 h of culture, CD56⁺ cells and infliximab (10 µg/ml) or human IgG1 (10 µg/ml) were added to the culture medium and incubated for the indicated times. The cells were collected and fixed with 1% paraformaldehyde. TUNEL assays were performed and the TUNEL-positive cells were analyzed by flow cytometry. (A) Histograms of cells treated with infliximab (filled histograms) or control human IgG1 (open histograms) at 24 h after the Ab addition. The gated areas (M1) represent the percentages of TUNEL-positive cells (infliximab vs. control IgG1). (B) Kinetic analysis of the percentages of TUNEL-positive cells. The indicated times refer to those after the addition of infliximab or control IgG1 to the culture medium. Representative results of two independent experiments are shown.

Fig. 1

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Fig. 2

Fig. 3

Fig. 4

Fig. 5