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IL-10 interferes directly with TCR-induced IFN-γ but not IL-17 production in memory T cells

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IL-10 is a potent immunoregulatory and anti-inflammatory cytokine. However, therapeutic trials in chronic inflammation have been largely disappointing. It is well established that IL-10 can inhibit Th1 and Th2 cytokine production *via* indirect effects on APC. Less data are available about the influence of IL-10 on IL-17 production, a cytokine which has been recently linked to chronic inflammation. Furthermore, there are only few reports about a direct effect of IL-10 on T cells. We demonstrate here that IL-10 can directly interfere with TCR-induced IFN-γ production in freshly isolated memory T cells in the absence of APC. This effect was independent of the previously described effects of IL-10 on T cells, namely inhibition of IL-2 production and inhibition of CD28 signaling. In contrast, IL-10 did not affect anti-CD3/anti-CD28-induced IL-17 production from memory T cells even in the presence of APC. This might have implications for the interpretation of therapeutic trials in patients with chronic inflammation where Th17 cells contribute to pathogenesis.

Key words: Cytokine · IFN-γ · IL-10 · IL-17 · T cells

Introduction

IL-10 was first described as a factor produced by Th2 cells, which inhibits cytokine production by Th1 cells [1]. Since then it has emerged that IL-10 is also produced by APC and that it can also inhibit cytokine production by Th0 and Th2 cells. Nevertheless, it is still unclear how exactly this inhibition is mediated on a molecular level and whether all T-cell subpopulations are equally affected by IL-10.

It has been demonstrated that a great amount of the inhibitory activity of IL-10 on T cells is indirect and results from its effects on APC. IL-10 down-regulates surface expression of MHC class II and of several co-stimulatory molecules on APC, such as CD80/86 and ICAM-1, and thereby strongly impairs their antigen-presenting capacity [2–4]. In addition, it influences the production of several soluble mediators including IL-1, IL-6, IL-12, IL-18 and TNF-α [5–7], which support T-cell activation and differentiation into Th1 cells. In line with this, we have previously demonstrated that the inhibitory effect of IL-10 on cytokine-induced IFN-γ production by T cells is entirely dependent on the presence of $CD14^+$ cells and that it can be overcome by the addition of exogenous cytokines [8]. However, T cells express IL-10 receptors and a few direct inhibitory effects of IL-10 on naïve T cells have been described, such as the inhibition of IL-2 production and more recently, the inhibition of CD28 signaling [9–11]. A transient activation of SHP-1 by IL-10 was suggested to mediate the inhibition of CD28 and also ICOS signaling [12].

Despite its broad anti-inflammatory profile, therapeutic trials with IL-10 to dampen an ongoing chronic inflammation have been largely disappointing (reviewed in [13]). The reasons for this are rather unclear. Chronic inflammation is thought to be driven by uncontrolled effector memory T-cell activation. It has been suggested that IL-10 has no direct effects on antigen-primed T cells because they would loose IL-10RI expression after

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activation [14]. In the past, many chronic inflammatory diseases were linked with an excessive Th1 response, with IFN-γ as the detrimental effector cytokine. However, later reports demonstrated that IFN-γ is necessary for the activity of regulatory T cells [15] and that it has also a role in limiting inflammation [16]. It now emerges that excessive production of IL-17 from Th17 cells might be an even more decisive factor in chronic inflammation (reviewed in [17, 18]). Whereas several factors which negatively regulate Th17 cell development have been described [19, 20], it is unknown which factors can restrict an already established memory IL-17 production.

Therefore, we analyzed the effect of IL-10 on various T-cell subpopulations regarding their TCR-induced IFN-γ and IL-17 production. In contrast to earlier reports [11, 12, 21], we found that IL-10 can inhibit TCR-induced IFN-γ production in freshly isolated memory T cells in the absence of APC. However, we could not detect any direct inhibitory effect on Th17 cells regarding their anti-CD3-induced IL-17 production. Furthermore, in the presence of APC, IL-10 had a lower capacity to inhibit antigen-induced IL-17 production compared with IFN-γ production in T cells. These results may help to understand why the success of IL-10 therapy in an ongoing chronic inflammation has its limitations. IL-10 inhibits IFN-γ stronger then IL-17 after antigen presentation *via* APC.

Results

IL-10 inhibits IFN-γ and to a lesser extent, IL-17 after antigen presentation *via* APC

IL-10 is known to inhibit the production of a wide range of T-cell cytokines by reducing antigen presentation and co-stimulation mediated by APC. To test whether this also applies for IL-17 production, we compared the ability of IL-10 to inhibit antigen-dependent production of IFN-γ and IL-17, respectively. We choose a fungal stimulus as antigen because they are known inducers of IL-17. Therefore, we stimulated PBMC with *Candida albicans* antigen in the absence or presence of IL-10 for 72 h (Fig. 1A). Under these conditions, IL-10 can exert its inhibitory effect on APC and T cells. Antigen-induced IFN-γ and IL-17 production in the absence of IL-10 were comparable between IFN-γ and IL-17 with a median of around 350 pg/mL. As expected, IL-10 was able to inhibit both IFN-γ and IL-17 production (each with $p < 0.05$, Fig. 1A). However, the inhibitory effect on IFN-γ production was significantly stronger than on IL-17 production ($p < 0.05$, Fig. 1A). This indicates that IL-10 might use different mechanisms for inhibiting IFN-γ and IL-17 production, respectively.

IL-10 interferes with TCR-induced IFN-γ but not IL-17 in freshly-isolated PBMC

We next asked whether IL-10 would be able to inhibit IFN-γ and/or IL-17 production independently of its ability to down-regulate antigen presentation and co-stimulatory molecules on APC. Hence, we provided TCR- and co-stimulation through plate-bound antibodies against CD3 and CD28. PBMC were stimulated for 24 h in the presence or absence of IL-10 before IFN-γ and IL-17 production were measured in the supernatants by ELISA. IL-10 significantly inhibited TCR-induced IFN-γ production ($p<0.05$, Fig. 1B), but only had a weak and non-significant inhibitory effect on IL-17 production (Fig. 1B). Similar effects were seen on the mRNA level: IL-10 inhibited TCR-induced IFN-γ mRNA production by 80% ($p<0.05$, Fig. 1C), but did not affect IL-17 mRNA induction (Fig. 1C).

These data were confirmed by assessing intracellular cytokine production in anti-CD3/anti-CD28-stimulated PBMC. For this, we added Brefeldin A only during the last 6 h of TCR-stimulation in order to avoid any interference of Brefeldin A with the inhibitory activity of IL-10. IL-10 reduced the percentage of IFN-γ-producing $CD4^+$ as well as $CD8^+$ T cells, but did not affect IL-17-producing T cells (Fig. 1D). IL-10 did not affect the activation of T cells in general, as we saw no difference in the percentage of CD25-expressing T cells in the presence or absence of IL-10 (data not shown). Other T-cell activation markers such as HLA-DR, another marker for acute activation and CD57, a marker for chronic activation, were also largely unaffected by IL-10 (data not shown).

Inhibitory effect of IL-10 on TCR-induced IFN-γ is independent of IL-2

IL-2 is an important cofactor for IFN-γ production by enabling mRNA transport and translation [22, 23]. Furthermore, it has been reported that exogenous addition of IL-2 abrogates the inhibitory effects of IL-10 on T-cell proliferation [10]. Indeed, we could confirm that the presence of exogenous IL-2 abrogated the anti-proliferative effect of IL-10 on T cells (Fig. 2A). In contrast, it did-not influence the inhibitory effect of IL-10 on IFN-γ production (Fig. 2B). Furthermore, exogenous addition of IL-2 did neither influence IL-17 production in presence nor absence of IL-10 (data not shown). These results pointed toward a so far unknown direct effect of IL-10 on IFN-γ production independent of IL-2 repression. Furthermore, because the amount of IFN-γ induction was higher in the presence of IL-2, we decided to add IL-2 throughout the following experiments.

IL-10 directly inhibits TCR-induced IFN-γ production in the absence of APC

The IL-10-induced inhibition on IFN-γ production after direct T-cell-stimulation with plate-bound anti-CD3/anti-CD28 mAb in the presence and absence of exogenous IL-2 suggested an (i) APC- and (ii) IL-2-independent mode of inhibition. However, in our previous work, we demonstrated the necessity of $CD14^+$ monocytes for the inhibitory effect of IL-10 on cytokine-induced IFN-γ production by T cells [8]. We therefore wondered whether

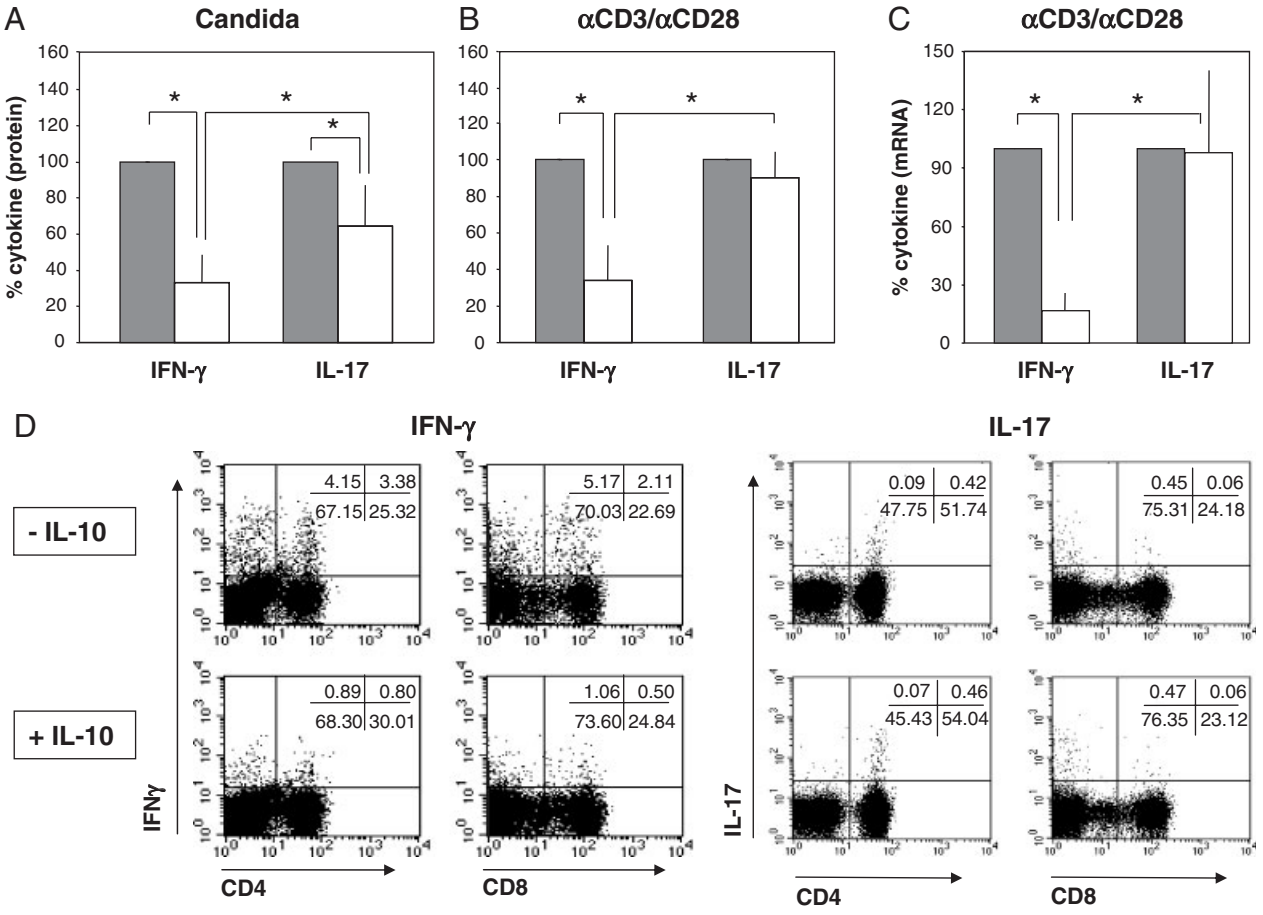

Figure 1. IL-10 does inhibit TCR-induced IFN-γ but not IL-17 production in freshly isolated PBMC. (A) Human PBMC from healthy blood donors were activated with *C. albicans* antigen for 72 h in the presence (white bars) or absence (gray bars) of IL-10 (10 ng/mL). Supernatants were collected for IFN-γ and IL-17 analyses by ELISA. The amount of IFN-γ or IL-17 in the absence of IL-10 was set to 100% for each individual donor, and IL-10 inhibition related to it. The absolute values ranged from 173 to 4453 pg/mL for IFN-γ and from 235 to 513 pg/mL for IL-17 in the non-inhibited samples. Results shown are averages from five independent experiments (±SD). (B) Human PBMC from healthy blood donors were activated with immobilised anti-CD3/anti-CD28 mAb (1 μg/mL each) and IL-2 (100 U/mL) for 24 h in the presence (white bars) or absence (gray bars) of IL-10 (10 ng/mL). Supernatants were collected thereafter and IFN-γ and IL-17 levels analyzed by ELISA. The amount of IFN-γ or IL-17 after anti-CD3/anti-CD28 treatment in the absence of IL-10 was set to 100% for each individual donor, and IL-10 inhibition related to it. The absolute values ranged from 9.4 to 427 ng/mL for IFN-γ and from 0.9 to 1.5 ng/mL for IL-17 in the non-inhibited samples. Results shown are averages from five independent experiments (±SD). (C) Following the same stimulation setup as described for (B), RNA was extracted from cells and IFN-γ and IL-17 mRNA levels were determined by Real-Time RT-PCR and normalized to the expression of the house keeping gene HPRT. The amount of IFN-γ or IL-17 mRNA following anti-CD3/anti-CD28 treatment without IL-10 incubation (gray bars) was set to 100% for each individual sample and IL-10 inhibition (white bars) related to this. Results shown are averages from five independent experiments (±SD). Significant differences as calculated by Wilcoxon test ($p<0.05$) are indicated by an asterisk (*). (D) PBMC were TCR-stimulated for 24 h by anti-CD3/anti-CD28 mAb (1 μg/mL each) and Brefeldin A added for the last 6 h. Cells were then stained with a mixture of CD3/CD4 and CD3/CD8 mAb, respectively, and intracellular IFN-γ and IL-17 production in $CD4^+$ and $CD8^+$ T cells was analyzed by flow cytometry. Data from one representative out of three experiments are shown.

this is also the case for TCR-induced IFN-γ production. Hence, we depleted $CD14^+$ monocytes from freshly isolated PBMC and then stimulated the remaining cells with anti-CD3/anti-CD28 mAb and IL-2 in the presence or absence of IL-10 for 24 h. IL-10 strongly inhibited TCR-induced IFN-γ production also in the absence of $CD14^+$ APC ($p<0.05$, Fig. 3A). To exclude an effect of $CD14^-$ APC such as B cells, we further purified the CD14-depleted PBMC by an additional step of $CD4^+$ selection. This procedure yielded highly purified $CD4^+$ T cells (on average 99%). IL-10 still significantly inhibited TCR-induced IFN-γ production, even though the effect was now less pronounced than with depletion of $CD14^+$ APC alone ($p<0.05$, Fig. 3A). Similar results were obtained after MACS™ purification of $CD4^+$ T cells either by direct $CD4^+$ selection or by CD4-untouched separation (data not shown). The observed inhibitory effect of IL-10 was not due to an inhibition of cell proliferation during the 24 h of stimulation, as was confirmed by ^{3}H-dTTP-incorporation (data not shown).

In conclusion, IL-10 had a direct inhibitory effect on TCR-induced IFN-γ production by $CD4^+$ T cells in the absence of APC and the presence of IL-2.

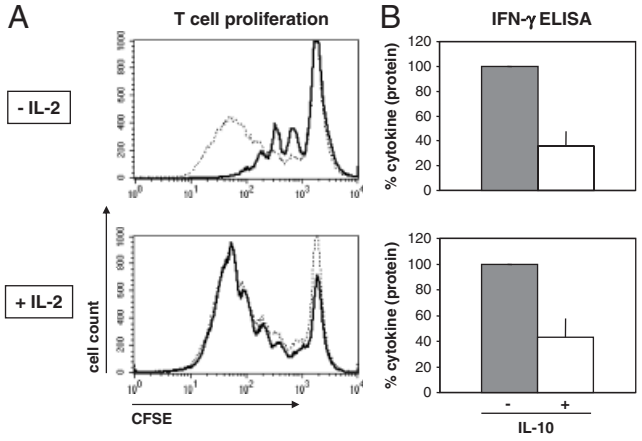

Figure 2. Exogenous IL-2 abrogates IL-10 inhibition of T-cell proliferation but has no influence on IL-10-induced inhibition of IFN-γ production. (A) Human PBMC were labeled with CSFE and proliferation was induced by TCR-stimulation with plate-bound anti-CD3/anti-CD28 mAb (1 μg/mL each) in the presence (bold lines) or absence (dotted lines) of IL-10 (10 ng/mL) and IL-2 (100 U/mL) as indicated. The degree of proliferation was assessed by analyzing the reduction of CSFE-label after cell devision by flow cytometry. One representative out of three experiments is shown. (B) Human PBMC were activated with immobilised anti-CD3/anti-CD28 mAb (1 μg/mL each) in the presence (white bars) or absence (gray bars) of IL-10 (10 ng/mL) and IL-2 (100 U/mL) as indicated. Supernatants were collected thereafter and IFN-γ production analyzed by ELISA. The amount of IFN-γ after anti-CD3/anti-CD28 treatment in the absence of IL-10 was set to 100% for each individual donor, and IL-10 inhibition related to it. The absolute values ranged from 2.7 to 87.6 ng/mL for IFN-γ in the non-inhibited samples. Results shown are averages from three independent experiments (±SD).

IL-10 inhibition of TCR-induced IFN-γ is not overcome by blocking SHP-1

In contrast to naïve T cells, antigen-primed T cells do not need co-stimulation for re-activation. Previous work suggested that IL-10 does not inhibit memory T cells, because T-cell proliferation induced by anti-CD3 in the absence of co-stimulation was not inhibited by IL-10 [11, 12, 21]. We therefore aimed to analyze the effect of IL-10 on TCR-induced IFN-γ and IL-17 production by memory T-cell subpopulations.

First, we investigated the effect of IL-10 on freshly isolated non-CD14-depleted and CD14-depleted PBMC that were TCR-stimulated in the absence of co-stimulation. Under these circumstances, only memory and effector T cells should produce IFN-γ. Surprisingly, IL-10 inhibited anti-CD3-induced IFN-γ production in non-CD14-depleted and CD14-depleted PBMC to a similar extent as anti-CD3/anti-CD28-induced IFN-γ production (both with $p < 0.05$, Fig. 3B). The same results were obtained for CD14-depleted and then $CD4^+$ re-purified T cells, which ruled out an effect of contaminating APC ($p < 0.05$, Fig. 3B). On the other hand, anti-CD3-induced IL-17 production was not inhibited by IL-10 (data not shown). This suggested that IFN-γ, but not IL-17 production from memory T cells, was directly inhibited by IL-10.

To confirm this, we repeated the experiments with $CD4^+$ memory ($CD45RO^+$) and naïve ($CD45RA^+$) T-cell subpopulations. Again, IL-10 inhibited IFN-γ production in memory $CD4^+$ T cells to a similar extent as in highly re-purified $CD4^+$ cells ($p < 0.05$, Fig. 3C) but had no effect on anti-CD3-induced IL-17 production (Fig. 3C). Presence or absence of exogenous IL-2 did

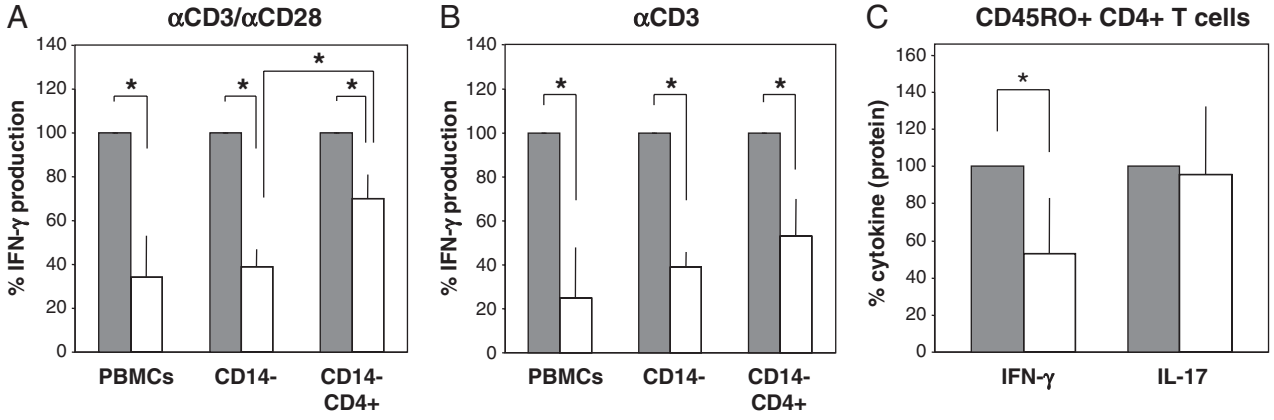

Figure 3. IL-10 directly inhibits TCR-induced IFN-γ production in the absence of APC and co-stimulation but has no influence on IL-17 production. PBMC from healthy blood donors were either used directly or depleted with anti-CD14-coated magnetic beads ($CD14^-$). $CD4^+$ T cells were further purified from CD14-depleted PBMC by $CD4^+$ selection to a purity of at least 99% ($CD14^-CD4^+$). For memory T-cell separation (C), $CD4^+$ cells were purified from PBMC by CD4-untouched magnetic separation. Then $CD45RO^+$ $CD4^+$ T cells were enriched by depleting $CD45RA^+$ cells. These different cell populations were activated with IL-2 (100 U/mL) and either (A) immobilised anti-CD3/anti-CD28 mAb (1 μg/mL each), or (B, C) immobilized anti-CD3 (1 μg/mL) for 24 h in the presence (white bars) or absence (gray bars) of IL-10 (10 ng/mL). Supernatants were collected thereafter and (A, B) IFN-γ production and (C) IFN-γ and IL-17 production was analyzed by ELISA. The amount of cytokine after respective stimulation without IL-10 was set to 100% for each individual donor and IL-10 inhibition related to this. In the absence of IL-10, the absolute values for IFN-γ (4–427 ng/mL for PBMC and 1.8–15.7 ng/mL for CD14-depleted and $CD4^+$ re-purified T cells) were comparable in the anti-CD3/anti-CD28-stimulated and only anti-CD3-stimulated samples. In memory $CD45RO^+$ $CD4^+$ T cells, in the absence of IL-10, the absolute values ranged from 4.6 to 15.5 ng/mL for IFN-γ and from 0.05 to 1.4 ng/mL for IL-17. Results shown are averages from five independent experiments (±SD). Significant differences as calculated by Wilcoxon test ($p < 0.05$) are indicated by an asterisk (*).

not alter these results, and naïve $CD4^+$ T cells did neither produce significant amounts of IFN-γ nor IL-17 (data not shown).

Naïve and central memory T cells express CD28, but it becomes down-regulated in effector memory or effector T cells ([24, 25], and data not shown). Recently, *Joss et al*. postulated that the inhibitory effect of IL-10 on T cells is mediated *via* inhibition of CD28 signaling, indicating that only $CD28^+$ T cells would be inhibited by IL-10 [11]. To test this, we separated $CD28^-$ and $CD28^+$ cells from CD14-depleted PBMC, and determined IFN-γ concentrations in the supernatants of these cells after TCR-stimulation by anti-CD3/anti-CD28 incubation. Surprisingly, $CD28^-$ T cells produced a significant amount of IFN-γ after TCR-stimulation and were susceptible to inhibition by IL-10 (Fig. 4A). This suggested that IL-10 also affects the $CD28^-$ effector memory and effector T-cell subsets. To confirm these data by another approach, we stained CD28 and intracellular IFN-γ in TCR-stimulated, CD14-depleted PBMC (Fig. 4B). IL-10 reduced the number of IFN-γ-producing T cells within the $CD28^-$ T-cell population (3.1% of T cells compared with 8.3%) as much as in the $CD28^+$ T-cell population (3.2% of T cells compared with 7.0%) (Fig. 4B). However, IL-10 did not seem to significantly alter the amount of IFN-γ produced *per* cell (as measured by MFI, data not shown). We also analyzed the effect of IL-10 on IFN-γ production by the T-cell subsets expressing the activation markers CD25, HLA-DR and CD57, respectively. Similar to the results for CD28, the inhibition of IFN-γ production by IL-10 was comparable in T-cell subsets either positive or negative for these activation markers (data not shown).

In summary, in contrast to IL-17, TCR-induced IFN-γ production in $CD4^+$ memory T cells was inhibited by IL-10. Furthermore, the ability of IL-10 to inhibit IFN-γ production in $CD28^-$ T cells suggests a molecular mechanism, which is independent of CD28 co-stimulation.

IL-10 inhibition of TCR-induced IFN-γ production is not overcome by blocking SHP-1

A very recent study suggested that transient activation of the tyrosine phosphatase SHP-1 mediates the inhibitory effect of IL-10 on CD28- or ICOS-induced T-cell proliferation [12]. SHP-1 has also been described to interfere with TCR-induced ZAP-70 activation. Therefore, we wondered whether SHP-1 activation also plays a role in mediating the inhibition of anti-CD3-induced IFN-γ production by IL-10. To address this question, we first used the general phosphatase inhibitor pervanadate in our

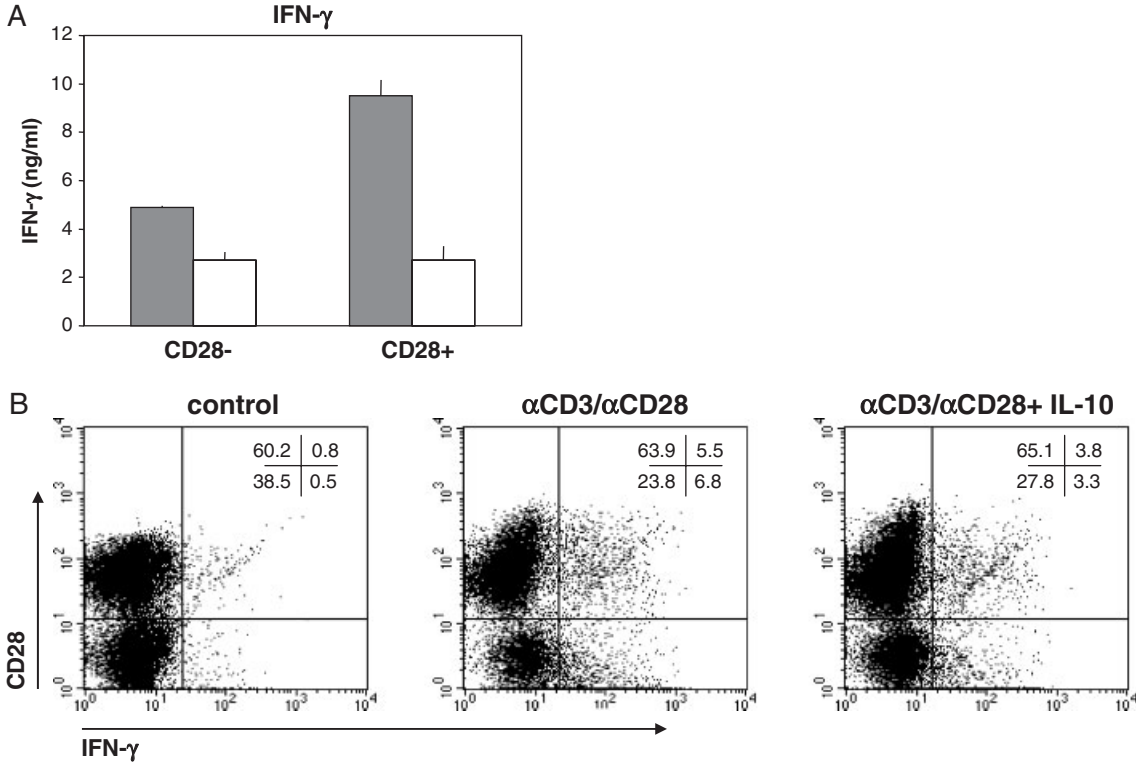

Figure 4. IL-10 inhibits TCR-induced IFN-γ production independently from CD28 expression. (A) Antigen-primed $CD28^-$ T cells were isolated by a two-step separation protocol. First, $CD14^+$ cells were depleted from PBMC and then $CD28^+$ cells were depleted from the remaining cells. The $CD28^+$ fraction was eluted for comparison. Then cytokine production was induced by immobilized anti-CD3/anti-CD28 (each 1 μg/mL) and IL-2 (100 U/mL) in the presence (white bars) or absence (gray bars) of IL-10 (10 ng/mL). Results shown are averages from three independent experiments (±SD). (B) CD14-depleted PBMC were stimulated for 30 h by immobilized anti-CD3 and anti-CD28 (each 1 μg/mL) and IL-2 (100 U/mL) in the presence or absence of IL-10 (10 ng/mL). After addition of Brefeldin A 6 h prior to the end of culture, cells were stained for surface anti-CD3 and anti-CD28 and intracellular IFN-γ expression and analyzed by flow cytometry. CD3-gated T cells are depicted from one out of two experiments. Control cells incubated with IL-10 only did not show IFN-γ production (data not shown).

experiments. Interestingly, non-stimulated T cells produced large amounts of IFN-γ in the presence of the inhibitor. In contrast, TCR-induced IFN-γ production was reduced to levels of IL-10 inhibition which both made it very difficult to interpretate these results (data not shown).

We therefore next used the specific, irreversible SHP-1 inhibitor sodium stibogluconate [26] to interfere only with SHP-1 activity. PBMC treated with this inhibitor did not produce IFN-γ spontaneously and responded normally toward TCR-stimulation. Inhibition of SHP-1 did not overcome the effect of IL-10 on TCR-induced IFN-γ production (Fig. 5A). This was the case for TCR-stimulation with anti-CD3 alone, as well as in the presence of additional co-stimulation with anti-CD28 (Fig. 5A). Even higher concentrations of sodium stibogluconate (50 μg/mL) did not abrogate the IL-10 inhibitory effect (data not shown). The activity of the inhibitor was shown by enhancement of IFN-α-induced STAT-1 activation in T-cells (Fig. 5B). These data suggest that IL-10 uses a SHP-1-independent mechanism for inhibiting TCR-induced IFN-γ production.

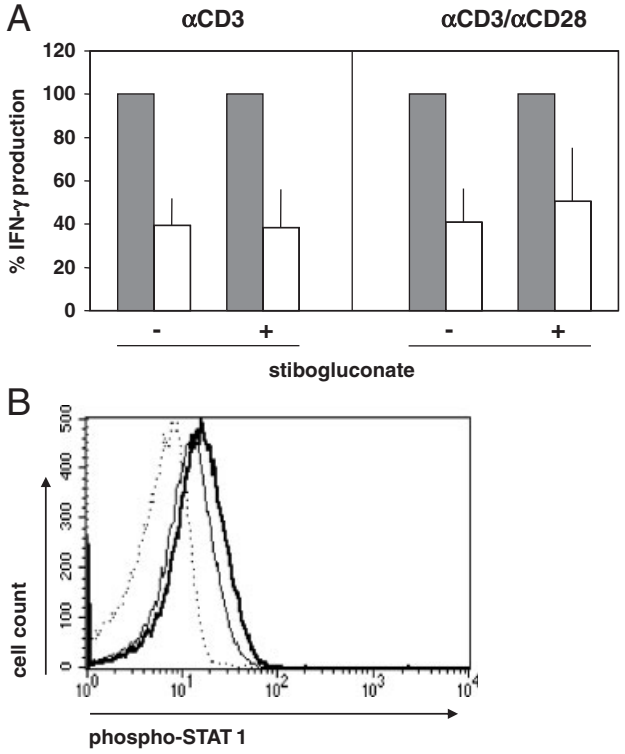

Figure 5. Inhibition of SHP-1 does not abolish IL-10-induced inhibition on TCR-stimulated IFN-γ production. (A) Human PBMC from healthy blood donors were left untreated or incubated with the irreversible SHP-1 inhibitor sodium stibogluconate (10 μg/mL) for 10 min. The cells were then activated with immobilised anti-CD3/anti-CD28 mAb (1 μg/mL each) or anti-CD3 (1 μg/mL) and IL-2 (100 U/mL) for 24 h in the presence (white bars) or absence (gray bars) of IL-10 (10 ng/mL). Supernatants were collected thereafter and IFN-γ analyzed by ELISA. Because of donor-specific differences for absolute cytokine levels, the amount of IFN-γ after anti-CD3/anti-CD28 or anti-CD3 treatment was set to 100% for each individual donor and IL-10 inhibition related to this. In the absence of IL-10, the absolute values for IFN-γ following anti-CD3 stimulation ranged from 3 to 31.5 ng/mL and similar values were obtained with anti-CD3/anti-CD28 stimulation and in the presence of stibogluconate, respectively. Results shown are averages from four independent experiments (±SD). (B) Freshly isolated PBMC were either left untreated (thin line) or pre-incubated with the SHP-1 inhibitor sodium stibogluconate (10 μg/mL; bold line) for 10 min and then treated with IFN-α for 2 h. PBMC without IFN-α treatment served as control (dotted line). Cells were then fixed and lysed and STAT1-phosphorylation was assessed by flow cytometry after co-staining of surface CD3 expression and intracellular staining of p-STAT1.

IL-10 inhibition of IFN-γ is lost by *in vitro* culture despite intact signalling

Next, we wondered whether recently activated T cells, which occur in acute infection and to an even greater extent in chronic inflammation, are susceptible to the inhibitory effect of IL-10 on IFN-γ production. It has been suggested that IL-10RI expression is down-regulated after TCR activation [14], which should lead to a loss of IL-10 responsiveness in recently activated T cells. Therefore, we incubated PBMC for 24–48 h in the presence of anti-CD3/anti-CD28 mAb and IL-2 to achieve pre-activation, and then studied the influence of IL-10 on IFN-γ production after re-stimulation with anti-CD3 and anti-CD28 mAb. In agreement with other reports, we did not observe an inhibitory effect of IL-10 on IFN-γ production by these freshly pre-activated T cells (Fig. 6A). Surprisingly, T cells cultured *in vitro* without pre-stimulation also lost their responsiveness toward IL-10. This led us to the conclusion that the unresponsiveness to the inhibitory effect of IL-10 on freshly pre-activated T cells is likely to be an *in vitro*-culture artifact rather than a stimulation-induced effect. Interestingly, despite the lack of an effect of IL-10 on IFN-γ production, the *in vitro* pre-activated T cells responded normally to IL-10-induced SOCS-3 mRNA induction (Fig. 6B) and STAT3 activation (Fig. 6C).

Th17 cells have intact IL-10 signalling

As we could observe an inhibitory effect of IL-10 on IFN-γ but not on IL-17 production from freshly isolated antigen experienced T cells, we next wondered whether Th17 cells might be unresponsive to IL-10 because they would have, *e.g.* lost IL-10 receptor expression during differentiation. We tried to address this question by measuring STAT3 activation in Th17 cells by intracellular FACS-staining with phospho-specific antibodies after 20 min of IL-10 stimulation. Several surface markers have been described to identify human Th17 cells with the IL-23 receptor expressing CCR4/CCR6 double-positive T cells being highly specific for IL-17 production [27–29]. We used therefore either antibodies against surface IL-23 receptor on $CD3^+$ T cells or gated for CCR4/CCR6 double-positive T cells to measure intracellular STAT3 activation in Th17 cells by IL-10. Surprisingly, Th17 cells mounted a normal STAT3 activation upon IL-10 stimulation (Fig. 7A). In order to analyze IL-10 target gene expression in Th17 cells, we enriched for Th17 cells by magnetic beads with

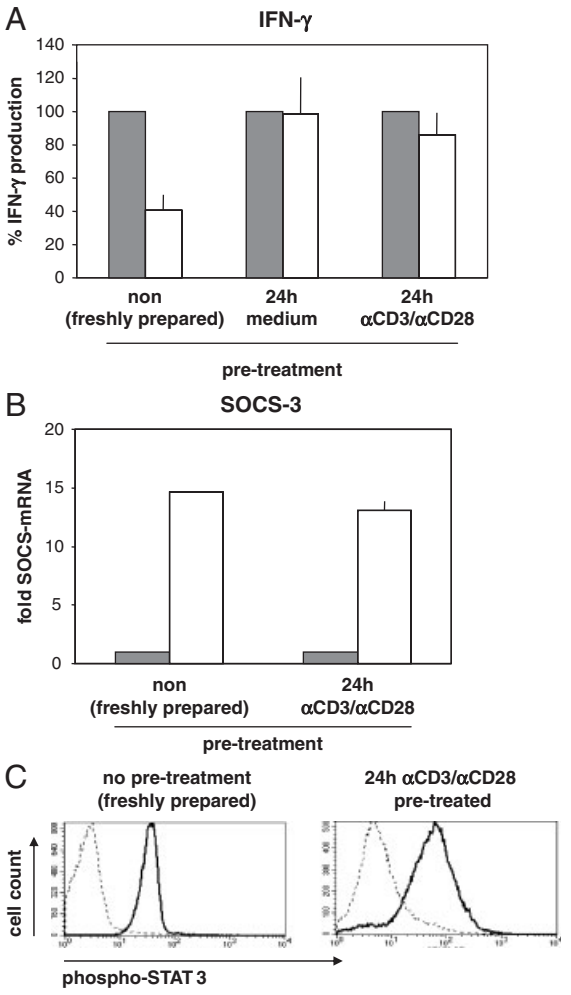

Figure 6. Loss of IFN-γ inhibitory effect of IL-10 after *in vitro* culture. (A) PBMC were either left untreated for 24 h (24 h medium) or pre-activated with immobilised anti-CD3/anti-CD28 mAb (1 μg/mL each) and IL-2 (100 U/mL) for 24 h (24 h anti-CD3/anti-CD28) followed by overnight starvation in 0.5% FCS-containing medium and washing. Production of IFN-γ was induced by immobilised 24 h anti-CD3/anti-CD28 mAb (1 μg/mL each) or anti-CD3 (1 μg/mL) and IL-2 (100 U/mL) for 24 h in the presence (white bars) or absence (gray bars) of IL-10 (10 ng/mL). As a positive control, freshly isolated PBMC from the same donor were stimulated directly. Supernatants were collected thereafter and IFN-γ measured by ELISA. The amount of IFN-γ after 24 h anti-CD3/anti-CD28 or anti-CD3 treatment was set to 100% for each individual donor and IL-10 inhibition was related to this. The absolute values for IFN-γ ranged from 9 to 115 ng/mL in the absence of IL-10 and were similar between freshly prepared and pre-treated cells. Results shown are averages from three independent experiments (±SD). (B) CD14-depleted PBMC were either used freshly prepared or pre-activated (24 h anti-CD3/anti-CD28) as described in (A). The cells were then either left untreated (gray bars) or incubated with IL-10 (10 ng/mL) for 1 h (white bars). Afterwards, $CD3^+$ cells were isolated and RNA was extracted from these cells. SOCS-3 levels were determined using Real-Time RT-PCR with HPRT as reference panel. The SOCS-3 mRNA levels of cells in the absence of IL-10 were set as 1 and the IL-10 induction of SOCS-3 related to this. Results shown are averages from two independent experiments (±SD). (C) Freshly isolated or 24 h anti-CD3/anti-CD28 pre-activated CD14-depleted PBMC as described in (A) were either left untreated (dotted lines) or incubated with IL-10 (10 ng/mL) in serum-free medium for 5 min (bold lines). Cells were then fixed and lysed and STAT3-phosphorylation was assessed by flow cytometry after co-staining of surface CD4 expression and intracellular staining of p-STAT3.

anti-IL-23R after CD14 depletion. Intracellular FACS-staining revealed that indeed these $CD3^+$ $IL\text{-}23R^+$ T cells are producers of IL-17 and that they produce little, if any, IFN-γ (Fig. 7B). In line with the observation that IL-10 is able to induce STAT3 activation in Th17 cells, we also observed an induction of its target gene SOCS-3 in these IL-23R-enriched T cells (Fig. 7C). These results together suggest that Th17 cells are fully responsive toward IL-10 but this does not result in inhibition of IL-17 production.

Discussion

The anti-inflammatory properties of IL-10 have been well described in the past. It targets the induction of various pro-inflammatory mediators as well as antigen presentation and thereby indirectly inhibits T-cell responses. *In vivo*, the lack of IL-10 in IL-10-deficient mice leads to the development of chronic inflammation in the form of colitis [30]. Because of its potent anti-inflammatory properties, IL-10 was used with high expectations in therapeutic trials for a number of chronic inflammatory diseases, which were thought to be driven by an excessive Th1 response (reviewed in [13]). However, despite some beneficial effects – particularly in psoriasis [31]– IL-10 therapy did not compare favorably to other biologicals, such as for example, anti-TNF-α therapy. This was rather surprising because IL-10 targets a wide range of inflammatory mediators in addition to TNF-α.

However, most *in vitro* studies have described inhibitory effects of IL-10 on the induction of an inflammatory immune response, but not on an ongoing or chronic inflammation. We therefore wanted to analyze whether IL-10 is able to inhibit an already established memory or effector T-cell response. In contrast to naïve T cells, memory and effector T cells respond to TCR engagement in the absence of co-stimulation. Furthermore, effector cytokine production occurs rapidly within 24 h after stimulation, because necessary epigenetic changes have already been established [32, 33]. To our surprise, IL-10 was able to directly inhibit TCR-induced IFN-γ production in memory T cells. This was evident from several observations: (i) The time frame for IFN-γ production in our direct TCR-triggered model was 24 h. Within this time frame, mainly memory and effector T cells respond, because naïve T cells have to proliferate first before they can produce IFN-γ. (ii) In contrast to previous reports on proliferation [11, 12], IL-10 inhibited IFN-γ production induced by anti-CD3 cross-linking without co-stimulation – a condition where only memory T cells should respond. (iii) In agreement with this, under the same conditions, isolated memory T cells (either $CD4^+$ $CD45RO^+$ T cells or $CD28^-$ effector/effector memory T cells) were inhibited by IL-10. (iv) Intracellular staining for IFN-γ production revealed that all of the investigated T-cell subsets were susceptible to inhibition by IL-10, including T cells with activation and memory markers. The latter is especially interesting with respect to $CD28^-$ T cells, as uncoupling of CD28 signaling has been suggested to mediate the direct inhibitory effect of IL-10 on T cells [11, 12]

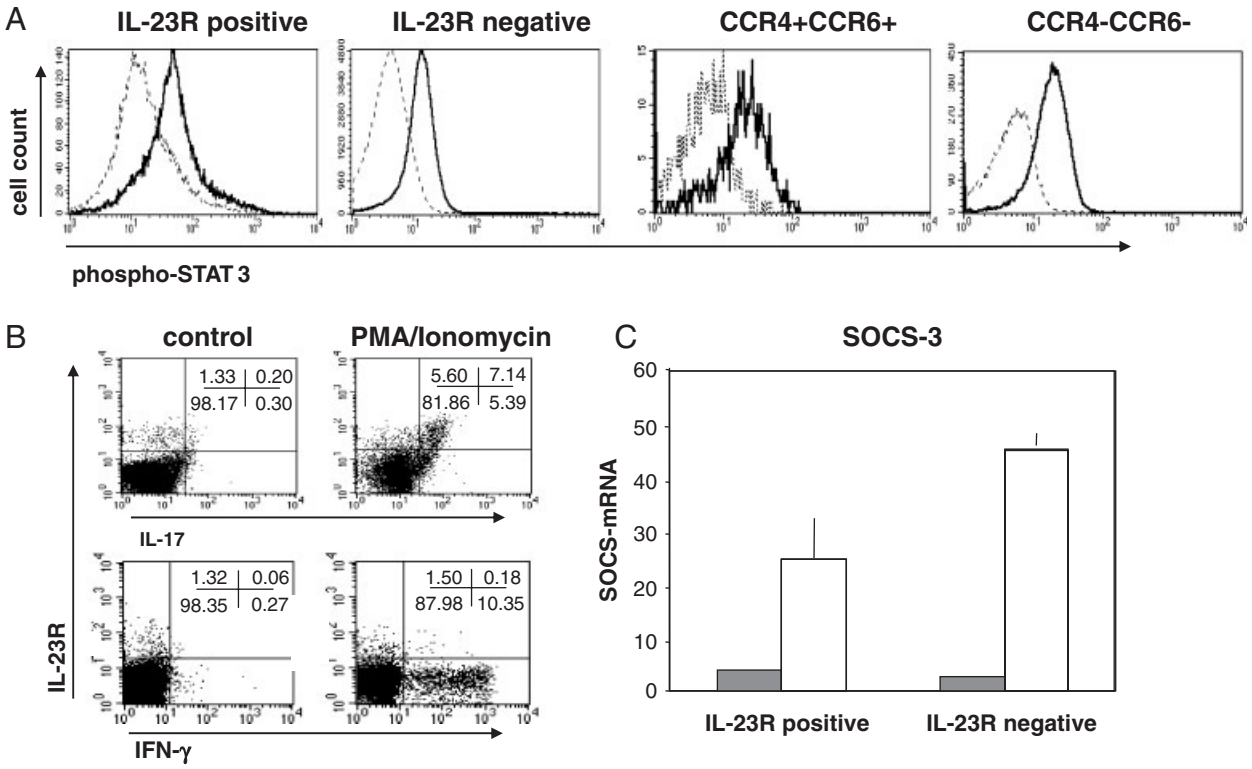

Figure 7. IL-10 induces STAT3 activation in Th17 cells. (A) Freshly isolated PBMC were either left untreated (dotted lines) or incubated with IL-10 (10 ng/mL) in serum-free medium for 20 min (bold lines) at 37°C. STAT3-phosphorylation was assessed by flow cytometry after co-staining of surface CD3 and IL-23R or CCR4 and CCR6 expression and intracellular staining of p-STAT3. Results are depicted in a histogram, which represents one out of three independent experiments. (B) Freshly isolated PBMC were either left unstimulated or stimulated with PMA and ionomycin for 15 h. The production of IFN-γ and IL-17 was assessed by flow cytometry after co-staining of surface CD3 and IL-23R expression and intracellular staining of IFN-γ and IL-17. CD3-gated T cells are depicted from one out of three experiments. (C) CD14-depleted and then IL-23 receptor-positive separated PBMC were either left untreated (gray bars) or incubated with IL-10 (10 ng/mL) for 1 h (white bars). Afterwards, RNA was extracted from these cells. SOCS-3 levels were determined using Real-Time RT-PCR with HPRT as reference panel. Results shown are averages from two independent experiments (±SD).

We furthermore showed that inhibition of anti-CD3-induced IFN-γ production by IL-10 (i) is independent from APC, which is in contrast to IL-12/IL-18-induced IFN-γ production [8], and (ii) is independent from IL-2 inhibition which was suggested to be another mechanism by which IL-10 directly inhibited T cells [9, 10, 34]. Fitting to the missing effect of IL-10 on IL-17 production (see below), receptor-proximal signaling events by IL-10 were not abrogated by the selective SHP-1 inhibitor stibogluconate, and we hypothesize a specific direct inhibitory mechanism of IL-10 on TCR-mediated IFN-γ production independent from (i) CD28 signaling, and (ii) inhibition of IL-2 production, which remains to be elucidated.

In contrast to IFN-γ, TCR-induced IL-17 production was unaffected by IL-10. Interestingly, Th17 cells did respond toward IL-10 stimulation with the activation of STAT3 excluding general unresponsiveness of Th17 cells toward IL-10. It has remained elusive why IL-10 therapy in chronic inflammatory diseases has been rather disappointing. In recent years, evidence is emerging that IL-17 production by Th17 cells, rather than IFN-γ production by Th1 cells, greatly accounts for the pathology in chronic inflammation (reviewed in [17, 18]). Our data suggest that the ability of IL-10 to stop a chronic inflammation caused by Th17 memory cells might be limited. However, it is well established that IL-10 can repress the production of major inducers of a Th17 response. IL-10 inhibits IL-6 expression [6] and the expression of the common p40 subunit [7] shared by IL-12 and IL-23 [35]. Interestingly, colitis caused by IL-10 deficiency can be prevented by depleting IL-23 [36] demonstrating that IL-10 can control the induction of an inflammatory IL-17 response. In contrast to IL-6 and IL-23, IL-10 does not inhibit TGF-β production but rather induces its activity [37, 38] and TGF-β has been associated with the generation of regulatory T cells (reviewed in [39]). Thus we speculate that although IL-10 may only partly be able to interfere with an already established inflammatory process, it may favor the induction of regulatory T cells instead of Th17 cells in the primary response to an inflammatory stimulus. Compatible with this hypothesis are findings in psoriatic patients treated with IL-10 for relapse prevention: a significant, decreased relapse rate could be seen, indicating that IL-10 application may be successful

in preventing rather than in reverting an already established inflammatory immune response [40].

In conclusion, our results suggest that in contrast to inhibition of Th1 responses, IL-10 has its limitation in inhibiting an already established and ongoing Th17-driven chronic inflammation. Taken together with data published by other groups, IL-10 however may be able to prevent the induction of a Th17 response. These results should have implications for the therapeutic use of IL-10 in inflammatory diseases. Firstly, they suggest that IL-10 therapy should be promising for inflammatory diseases that are solely caused by an ongoing excessive Th1 response. Secondly, it might be still possible to use IL-10 in Th17-driven diseases, but only in combination with conventional or anti-TNF therapy stopping the ongoing inflammatory process. In that case, IL-10 should be able to prevent a relapse.

Materials and methods

Cell culture and stimulation

Human PBMC were isolated from citrate anti-coagulated blood of healthy volunteers by Ficoll-Paque density gradient centrifugation and cultured at a cell density of 1×10^6 cells/mL in *Roswell Park Memorial Institute* 1640 medium supplemented with FCS (10% v/v), L-glutamine (2 mM) and penicillin/streptomycin (each at 10 000 U/mL) (all Biochrom KG, Germany). All healthy volunteers participated on a voluntary basis and gave written informed consent.

Antigen-dependent stimulation of cytokine production was performed by stimulating PBMC (2×10^6/mL) with *C. albicans* (dilution of 1/1000; Stallergenes GmbH, Germany) in the presence or absence of IL-10 (10 ng/mL; R&D Biosystems, Germany). After 72 h, supernatants were harvested and IFN-γ and IL-17 concentrations were determined using commercially available ELISA (R&D Biosystems). For TCR-induced cytokine production, PBMC or isolated T cells (1×10^6 cells/mL) were stimulated with plate-bound anti-CD3 (OKT3, 1 μg/mL; Jansen-Cilag GmbH, Germany) alone or when indicated in combination with plate-bound anti-CD28 (L293, 1 μg/mL; BD Bioscience, Germany) and IL-2 (100 IU/mL; R&D Biosystems) in the presence or absence of IL-10 (10 ng/mL). After 24 h, supernatants were harvested and IFN-γ and IL-17 concentrations were determined using commercially available ELISA (R&D Biosystems).

If pre-activated PBMC were used, PBMC were pre-stimulated for 24 h with immobilized anti-CD3/anti-CD28 mAb (1 μg/mL each) and IL-2 (100 IU/mL). They were then washed and starved overnight in *Roswell Park Memorial Institute* medium containing 0.5% FCS v/v before being re-stimulated with anti-CD3/anti-CD28 mAb in the presence or absence of IL-10 (10 ng/mL). As control, instead of pre-stimulation, cells were left untreated for 24 h and then re-stimulated under the same conditions. Twenty-four hours after re-stimulation, cells were harvested and supernatants were analyzed for IFN-γ or IL-17 production by ELISA.

Cell separation

Monocytes were depleted from PBMC by using anti-CD14-coated magnetic MACS™ beads (Miltenyi Biotech GmbH, Germany) following the manufacturer's protocol. Cell purity was confirmed by flow cytometry ($CD14^+$ cells $< 0.5\%$). For highly purified $CD4^+$ T cells, CD14-depleted PBMC were re-purified with anti-CD4-coated MACS™ beads (Miltenyi Biotech GmbH). Purity of $CD4^+$ T cells was $> 99\%$ in the life cell gate. For isolation of $CD4^+$ memory T cells, first $CD4^+$ T cells were separated from PBMC with CD4-untouched MACS™ beads and then further separated either for $CD45RO^+$ cells (memory $CD4^+$ T cells) *via* depletion of $CD45RA^+$ cells or for $CD45RA^+$ cells (naïve $CD4^+$ T cells) *via* depletion of $CD45RO^+$ cells using the MACS™ system (Miltenyi Biotech GmbH). Separation of $CD28^-$ and $CD28^+$ cells was performed with anti-CD28-coated MACS™ beads (Miltenyi Biotech GmbH) after CD14 depletion from PBMC. Separation of IL-$23R^+$ cells was performed with anti-FITC-coated MACS™ beads (Miltenyi Biotech GmbH) after CD14 depletion from PBMC and labeling with anti-IL-23R-FITC antibody (R&D Biosystems).

Measurement of PBMC proliferation with CFSE

PBMC were re-suspended at 1×10^7 cells/mL in PBS containing 5 μM CFSE (Molecular Probes/Invitrogen, USA). After 5 min incubation at room temperature, cells were washed twice with culture medium. Then cells were stimulated at a concentration of 1×10^6 cells/mL with immobilized anti-CD3 Ab (1 μg/mL) and anti-CD28 Ab (1 μg/mL), with or without IL-2 (100 U/mL), in the presence or absence of IL-10 (10 ng/mL) for 5 days. Cells were finally analyzed using a FACScalibur flow cytometer (Becton Dickinson, Germany) and CellQuest software (Becton Dickinson).

Analysis of intracellular cytokine production by flow cytometry

PBMC (2×10^6) or respective isolated T-cell subsets were stimulated with immobilized anti-CD3/anti-CD28 (each at 1 μg/mL) and IL-2 (100 U/mL), in the presence or absence of IL-10 (10 ng/mL) for 30 h. After 24 h, Brefeldin A (10 μg/mL; Sigma, Germany) was added for the last 6 h. For analysis of IL-$23R^+$ T cells, PBMC were stimulated with PMA (10 ng/mL; Sigma), ionomycin (0.5 μg/mL; Sigma) and Brefeldin A for 15 h. Following stimulation, cells were harvested, washed in PBS and incubated for 30 min at 4°C in the dark with the following monoclonal antibodies: anti-HLA-DR (L243; Becton Dickinson), anti-CD3 (SK7; Becton Dickinson), anti-CD8 (SK1; Becton Dickinson), anti-CD25 (2A3; Becton Dickinson), anti-CD57 (NC1; Beckman-Coulter), anti-CD28 (CD28.2; Beckman-Coulter), anti-CD4 (SK3; Becton Dickinson), anti-CD45RO (Leu-45RO; Becton Dickinson), anti-CD45RA (HI100; Becton Dickinson), anti-IL-23R (R&D Biosystems). Fluorochrome

combinations were chosen to allow for analysis of co-staining of respective antigens. All antibodies were used in saturating amounts. Cells were then washed once in PBS containing 2% v/v FCS and 0.1% w/v NaN_2 prior to fixation and permeabilization with 500 μL Cytofix/Cytoperm solution (Becton Dickinson) for 10 min at room temperature in darkness. Cells were washed once again and incubated with blocking buffer (100 μL PBS/10% v/v human AB serum; both Sigma) for 20 min at 4°C in the dark to prevent unspecific binding of the anti-cytokine monoclonal antibody. After another washing step, 10 μL of anti-IFN-γ-FITC (25723.11; Becton Dickinson) or anti-IL-17A-PE (eBio64DEC17; eBioscience, Germany) were added and cells were incubated for 30 min at 4°C in darkness followed by a final washing step prior to measurement. Measurement was done using four-color flow cytometry on a FACS Calibur (Becton Dickinson; at least 50 000 $CD3^+$ lymphocytes were collected). Data were analyzed using CellQuest (Becton Dickinson). T-cell gating was done using side scatter and CD3-staining properties.

Analysis of phospho-STAT3 and phospho-STAT1 induction by flow cytometry

Freshly isolated PBMC or pre-activated PBMC (of the same donor) were incubated for 20 min with or without IL-10 (10 ng/mL). Then cells were stained with anti-IL-23 R-FITC and fixed with Cytofix Buffer (Becton Dickinson) for 10 min at 37°C and permeabilized with Phosflow Perm Buffer (Becton Dickinson) for 30 min on ice. For surface staining of CCR4 and CCR6, PBMC were first stained with anti-CCR6-PE (11A9, Becton Dickinson) and anti-CCR4-FITC (R&D Biosystems) and then incubated for 20 min with or without IL-10 (10 ng/mL), fixed with Cytofix Buffer for 10 min at 37°C and permeabilized with Phosflow Perm Buffer for 30 min on ice.

For induction of STAT1 tyrosine phosphorylation, PBMC were incubated with IFN-α2a (0.1 ng/mL; Miltenyi Biotech GmbH) for 2 h, then fixed with Cytofix Buffer for 10 min at 37°C and permeabilized with Phosflow Perm Buffer for 30 min on ice. Cells were washed and anti-CD3-PerCP (SK7, Becton Dickinson) and anti-Stat3 (pY705)-PE (4/P Stat3, Becton Dickinson) or anti-Stat3 (pY705)-FITC (4/P Stat3, Becton Dickinson) or anti-Stat1 (pY701)-FITC (Becton Dickinson) were added and incubated for 30 min at room temperature in the dark. Cells were analyzed using a FACS Calibur flow cytometer and CellQuest software.

Analysis of cytokine and SOCS-3 mRNA induction

Total RNA was isolated for cytokine mRNA quantification with Absolute mRNA Micro Kits (Stratagene, Germany) after 24 h of TCR-stimulation by immobilized anti-CD3/anti-CD28 (each 1 μg/mL) in the presence or absence of IL-10 (10 ng/mL). mRNA was transcribed into cDNA with Moloney murine leukemia virus Reverse Transcriptase (Gibco BRL, US) and oligodT Primers (GE Healthcare, Germany). Quantitative Real-Time RT-PCR for IFN-γ and IL-17 was performed with specific primers and probes from Applied Biosystems (Germany) using the ABI prism 7700 sequence detector (Applied Biosystems) and hypoxanthine phosphoribosyltransferase (HPRT) as reference panel. For analysis of SOCS-3 induction, PBMC were stimulated with anti-CD3/anti-CD28 (1 μg/mL) and IL-2 (100 U/mL) for 24 h. They were then washed and starved overnight before stimulation with anti-CD28 and/or anti-CD3 mAb in the presence or absence of IL-10 (10 ng/mL). Cells were harvested after 1 h, $CD3^+$ cells were isolated by magnetic MACS™ beads and total RNA was extracted from these cells. For analysis of SOCS-3 induction in separated $IL\text{-}23R^+$ cells, the cells were stimulated with anti-CD3/anti-CD28 mAb in the presence or absence of IL-10 (10 ng/mL) for 1 h and then total RNA was extracted. SOCS levels were determined after reverse transcription by Real-Time RT-PCR with HPRT as reference panel. SOCS-3 sense: ctttctgatccgcgacagct, SOCS-3 anti-sense: tcacactggatgcgcaggt, SOCS-3 probe: ccagcgccacttcttcacgctcag.

Statistical analysis

If not stated otherwise, data are presented as mean ± SD values. For ELISA data, cytokine production of TCR-stimulated cells was set at 100% in each individual experiment. All other samples from the same experiment were expressed as percentages relating to this. This was done because of large inter-individual variations in absolute cytokine levels. The average and standard deviation was determined from 3–5 independent experiments. Statistical analysis was done for experiments with at least five independent donors by using the Wilcoxon matched-pairs signed-ranked test and SPSS software (USA). *p*-values (two-tailed) below 0.05 were considered as significant.

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Abbreviation: **HPRT:** hypoxanthine phosphoribosyltransferase

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