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Different Modes of IL-10 and TGF-β to Inhibit Cytokine-Dependent IFN-γ Production: Consequences for Reversal of Lipopolysaccharide Desensitization[1]

Martina Schröder, Christian Meisel, Katharina Buhl, Nina Profanter, Nadine Sievert, Hans-Dieter Volk, and Gerald Grütz[2]

LPS hyporesponsiveness is characterized by a diminished production of proinflammatory cytokines which can be caused by pretreatment with either LPS (=LPS desensitization) or the combination of the anti-inflammatory cytokines IL-10 and TGF-β. However, the resulting hyporesponsive states differ regarding their reversibility by the IFN-γ-inducing cytokine IL-12. Therefore, we aimed at studying the reasons for this differential IL-12 responsiveness of IFN-γ-producing cells and its consequences for LPS hyporesponsiveness in more detail. In an in vitro IL-12/IL-18 responsiveness model, we demonstrated that IL-10, if permanently present, does not directly inhibit IL-12/IL-18 responsiveness in T/NK cells but indirectly interferes with IFN-γ production in the presence of monocytes. In contrast, TGF-β acted directly on IFN-γ-producing cells by interfering with IL-12/IL-18 responsiveness. After removal of IL-10 but not of TGF-β, LPS hyporesponsiveness can be reverted by IL-12/IL-18. Consequently, the addition of recombinant TGF-β during LPS desensitization rendered PBMCs hyporesponsive to a reversal by IL-12/IL-18. Our data suggest that the persistence of IL-10 and the presence of TGF-β determine the level of IFN-γ inhibition and may result in different functional phenotypes of LPS desensitization and LPS hyporesponsiveness in vitro and in vivo. ***The Journal of Immunology*****, 2003, 170: 5260–5267.**

An excessive proinflammatory reaction as observed in sepsis and after major surgery is often followed by a dominant compensatory anti-inflammatory response syndrome. Moreover, stress reactions triggered by head trauma can directly lead to an anti-inflammatory response. All types can result in an immunoparalytic state ("immunoparalysis"), if the anti-inflammatory response is strong and long-lasting (1, 2). Monocytes from immunoparalytic patients show an impaired production of proinflammatory cytokines when stimulated with the bacterial cell wall component endotoxin (LPS) ex vivo (3). Long-lasting immunoparalysis may therefore lead to a fatal outcome in critically ill patients due to opportunistic infections (4, 5).

LPS desensitization is considered to be an experimental model for immunoparalysis (6, 7). It describes the fact that monocytes incubated with a low dose of LPS have a diminished capacity to produce TNF-α upon restimulation with a second higher dose of LPS (8, 9). In addition, the production of IFN-γ by T/NK cells is also reduced (10–12). We and others have previously shown that the anti-inflammatory cytokines IL-10 and TGF-β are involved in mediating the process of LPS desensitization. Furthermore, IL-10 and TGF-β can even directly induce a state of LPS hyporesponsiveness (6, 13). Thus, low-dose LPS pretreatment and IL-10/TGF-β pretreatment both induce a similar phenotype of LPS hyporesponsiveness.

Since IFN-γ has activating effects on monocytes, its decreased expression in LPS hyporesponsiveness was likely to contribute to impaired monocyte function. Indeed, monocyte dysfunction induced by either LPS or IL-10/TGF-β pretreatment was reversible by exogenous IFN-γ (7). In addition, IFN-γ was able to revert LPS tolerance in mice (14), to restore the diminished ex vivo TNF-α production of immunoparalytic patients, and to improve their clinical outcome (4). These results clearly suggest IFN-γ as a key regulator in this system. After LPS treatment, several IFN-γ-inducing stimuli are produced by monocytes. One of these factors is IL-12, which is strongly diminished during LPS desensitization and has been demonstrated to be the main IFN-γ-inducing factor in this system (11). It was therefore surprising that IL-12 was able to restore LPS responsiveness only in LPS-desensitized PBMCs but not in IL-10/TGF-β-pretreated PBMCs nor in cells from septic patients (7, 15). This observation suggested differences in IL-12 responsiveness of IFN-γ-producing cells and raised the question which of the in vitro LPS hyporesponsiveness models reflects the situation in immunoparalytic patients best.

IL-10 is known to modulate T cell responses mainly via its suppressive effects on APCs, e.g., by down-regulation of HLA-DR expression (16–18) and by the inhibition of proinflammatory cytokines including IL-12 (19, 20). However, direct inhibitory effects of IL-10 on T cell function and differentiation have also been described (21), including inhibition of IL-2 production (22, 23), suppression of IL-12R up-regulation (24), and inhibition of CD28 signaling (25, 26). For TGF-β, similar direct inhibitory effects on T cells have been described (27–29). Contrasting reports have been published on the interference of TGF-β with IL-12 signaling (30–33). TGF-β can inhibit T cell function also indirectly via inhibitory effects on APCs, but it seems to have paradoxical effects on proinflammatory cytokine production depending on the cellular context (34).

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By dissecting the mechanisms of IL-10 and TGF-β in the generation of LPS hyporesponsiveness and the role of IFN-γ-producing cells in the desensitization process, we were able to demonstrate that IL-10 and TGF-β use different ways of suppressing IFN-γ production, which leads to the observed differences in reversibility of the respective LPS-hyporesponsive states.

Materials and Methods

Cell culture

Human PBMCs were isolated from blood of healthy volunteers by Ficoll-Paque (Amersham Biosciences, Freiburg, Germany) density gradient centrifugation and cultured at 1×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and penicillin/streptomycin (Biochrom, Berlin, Germany). Recombinant human IL-2, IL-10, IL-12, IL-18, and TGF-β were purchased from R&D Biosystems (Heidelberg, Germany). All solutions were tested regularly for endotoxin contamination by TNF-α release from human PBMCs. Only endotoxin-free solutions were used for cellular experiments. Monocytes were depleted by using CD14-coated MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's protocol. Cell purity was controlled using FACS staining ($CD14^+$ cells $<0.5\%$ after depletion).

LPS desensitization and reversal

Freshly isolated PBMCs were cultured in 48-well plates and incubated for 24 h with 500 pg/ml LPS (*Escherichia coli* 0127:B8, Sigma-Aldrich, Heidelberg, Germany) or 10 ng/ml IL-10 and/or TGF-β each. After this primary culture, cells were washed three times before IL-12 (10 ng/ml) and/or IL-18 (20 ng/ml) was added. Twenty-four hours later, cells were restimulated with 100 ng/ml LPS and supernatants were collected after 4 or 24 h to measure TNF-α and IFN-γ, respectively. For comparison of data, the response of nondesensitized cells treated with IL-12/IL-18 was set to 100% in each individual experiment because of donor-dependent differences.

IL-12/IL-18 response assay

Freshly isolated PBMCs were cultured at a concentration of 1×10^6 cells/ml in 96-well plates and stimulated simultaneously with plate-bound anti-CD3/anti-CD28 Abs (OKT3; Janssen-Cilag, Neuss, Germany/ L293, BD Biosciences, Heidelberg, Germany) at a concentration of 0.01 and 2 μg/ml, respectively, 100 U/ml IL-2, 10 ng/ml IL-12, 10 ng/ml IL-18 and IL-10, or 10 ng/ml TGF-β. Supernatants were collected after 48 h and IFN-γ concentration was measured by ELISA. For comparison of data, the IFN-γ production of IL-12/IL-18-stimulated cells was set to 100% in each individual experiment because of donor-dependent differences.

Blocking Abs against CD80/CD86 (L307.4/IT2.2; BD PharMingen, Heidelberg, Germany) were added at a concentration of 15 μg/ml simultaneously with the other reagents.

Analysis of cytokine production by flow cytometry and ELISA

PBMCs were stimulated with immobilized anti-CD3 mAb (0.01 μg/ml) and anti-CD28 mAb (2 μg/ml), IL-2 (100 U), and IL-12/IL-18 (10 ng/ml each) in the presence or absence of IL-10 (10 ng/ml) or TGF-β (10 ng/ml) for 30 h. After 24 h, brefeldin A (Sigma-Aldrich) was added at 5 μg/ml. Before fixation, cells were stained with the following mAbs: anti-CD16-PE (3G8), anti-CD8-PerCP (SK1), and anti-CD3-APC (UCHTC1; all from BD Biosciences). Cells were permeabilized and incubated with anti-IFN-γ-FITC mAb (25723.11; BD Biosciences) or a FITC-conjugated isotypic control mAb (BD Biosciences) both used at 3 μg/ml each. Samples were analyzed by four-color flow cytometry on a FACSCalibur using CellQuest software (BD Biosciences).

Concentrations of TNF-α and IFN-γ in the culture supernatants were determined using commercially available ELISA kits (R&D Biosystems). The detection limit for IFN-γ and TNF-α was 15 pg/ml.

Statistical analysis

Statistical analysis was done using the Friedman test followed by the Wilcoxon matched pairs signed-ranked test and SPSS software (Cary, NC).

Results

Failure of IL-12 to revert IL-10/TGF-β-induced LPS hyporesponsiveness is not corrected by IL-18

We have previously shown that the reduced TNF-α production in LPS-hyporesponsive monocytes could be restored to normal levels by the addition of IFN-γ regardless of the desensitizing stimulus

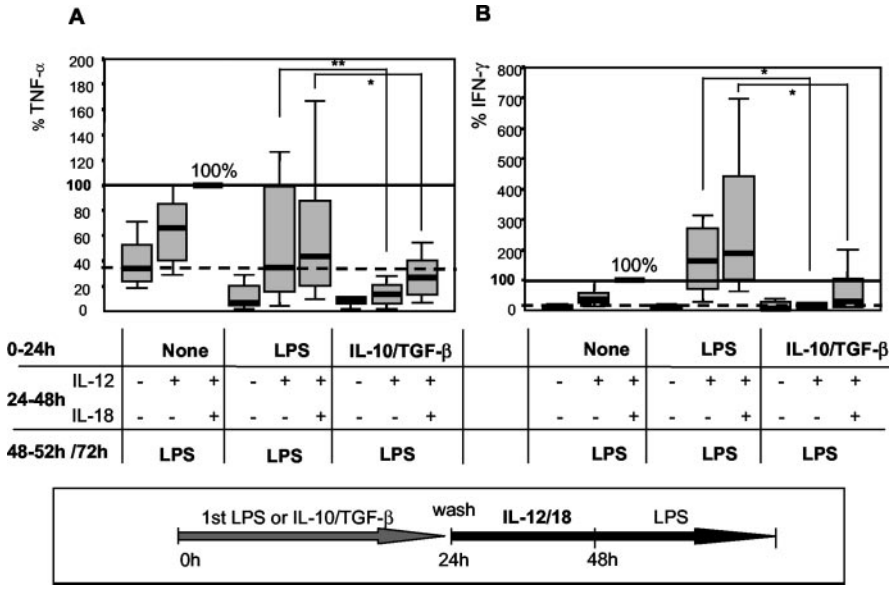

FIGURE 1. IL-18 does not overcome the desensitizing effect of IL-10/TGF-β on TNF-α and IFN-γ production. PBMCs were incubated with the desensitizing stimulus (LPS or IL-10/TGF-β) for 24 h, washed extensively, and then either left untreated or stimulated with IL-12 and/or IL-18 as indicated. After another 24 h, cells were restimulated with LPS and supernatants were collected after 4 or 24 h to measure TNF-α (*A*) and IFN-γ (*B*), respectively. The response of nondesensitized cells treated with IL-12/IL-18 was set to 100% in each individual experiment because of donor-dependent differences in TNF-α and IFN-γ levels. The absolute cytokine concentrations of IL-2/IL-18-treated cells (set to 100%) range from 1.4 to 15.9 ng/ml TNF-α and 0.25 to 14.9 ng/ml IFN-γ, respectively. Data of 11 independent experiments representing 8 individual blood donors (TNF-α) or 7 independent experiments representing 6 individual blood donors (IFN-γ) are shown as box plots with bars representing the medians. Boxes comprise the range between the 25th and the 75th percentile (interquartile range). The lines extending from the interquartile range include values within 1.5 times the interquartile range from the upper or lower quartile. Points at a greater distance from the median are omitted from the graphic (outliers). The broken line indicates cytokine production of untreated/unstimulated control cells to point out the desensitization effect. Significant differences are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$).

(LPS vs IL-10/TGF-β). The question remained why the IFN-γ-inducing cytokine IL-12 was able to restore LPS responsiveness only in LPS-desensitized PBMCs but not in cells pretreated with IL-10/TGF-β or cells from immunoparalytic patients (7, 15). IL-18 has been described as another important IFN-γ-inducing factor that acts synergistically with IL-12 (35, 36). Therefore, we asked whether IL-18 would be the missing factor for reversal of IL-10/TGF-β-induced LPS hyporesponsiveness.

As expected, the addition of IL-18 strongly enhanced LPS-induced TNF-α and IFN-γ production in untreated control cells compared with IL-12 alone (Fig. 1). LPS-pretreated cells produced even higher levels of IFN-γ than nondesensitized cells upon addition of either IL-12 or IL-12/IL-18, while their TNF-α production was only partially restored. IL-12/IL-18 in combination enhanced IFN-γ and TNF-α production also in IL-10/TGF-β-pretreated cells, whereas IL-12 alone did not have any significant effect. Nevertheless, this reversal is only partial and the production of TNF-α remains significantly diminished compared with untreated control cells (Fig. 1, dotted line, $p < 0.05$). This becomes even more obvious in comparison to control cells stimulated under the same conditions (with IL-12/IL-18 treatment, 100% line, $p < 0.01$). The differences in reversibility of TNF-α and IFN-γ production between LPS-desensitized and IL-10/TGF-β-pretreated cells remained significant (Fig. 1, $p < 0.05$) even in the presence of both IL-12 and IL-18. Therefore, the effect of IL-10 and TGF-β cannot entirely be explained by the inhibition of the IFN-γ-inducing cytokines IL-12 and IL-18 in LPS-hyporesponsive monocytes. Hence, we sought other explanations for the observed differences in reversibility.

Pretreatment with IL-12/IL-18 does not prevent IL-10/TGF-β-induced LPS hyporesponsiveness

One major difference between LPS-pretreated and IL-10/TGF-β-pretreated cells is the time point of exposure to IL-12/IL-18. During the normal course of LPS stimulation, monocytes first produce proinflammatory cytokines including IL-12 and IL-18 followed by the anti-inflammatory cytokines IL-10 and TGF-β. Therefore, T and NK cells first encounter IL-12, IL-18, and other proinflammatory cytokines before IL-10 and TGF-β are produced. This initial contact could prevent the inhibition of IFN-γ production by IL-10 and TGF-β and therefore enable IL-12 and IL-18 to revert LPS hyporesponsiveness.

To test this hypothesis, we preincubated PBMCs with IL-12 and IL-18 for 4 h before addition of IL-10 and TGF-β (Fig. 2). Prestimulation with IL-12/IL-18 did not prevent the establishment of IL-10/TGF-β-induced LPS hyporesponsiveness nor enabled its reversal by IL-12/IL-18 (Fig. 2). Therefore, initial contact with IL-12/IL-18 during the course of LPS desensitization does not provide an explanation for the observed difference in reversibility between the two models of LPS hyporesponsiveness.

IL-10-induced LPS hyporesponsiveness can be reverted by IL-12/IL-18, whereas TGF-β-treated cells remain unresponsive

It is likely that the concentrations of endogenous IL-10 and TGF-β produced during LPS desensitization differ from the amounts of recombinant cytokines we used to induce LPS hyporesponsiveness. Therefore, we investigated the individual influence of each cytokine on the induction and reversibility of LPS hyporesponsiveness separately.

Exogenous IL-12 only slightly increased IFN-γ production in IL-10- or TGF-β-pretreated PBMCs (Fig. 3*B*) but strongly enhanced IFN-γ production in LPS-pretreated cells. This lack of reversibility was comparable to cells pretreated with IL-10 and TGF-β in combination (Fig. 1*B*). However, the addition of IL-12/IL-18 in combination fully restored TNF-α and IFN-γ production in IL-10-pretreated PBMCs but not TGF-β-pretreated PBMCs (Fig. 3*A*, $p < 0.01$ IL-10 vs TGF-β, and Fig. 3*B*, $p < 0.05$ IL-10 vs TGF-β). Therefore, IL-18 seems to be the missing factor to overcome IL-10 but not TGF-β-induced LPS hyporesponsiveness.

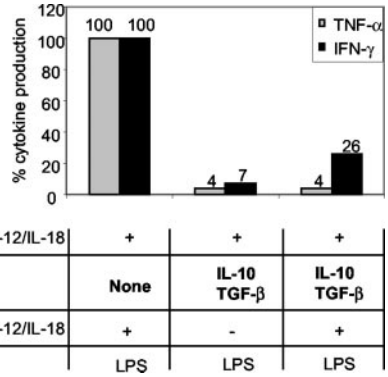

FIGURE 2. The different reversibility of LPS and IL-10/TGF-β-pretreated cells is not due to different kinetics of exposure to IL-12/IL-18. PBMCs were incubated with IL-12/IL-18 for 4 h, before the desensitizing stimulus (LPS or IL-10/TGF-β) was added. After 24 h, cells were washed extensively and then either left untreated or stimulated with IL-12 and/or IL-18 as indicated. At 48 h, cells were restimulated with LPS and supernatants were collected after 4 or 24 h to measure TNF-α (▫) and IFN-γ (■), respectively. The response of nondesensitized cells treated with IL-12/IL-18 was set to 100%. TNF-α production is depicted as gray bars and IFN-γ production as black bars. One representative experiment of two is shown.

IL-10 and TGF-β have similar inhibitory effects on IL-12/IL-18-induced IFN-γ production if permanently present

These results suggested differences in the mechanisms of IL-10 and TGF-β for inhibiting IFN-γ production. T cells and NK cells are the main producers of IFN-γ upon stimulation with LPS. This pathway of IFN-γ induction is mainly mediated by IL-12 and IL-18 but can be enhanced by other cytokines and costimulatory molecules (11). To further investigate possible effects of IL-10 and TGF-β on IL-12/IL-18 responsiveness, we tried to simplify the experimental setup and focused on IFN-γ-producing cells as targets in the process of LPS desensitization.

Freshly isolated PBMCs were stimulated simultaneously with anti-CD3/anti-CD28, IL-2, IL-12, and IL-18 in the absence or presence of IL-10 or TGF-β. In this experimental setup, which includes T cell activation, IFN-γ should be induced by memory T cells as well as NK cells. By providing rIL-12 and rIL-18, we focused on the effects of IL-10 and TGF-β on the responsiveness of IFN-γ-producing cells toward IL-12/IL-18.

In this experimental setup, IL-10 as well as TGF-β was able to inhibit IL-12/IL-18-induced IFN-γ production to a similar degree (Fig. 4). We confirmed these results at the single-cell level by flow cytometric analysis of intracellular IFN-γ expression. In contrast to a previous report (37), our results demonstrated that IL-10 and TGF-β do not only inhibit IFN-γ production in $CD3^+$ T cells but also in NK cells to a similar extent (data not shown).

At first sight, these results seem to be contradictory to the data obtained in the LPS hyporesponsiveness model, in which IL-10-mediated IFN-γ inhibition was reversible by IL-12/IL-18. However, one difference between the two models is the duration of IL-10 presence in the culture. In the LPS hyporesponsiveness model, inhibitory cytokines are removed before addition of IL-12/IL-18, whereas they remain constantly present in the direct IL-12/IL-18 response model. Therefore, we removed IL-10 and TGF-β in the direct IL-12/IL-18 response model after preincubation to

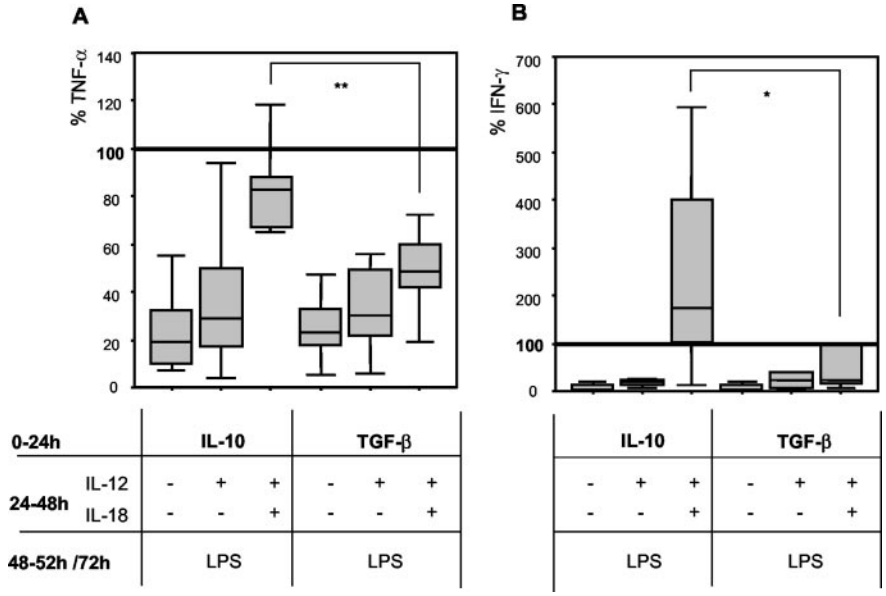

FIGURE 3. IL-12/IL-18 in combination are able to revert IL-10- but not TGF-β-induced LPS hyporesponsiveness. PBMCs were incubated with the desensitizing stimulus (LPS or IL-10/TGF-β) for 24 h, washed extensively, and then either left untreated or stimulated with IL-12 and/or IL-18 as indicated. After 48 h, cells were restimulated with LPS and supernatants were collected after 4 or 24 h to measure TNF-α (*A*) and IFN-γ (*B*), respectively. The response of nondesensitized cells treated with IL-12/IL-18 was set to 100% as described in Fig. 1 (indicated by the black line). Data of 10 independent experiments representing 7 individual blood donors (TNF-α) or 6 independent experiments representing 5 individual blood donors (IFN-γ) are shown as box plots with bars representing the medians. Boxes comprise the range between the 25th and the 75th percentile (interquartile range). The lines extending from the interquartile range include values within 1.5 times the interquartile range from the upper or lower quartile. Points at a greater distance from the median are omitted from the graphic (outliers).

have comparable conditions in both models. Indeed, IL-10 lost its inhibitory effect on IL-12/IL-18 responsiveness after removal, whereas the inhibitory effect of TGF-β was long-lasting even in its absence (data not shown).

We conclude from these data that IFN-γ-producing cells remain unresponsive to IL-12/IL-18 as long as IL-10 is present. However, these cells do not demonstrate an impaired IL-12/18-responsiveness after removal of IL-10 and therefore produce IFN-γ upon exogenous addition of IL-12/IL-18.

The inhibitory effect of IL-10 on IL-12/IL-18-induced IFN-γ production depends on the presence of monocytes

IL-10 is known to inhibit T cell function indirectly by impairing APC function. Additionally, it has recently been shown that macrophages from LPS-desensitized mice can transfer LPS hyporesponsiveness to nondesensitized NK/T cells, suppressing their IFN-γ production (11). We therefore aimed at investigating the role of monocytes for the inhibitory function of IL-10 and TGF-β.

To this end, we depleted $CD14^+$ monocytic cells from PBMCs using magnetic cell separation before setting up the IL-12/IL-18 response model. As shown in Fig. 5*A*, the inhibitory effect of IL-10 on IL-12/18-induced IFN-γ production was lost in the absence of $CD14^+$ monocytes. Reconstitution of CD14-depleted PBMCs with purified $CD14^+$ cells re-established the inhibition of IFN-γ production by IL-10, which demonstrates that the inhibitory effect of IL-10 depends on the presence of monocytes (Fig. 5*B*). In contrast, the inhibitory action of TGF-β was not affected by the depletion of $CD14^+$ cells and is therefore independent of monocytes.

In our model, we tried to rule out all known effects of APCs on IFN-γ production by providing these stimuli exogenously (anti-CD3/anti-CD28, IL-2 and IL-12, and IL-18). Nevertheless, we noticed a requirement for monocytes for the inhibitory action of IL-10 and observed a decreased IFN-γ production in CD14-depleted cells compared with PBMCs. Therefore, we sought for additional monocyte-derived IFN-γ-inducing factors that could constitute a target of IL-10 action.

Inhibition of IL-1β and TNF-α expression, but not modulation of costimulatory molecules contributes to the inhibitory effect of IL-10

First, we investigated the role of costimulatory molecules. We determined the levels of CTLA-4, inducer costimulator molecule, and CD40 ligand in IL-12/18-stimulated T cells by FACS analysis. Neither IL-10 nor TGF-β inhibited the activation-induced up-regulation of these molecules in our system (data not shown). Furthermore, the addition of blocking Abs to CD80 and CD86 neither inhibited the production of IFN-γ in response to IL-12/IL-18 nor its down-regulation by IL-10 and TGF-β (Fig. 6).

Next, we looked for possible soluble mediators to enhance IFN-γ production apart from IL-2, IL-12, and IL-18. We investigated the involvement of IL-1β and TNF-α. Both cytokines are described as proinflammatory cytokines to be down-regulated by

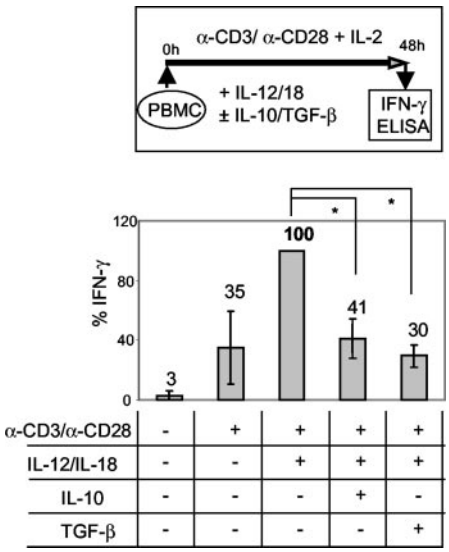

FIGURE 4. IL-10 and TGF-β inhibit IL-12/IL-18-induced IFN-γ production in PBMCs. Freshly isolated PBMCs were stimulated with anti-CD3/anti-CD28 Abs, IL-2, and IL-12/IL-18 as indicated. IL-10 or TGF-β was added simultaneously with the other stimuli and supernatants were collected after 48 h to measure IFN-γ. Results are shown as mean values $\pm$ SD from nine independent experiments with different blood donors. IFN-γ production of IL-12/IL-18-stimulated cells was set to 100% in each individual experiment because of large interindividual variation of IFN-γ production. Absolute concentrations of IFN-γ ranged from 13.7 to 47.3 ng/ml. Significant differences are indicated by an asterisk ($*$, $p < 0.05$) and were determined in comparison to IL-12/18-stimulated cells.

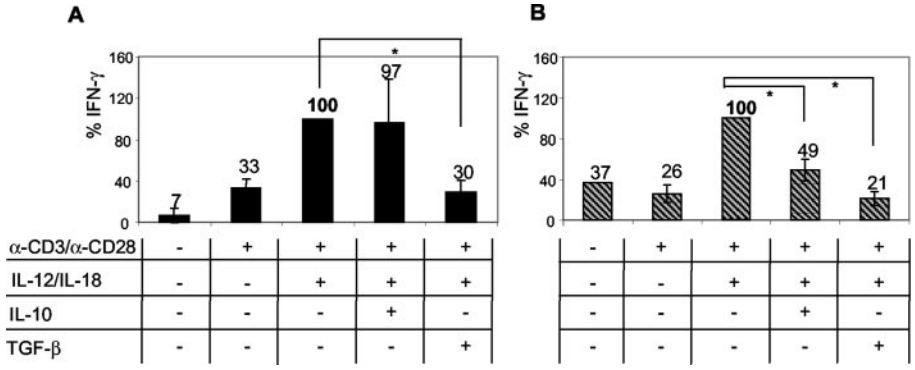

FIGURE 5. The inhibitory effect of IL-10 on cytokine-induced IFN-γ production depends on the presence of monocytes, whereas TGF-β exerts its effect directly on T cells. $CD14^+$ cells were depleted from freshly isolated PBMCs by using anti-CD14 MACS beads before setting up the simultaneous stimulation assay (*A*). As control, we re-added $CD14^+$ cells to the depleted cells (*B*). In both setups, cells were stimulated with anti-CD3/anti-CD28 Abs and IL-12/IL-18, and IL-10 or TGF-β was added simultaneously. Results are shown as mean values from six independent experiments with different blood donors. IFN-γ production of IL-12/IL-18-stimulated cells was set at 100% in each individual experiment and for *A* and *B* independently. Absolute IFN-γ concentrations ranged from 4.1 to 29.7 ng/ml (*A*) and from 10.8 to 72.0 ng/ml (*B*). Significant differences are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$) and were calculated in comparison to IL-12/IL-18-treated cells.

IL-10 in monocytes and were shown to synergize with IL-12 for IFN-γ production in T cells, similar to IL-18 (38, 39). We noticed that TNF-α and IL-1β are produced at significant levels in our IL-12/IL-18 stimulation model, which could be caused by cell-cell contact between activated T cells and monocytes (40, 41). The production of both cytokines was inhibited by IL-10 but not by TGF-β (data not shown). The addition of IL-1β and TNF-α to CD14-depleted cells resulted in a strong increase in IFN-γ production, so that these cells reached cytokine levels of nondepleted cells (Fig. 7).

Therefore, we asked whether the inhibition of IL-1β and TNF-α by IL-10 contributes to its inhibitory effect on cytokine-dependent IFN-γ production and added rIL-1β and TNF-α exogenously to compensate for their inhibition by IL-10. Fig. 8 shows that the inhibitory effect of IL-10 was decreased from 70% suppression in IL-12/IL-18-stimulated cells to 30% in cells stimulated additionally with exogenous IL-1β and TNF-α. Nevertheless, the residual inhibitory effect of IL-10 remained significant ($p < 0.05$) even when all IFN-γ-inducing factors (IL-12, IL-18, TNF-α, IL-1β) were added in combination. This suggested that an additional IFN-γ-inducing factor is produced in our system which is a target for the inhibitory action of IL-10.

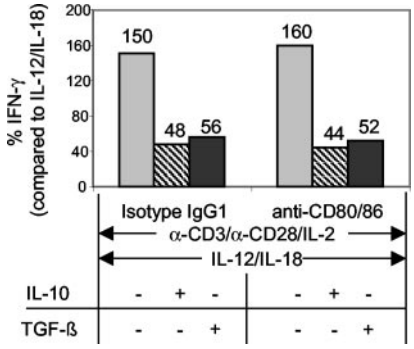

FIGURE 6. CD80/CD86 are not involved in IFN-γ induction or the inhibitory effect of IL-10 or TGF-β. PBMCs were simultaneously incubated with anti-CD3/anti-CD28, IL-2, and IL-12/IL-18 in the presence or absence of IL-10 or TGF-β as indicated. Blocking Abs against CD80 and CD86 or an isotypic control Ab (15 μg/ml each) were added at the beginning of the assay. IFN-γ levels were measured after 48 h by ELISA and are shown as percentage of the IFN-γ response induced by IL-12/IL-18, which was set to 100%. One representative experiment of two is shown.

IL-15 was recently described to enhance IFN-γ production (42, 43). However, in our experimental setup, exogenous IL-15 did not enhance IFN-γ production in the presence of IL-12/IL-18 nor reduce the inhibitory effect of IL-10 (data not shown). This might be due to the presence of high amounts of IL-2 in our model, which can be redundant to IL-15.

Exogenous addition of TGF-β during LPS desensitization prevents reversibility of LPS hyporesponsiveness by IL-12/IL-18

Our data show that only TGF-β but not IL-10 can directly interfere with IL-12/IL-18 responsiveness in IFN-γ-producing cells. In conclusion, a sufficient presence of active TGF-β should inhibit IFN-γ production in response to IL-12/IL-18 and therefore abolish reversibility by IL-12/IL-18 also in the LPS desensitization model. Nevertheless, the fact that LPS desensitization can be reverted by IL-12/IL-18 demonstrates that IFN-γ-producing cells remain responsive to IL-12/18 in this case. Therefore, we wondered whether

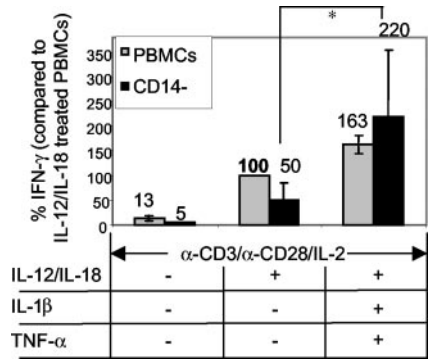

FIGURE 7. IL-1β and TNF-α enhance IFN-γ production in PBMCs and CD14-depleted cells. PBMCs were simultaneously incubated with anti-CD3/anti-CD28, IL-2, IL-12/IL-18, and IL-10 or TGF-β as indicated. In addition, IL-1β and/or TNF-α were added at a concentration of 10 ng/ml each. The IFN-γ response induced by IL-12/IL-18 in PBMCs is set at 100% in each individual experiment and all other results are shown in relation to this. Absolute values range from 0.25 to 14.9 ng/ml IFN-γ. IFN-γ production of CD14-depleted cells (■) is depicted compared with PBMCs (▨). Results are shown as mean values ± SD from five independent experiments with different blood donors. For statistical analysis, values of PBMCs or CD14-depleted cells were compared with the respective cell type treated with IL-12/18 alone. Significant differences are indicated by an asterisk (*, $p < 0.05$).

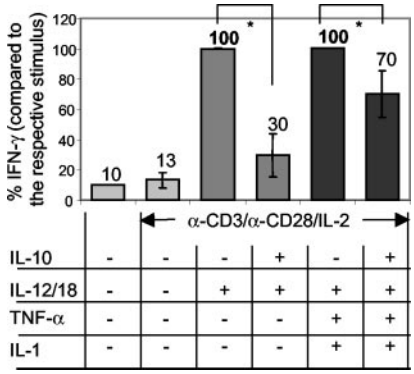

FIGURE 8. IL-1β and TNF-α contribute to the inhibitory effect of IL-10 but do not entirely account for it. PBMCs were simultaneously incubated with anti-CD3/anti-CD28, IL-2, IL-12/IL-18, and IL-10 or TGF-β as described in Fig. 4. In addition, IL-1β and/or TNF-α were added at a concentration of 10 ng/ml each. IFN-γ levels of IL-12/IL-18 alone (□) and plus IL-1β/TNF-α (■) were set at 100% in each case to point out the inhibitory effect of IL-10 for each stimuli. Results are shown as mean values ± SD from five independent experiments with different blood donors. Significant differences are indicated by an asterisk (*, $p < 0.05$).

sufficient amounts of TGF-β are actually produced during LPS pretreatment as originally suggested (6, 13).

To test this hypothesis, we added the same amount of activated rTGF-β (10 ng/ml) during LPS pretreatment as we had used for the induction of LPS hyporesponsiveness by TGF-β alone. Indeed, the exogenous addition of TGF-β during LPS desensitization rendered these cells unresponsive to a reversal of TNF-α production by IL-12/IL-18 (Fig. 9*A*, $p < 0.05$ LPS/TGF-β vs LPS). Surprisingly, we observed a significant IFN-γ production by these cells after incubation with IL-12/IL-18, which was comparable to untreated control PBMCs under the same conditions (Fig. 9*B*). Nevertheless, IFN-γ production in LPS/TGF-β-pretreated cells was significantly ($p < 0.05$) lower compared with LPS-pretreated cells whose function was fully restored by IL-12/IL-18. Thus, residual IFN-γ production after LPS/TGF-β pretreatment and IL-12/IL-18 stimulation does not seem to be sufficient to overcome LPS hyporesponsiveness.

Therefore, we conclude that TGF-β is the major factor to prevent reversibility of LPS hyporesponsiveness by IL-12/IL-18 due to its direct interference with IL-12/IL-18 responsiveness of IFN-γ-producing cells. The amount of TGF-β produced or activated by low-dose LPS pretreatment is obviously not sufficient to inhibit the response to exogenous IL-12/IL-18.

Discussion

The present study aimed at further investigating the role of the anti-inflammatory cytokines IL-10 and TGF-β in LPS desensitization, which is considered to be an experimental model for the immunoparalytic state observed in late-stage septic patients. Our results suggest that the model of LPS desensitization only partially reflects the dysfunction of immune cells (particularly of T cells) observed in immunoparalytic patients.

LPS desensitization was reported to be mediated by endogenous IL-10 and TGF-β and both cytokines can also directly induce a similar state of LPS hyporesponsiveness. (6, 13). However, these models were found to differ regarding their reversibility by IL-12 (7, 15). Remarkably, PBMCs from immunoparalytic patients rather resembled the phenotype of IL-10/TGF-β-pretreated cells than that of LPS-desensitized cells, since LPS hyporesponsiveness of these cells was not reversible by IL-12 (15). Therefore, it seemed important to elucidate the differences between the individual models and to investigate the mechanisms of IL-10 and TGF-β for inducing a LPS-hyporesponsive state. Our data on the different mechanisms of IL-10 and TGF-β for inhibiting IL-12/IL-18 responsiveness in T/NK cells helped to explain the observed phenotypes of IL-12/IL-18 reversibility in the different LPS hyporesponsiveness models (summarized in Fig. 10).

We demonstrated that the inhibitory effect of IL-10 on IFN-γ-producing cells is mainly due to the suppression of IFN-γ-inducing cytokines produced by monocytes. This explains why the substitution of these factors enables the reversal of IL-10-induced LPS hyporesponsiveness in monocytes. However, we noticed that, even

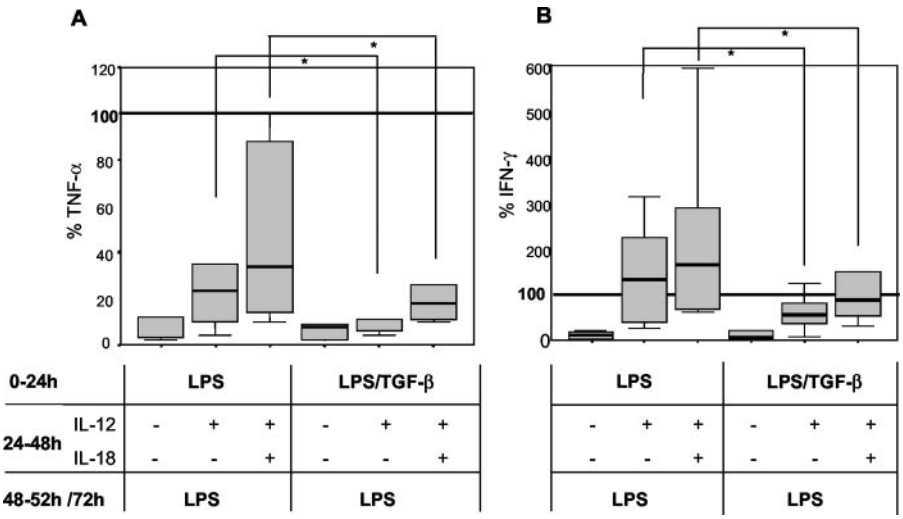

FIGURE 9. TGF-β has an additional desensitizing effect when given simultaneously with LPS. PBMCs were incubated with the desensitizing stimulus (LPS or LPS/TGF-β) for 24 h, washed extensively, and then either left untreated or stimulated with IL-12 and/or IL-18 as indicated. Four and 24 h after restimulation with LPS, supernatants were collected to measure TNF-α and IFN-γ levels, respectively. Results are shown as percentage of cytokine production by IL-12/IL-18-stimulated control cells. Data of six independent experiments from five different blood donors are depicted as box plots with bars representing the medians. Boxes comprise the range between the 25th and the 75th percentile (interquartile range). The lines extending from the interquartile range include values within 1.5 times the interquartile range from the upper or lower quartile. Points at a greater distance from the median are omitted from the graphic (outliers). The black line indicates cytokine production of untreated control cells stimulated with IL-12/IL-18, which was set to 100% as described in Fig. 1.

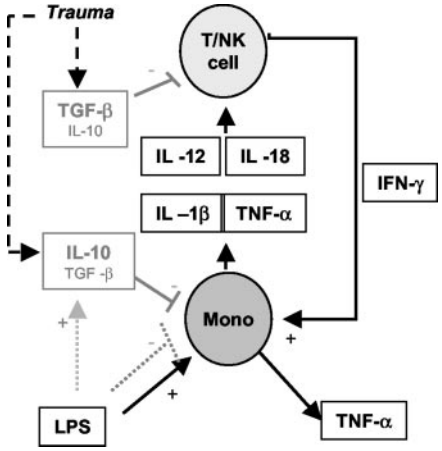

FIGURE 10. Schematic summary of results. LPS stimulation of monocytes (black arrows) leads to the production of proinflammatory cytokines (IL-12, IL-18, IL-1β, TNF-α) which act on T/NK cells to induce IFN-γ. This provides a positive feedback loop which amplifies TNF-α production in monocytes. The anti-inflammatory cytokines (gray arrows) IL-10 and TGF-β act in different ways to interfere with the normal response to LPS. IL-10 targets mainly the production of IFN-γ-inducing cytokines in monocytes but not T/NK cells. Therefore, exogenous IFN-γ-inducing cytokines in combination can restore the normal IFN-γ and TNF-α response to LPS. In contrast, TGF-β interferes directly with the response of T/NK cells to IFN-γ-inducing cytokines. Therefore, it prevents restoration of a normal response to LPS by exogenous IFN-γ-inducing cytokines. In this case, monocytic dysfunction can only be restored by exogenous IFN-γ. LPS desensitization (dotted gray arrows) acts mainly via induction of IL-10 and additional intrinsic effects on monocytes, but does not affect T/NK cell responsiveness. In critically ill patients, activation of TGF-β (and IL-10) by trauma (dotted black arrows) in addition to endotoxin translocation can occur which may lead to a development of immunoparalysis by the combined effects of IL-10 and TGF-β.

in the presence of all IFN-γ-inducing cytokines used in this study (IL-12, IL-18, IL-1β, IL-2, IL-15, and TNF-α), IL-10 still significantly inhibited IFN-γ production. This suggests that either an as yet unknown inhibitory factor is induced by IL-10 or that additional monocytic factors are involved in cytokine-dependent IFN-γ production and that the production of these factors is inhibited by IL-10. In this regard, interesting novel candidates, including the IL-12 homologues IL-23 and IL-27, have recently been described (44, 45), but further studies are required to determine their relevance for LPS desensitization.

In contrast to IL-10, TGF-β directly inhibits IFN-γ production by T cells and NK cells and the LPS-hyporesponsive state induced by TGF-β is therefore not reversible by IL-12/IL-18. Because LPS desensitization is reversible by IL-12/IL-18, a major involvement of TGF-β in its induction became rather unlikely. Indeed, addition of recombinant TGF-β during the process of LPS desensitization rendered this model unresponsive to a reversal by IL-12/IL-18. This suggests active TGF-β as the major factor determining the reversibility of an LPS-hyporesponsive state. Therefore, the in vitro model of LPS desensitization is probably rather mediated by a temporary presence of IL-10 than by TGF-β. Apart from endogenous IL-10, other intrinsic mechanisms are likely to be involved in LPS desensitization. Described potential mechanisms include alteration of G protein binding (46, 47), impaired mitogen-activated protein kinase (48), or Toll-like receptor 4 signaling involving the induction of IL-1R-associated kinase M (49) and suppressor of cytokine signaling 1/3 (50–52), as well as increased expression of the p50 subunit of NF-κB (53, 54). These intrinsic mechanisms might explain why the restoration of TNF-α production did not completely correlate to the restored IFN-γ levels in our experiments.

We believe that our results from this in vitro model have important implications also for the interpretation of results obtained from in vivo LPS desensitization models as well as of clinical observations. Recent findings in an in vivo LPS desensitization model by Varma et al. (11) resemble several aspects of our study. In their model, the suppression of IFN-γ production was also dependent on the presence of macrophages, which points to an involvement of IL-10 according to our results. Furthermore, T/NK cells from LPS-tolerant mice remained fully responsive to IL-12/IL-18 regarding IFN-γ production, which supports the view that at least in this in vivo model of LPS desensitization IL-10 and not TGF-β is the dominant mediator of endotoxin tolerance.

IL-10 by itself cannot induce an immunoparalytic state in patients. We observed only a temporary immune suppression during the treatment of psoriatic patients with IL-10 (55). These data imply that, in addition to IL-10, other immunosuppressive factors have to be involved in the pathogenesis of immunoparalysis.

TGF-β is mainly regulated at the level of activation via proteolytic processes rather than at the transcriptional level, and large amounts of inactive TGF-β can be found in human plasma. TGF-β can be activated by stimulated thrombocytes during inflammation and wound healing. This process might be missing in simple models of LPS shock or desensitization. However, patients with immunoparalysis usually suffer from severe trauma, which can trigger the activation of TGF-β in vivo. Therefore, the severity of trauma might be an additional factor contributing to the level of immunoparalysis in patients. However, the proof for an involvement of TGF-β in the establishment of immunoparalysis has been difficult to provide so far. This could be due to the fact that a local activation of high amounts of TGF-β might be sufficient to impair T and NK cell functions. Furthermore, these investigations could be confounded by the fact that the inhibitory effect of TGF-β is long-lasting and persists for at least 20 h.

Preliminary experiments suggest that PBMCs from septic patients with immunoparalysis show a varying responsiveness to IL-12/IL-18 stimulation (most of them are resistant to IL-12/IL-18), which might be due to a different level of exposure to TGF-β in vivo (our unpublished data). We are currently expanding these investigations and their impact for prognosis in a clinical study.

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