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IL-18-independent cytotoxic T lymphocyte activation and IFN-γ production during experimental acute graft-versus-host disease

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Abstract

Acute graft-versus-host disease (GvHD) is a serious complication after allogeneic bone marrow transplantation. Donor-derived T cells infiltrate recipient target organs and cause severe tissue damage, often leading to death of the affected patient. Tissue destruction is a direct result of donor $CD8^+$ T cell activation and cell-mediated cytotoxicity. IL-18 is a novel pro-inflammatory cytokine with potent T_h1 immune response-promoting and cytotoxic T lymphocyte (CTL)-inducing activity. IL-18 is strongly induced in experimental mouse models and human patients with acute GvHD. However, the precise role of IL-18 in the development of acute GvHD is still unknown. In this study, we have used IL-18-binding protein, a soluble IL-18 decoy receptor, to specifically neutralize IL-18 *in vivo* and *in vitro*. Our results demonstrate that IL-18 is induced during GvHD. However, its effect in the induction of GvHD appears to be redundant, since neutralization of IL-18 does not alter any disease parameter analyzed. Our study further shows that IFN-γ production and CTL induction upon activation by T cell mitogens or by alloantigen does not involve IL-18-mediated amplification, in contrast to lipopolysaccharide-induced IFN-γ production. We conclude that IL-18 expression correlates with the course of GvHD; however, its effect is dispensable for IFN-γ and CTL induction for the initiation phase of this disease, most likely due to direct, IL-18-independent, CTL activation.

Introduction

Acute graft-versus-host disease (GvHD) is one of the most common complications after allogeneic bone marrow transplantation. Recognition of alloantigen leads to the activation of donor T cells and the development of cytotoxic effector functions results in severe tissue damage, which may even lead to the death of the affected patient. The two major cytotoxic mechanisms in T cell-mediated killing of target cells involve either the release of cytotoxic granule proteins, such as perforin and granzymes, or the expression of the apoptosis-inducing Fas (CD95/APO-1) ligand (FasL) [reviewed in (1–3)]. Both pathways appear to be important in the pathogenesis of systemic GvHD in experimental mouse systems. Inhibition of the FasL activity (4–9) or perforin (8) ameliorates certain aspects of GvHD-associated pathology, whereas inhibition of both cytotoxic effector mechanisms abrogates the development of lethal GvHD (10).

IL-18 is a recently described member of the IL-1 cytokine family. Initially characterized as an IFN-γ-inducing cytokine, subsequent studies have demonstrated a multitude of functions beyond lymphocyte activation [reviewed in (11–13)]. IL-18 is expressed by a variety of cell types, including activated monocytes/macrophages, dendritic cells, keratinocytes and

tissue macrophages, such as Kupffer cells [reviewed in (11–13)]. Similar to IL-12, IL-18 supports T_h1 cell differentiation, induces cytokine production, such as IFN-γ, tumor necrosis factor-α and granulocyte macrophage colony stimulating factor, and enhances both FasL- and perforin-dependent cytotoxic effector functions of T cells and NK cells (14–18). The *in vivo* functions of IL-18 in acute and chronic immune responses are as yet insufficiently understood. During experimental cytomegalovirus infection, IL-18 contributes together with IL-12 to IFN-γ induction and T cell-mediated anti-viral immune response (19). Accordingly, NK cell activity and T_h1 responses appear to be impaired in IL-18-deficient mice (18). Thus, these reports suggest an important role for IL-18 in the development of T_h1-mediated immune responses and cell-mediated cytotoxicity.

Acute GvHD is a largely T_h1-dominated immune response, where $CD8^+$ T cell-mediated cytotoxicity causes severe tissue damage. IFN-γ, produced during the initiation phase of the disease, appears to be involved in the development of these cytotoxic effector T cells and thus the pathogenesis of acute GvHD, since neutralization of IFN-γ impairs cytotoxic T lymphocyte (CTL) development and FasL expression (6,20). The role of IL-18 as an IFN-γ-inducing and T_h1-polarizing cytokine in the pathogenesis of acute GvHD is currently unknown. Recent studies in mice and human patients have shown that elevated IL-18 expression is detected in serum and spleen of acute GvHD-affected patients and animals, correlating with the disease progression and serum IFN-γ levels (21–23). Therefore, IL-18 may play a disease-promoting, pro-inflammatory role in the onset of acute GvHD. In contrast to acute GvHD, chronic GvHD is associated with diminished donor anti-host CTL activity, the expansion of auto-reactive recipient $CD4^+$ T cells and B cells, and subsequent auto-antibody production [reviewed in (24)]. Most interestingly, therapeutic IL-18 administration can prevent the development of chronic GvHD by the induction of regulatory alloreactive $CD8^+$ cytotoxic donor T cells (25).

In this study, we have investigated in an animal model the expression of IL-18 during the onset of acute GvHD, and its role in GvHD-associated IFN-γ production and CTL development. IL-18 was specifically neutralized by *in vivo* administration of recombinant IL-18-binding protein (IL-18 BP), a recently described soluble high-affinity decoy receptor for IL-18 (26–28). While IL-18 expression was found to be readily increased during the course of acute GvHD, its neutralization did not inhibit donor T cell expansion, IFN-γ serum levels or anti-host CTL activity. *In vivo* and *in vitro* experiments further revealed that IFN-γ production by T cells upon activation by alloantigen or T cell mitogens does not involve IL-18-mediated amplification. We conclude that IL-18-mediated effects are redundant or non-essential for the development of acute GvHD.

Methods

Mice and reagents

Female C57BL/6 ($H2^b$) and C57BL/6 × DBA2 F_1 (B6D2F1) ($H2^{b/d}$) mice (7–10 weeks old) were purchased from BRL (Fuellinsdorf, Switzerland). Anti-CD8α–CyChrome, anti-CD8β–phycoerythrin (PE), anti-CD3–FITC, anti-TCRαβ–FITC, anti-TCRγδ–FITC and anti-$H2^d$–biotin were obtained from PharMingen (La Jolla, CA). Streptavidin–PE was from Southern Biotechnology (Birmingham, AL). Recombinant 6-histidine-tagged human IL-18 BP, isoform a, was produced in COS cells. Purification and assessment of its IL-18-neutralizing activity was performed as described by others previously (26,29). Recombinant mouse IL-18 was obtained from R & D Systems (Minneapolis, MN), and lipopolysaccharide (LPS), staphylococcal enterotoxin B (SEB) and concanavalin A (Con A) from Sigma (St. Louis, MO). [^{3}H]Thymidine was purchased from Amersham Pharmacia (Duebendorf, Switzerland). Cyclosporin A (Sandimmun) was from Novartis (Basel, Switzerland). Culture medium consisted of RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol and 20 mM HEPES, pH 7.4. HEPES-buffered salt solution (HBS) consisted of 10 mM HEPES, pH 7.2, 25 mM $NaHCO_3$, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 137 mM NaCl and 5.6 mM D-glucose. Percoll and Ficoll were purchased from Amersham Pharmacia (Uppsala, Sweden). Isotonic Percoll solution was made by adding 1/10 of the volume of 10 times concentrated HBS.

Induction of experimental acute GvHD

Experimental acute GvHD was induced as described previously (4). Briefly, spleen cells from C57BL/6 or B6D2F1 mice were isolated by dissociation of the spleen between frosted microscopy slides, followed by hypotonic lysis. GvHD was induced by injection of 10^8 C57BL/6 spleen cells into the tail vein of B6D2F1 recipients. Control mice received equal numbers of syngeneic B6D2F1 spleen cells. In some experiments mice received daily treatment of 150 μg IL-18 BP or PBS control by i.p. injection. After 4, 8 and 12 days mice were sacrificed, serum was collected, and spleen, small and large intestine, and liver were isolated. Some tissue samples were either embedded in Tissue Tek cryosection medium (Sakura Finetek, Zoeterwoude, Netherlands) or fixed in 4% paraformaldehyde in PBS and embedded in paraffin.

Isolation of T cells

Intraepithelial lymphocytes (IEL) from small bowel were isolated as described before (4). Briefly, epithelial cells and IEL were dissociated in HBS containing 1 mM DTT and separated on a 40/70% Percoll gradient. The interphase containing enriched IEL (usually between 40 and 70% $CD8^+$ cells) was washed, resuspended in culture medium and used for further experiments. Spleen cells were isolated as described above.

Assessment of T cell subsets

After isolation, spleen cells or IEL from small intestine were washed in PBS, 1% calf serum, 0.05% sodium azide (wash buffer), and stained with anti-CD8α, anti-CD8β, anti-TCRαβ, anti-TCRγδ, anti-CD3 and anti-$H2^d$. After two washes, cells were fixed in 1% paraformaldehyde in PBS and analyzed by flow cytometry on a FACScan using CellQuest software (Becton Dickinson, San Jose, CA). An electronic gate was set around the lymphocyte population based on forward and side scatter properties, and individual lymphocyte subpopulations were compared to total numbers of lymphocytes.

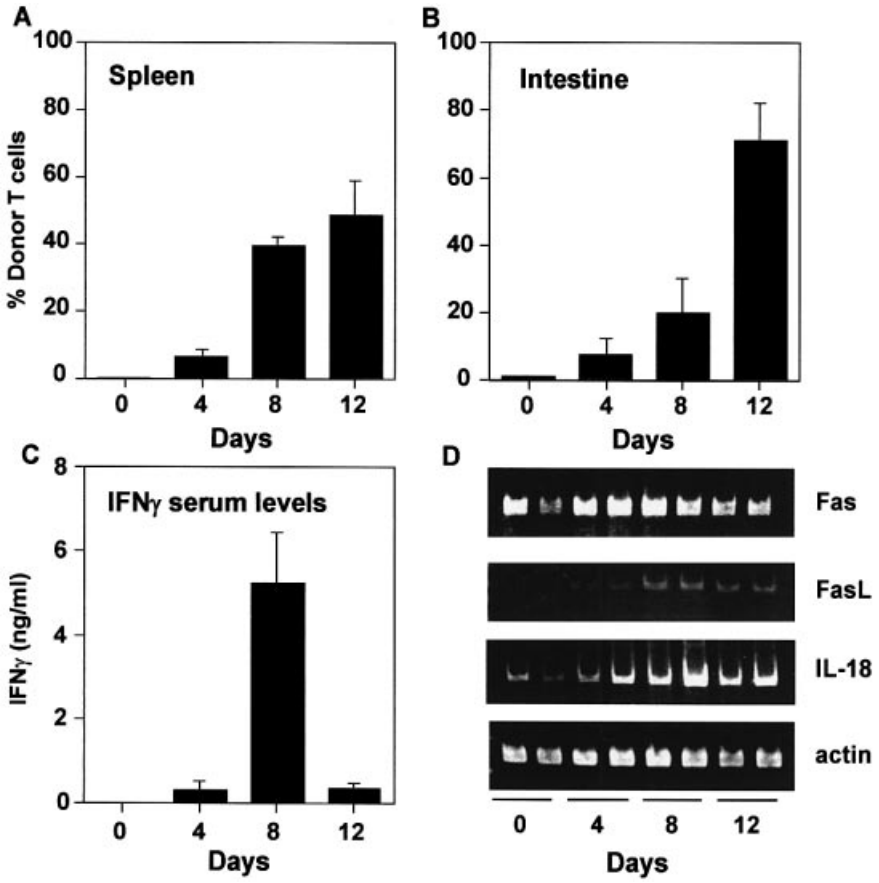

Fig. 1. Kinetics of acute GvHD and associated factors. Acute GvHD was induced as described in Methods. At indicated time points, mice were analyzed for donor T cell infiltration and gene expression. Percentage of donor T cells ($H2^{d-}$ $CD3^{+}$) in spleen (A) and small intestinal epithelial layer (B). (C) Serum levels of IFN-γ. Four mice were analyzed per time point. Mean values ± SD are shown. (D) Fas, FasL, IL-18 and actin expression in the spleen was analyzed by RT-PCR. Two mice per time point were analyzed.

Assessment of gene expression by RT-PCR

Fas, FasL, IFN-γ and IL-18 expression was detected by semiquantitative RT-PCR. Spleens from control animals or animals with ongoing GvHD were isolated and frozen in liquid nitrogen until further use. Total RNA was then isolated using Tri-reagent (Sigma, St Louis, MO) and the manufacturer's suggested protocol. cDNA was synthesized from 2 μg total RNA using a commercial cDNA kit (Promega, Madison, WI), oligo(dT) primers and the manufacturer's suggested conditions. PCR for Fas, FasL, IFN-γ, IL-18 and actin as a positive control was performed as described previously (20,23,30). The following primers were used: mFas forward 5′-GAGGACTGCAAAATGAATGGGG-3′, mFas reverse 5′-ACAACCATAGGCGATTTCTGGG-3′; mFasL forward 5′-CAGCAGTGCCACTTCATCTTGG-3′, mFasL reverse 5′-TTCACTCCAGAGATCAGAGCGG-3′; mIL-18 forward 5′-ACTGTACAACCGCAGTAATACGG-3′, mIL-18 reverse 5′-AGTGAACATTACAGATTTATCCC-3′; mIFN-γ forward 5′-CTTCTTCAGCAACAGCAAGGCGAAAA-3′, mIFN-γ reverse 5′-CCCCCAGATACAACCCCGCAATCA-3′; actin forward 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, actin reverse 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′. PCR was performed in a PTC-100 thermocycler (MJ Research, Watertown, MA). cDNAs were amplified for 29 cycles (IFN-γ), 32 cycles (FasL), 30 cycles (Fas) or 20 cycles (IL-18 and actin) at 1 min 94°C, 1 min 55°C, 1 min 72°C, followed by 10 min extension at 72°C. PCR products were resolved on a 4% acrylamide–1 × TBE gel and visualized by ethidium bromide staining.

Cytotoxicity assay

Anti-host cytotoxicity was assessed by the ability of donor CTL to cause DNA fragmentation in $H2^{d+}$ target cells. Fas-transfected L1210 [L1210-Fas, $H2^{d+}$ (4,31,32)] were labeled for 2 h with 5 μCi/ml [^{3}H]thymidine (Amersham Pharmacia) at 37°C in culture medium. After two washes in PBS, cells were resuspended in culture medium and co-cultured at 20,000 cells/well in a flat-bottom 96-well tissue culture plate with effector T cells at increasing effector:target ratios. After 16 h, unfragmented target cell DNA was harvested on glass fiber filters and cytotoxicity was calculated as follows: DNA fragmentation (%) = 100 × (1 – c.p.m. experimental group/c.p.m. control group). All assays were done in triplicates.

In vitro IL-18 neutralization assay

Spleen cells from C57BL/6 mice were isolated as described above. Cells (3×10^{6}/ml) were then cultured in 24-well plates, either unstimulated or stimulated with 1 μg/ml LPS, 10 ng/ml

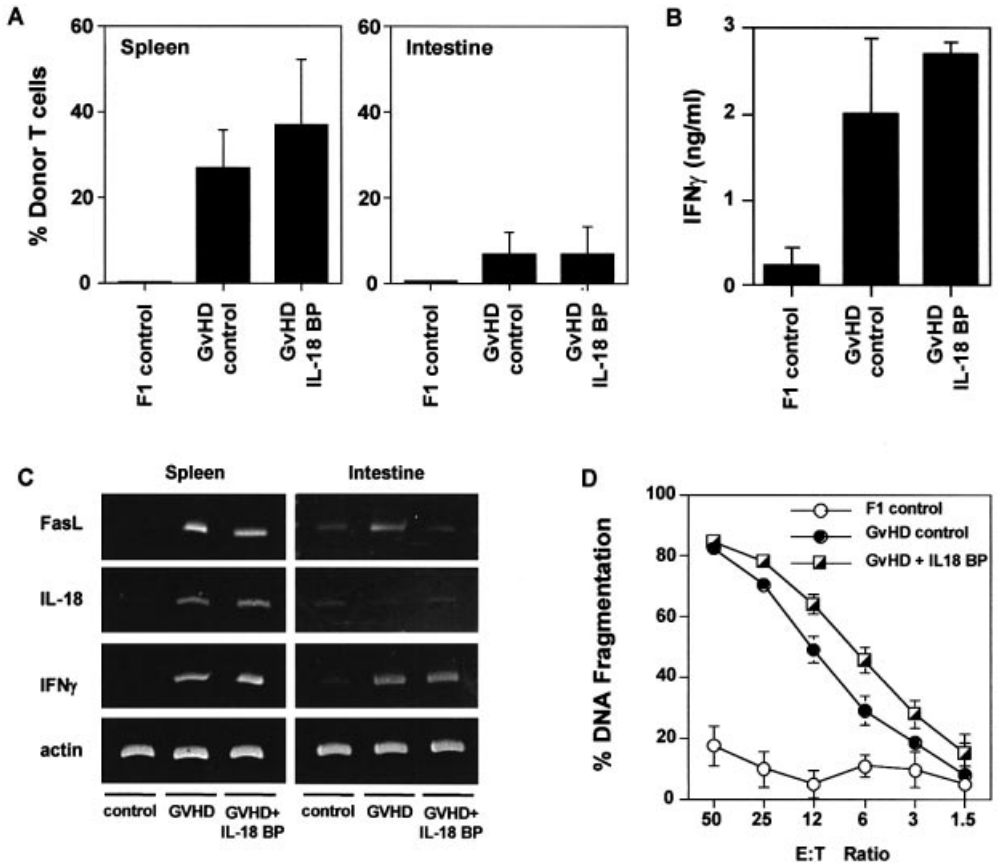

Fig. 2. Effect of IL-18 BP on the development of acute GvHD. Mice were induced to develop acute GvHD and treated by daily i.p. injection of PBS (F_1 control, GvHD control) or 6 mg rIL-18 BP/kg body wt (GvHD + IL-18 BP). After 8 days mice were sacrificed and analyzed for donor T cell ($H2^{d-}$ $CD3^+$) infiltration in spleen and small intestinal epithelium (intestine) (A), and IFN-γ serum levels (B). Three mice per group were analyzed. Mean values ± SD are shown. (C) FasL, IL-18, IFN-γ and actin mRNA expression in spleen and small intestine was analyzed by RT-PCR. (D) Anti-host ($H2^d$) cytotoxicity. Spleen cells from control animals, GvHD animals or GvHD animals treated with IL-18 BP were incubated at different effector:target (E:T) ratios with $H2^{d+}$ target cells and target cell DNA fragmentation was calculated. Three mice per group were analyzed. Mean values ± SD are shown. All data shown in (A)–(D) originate from the same experiment. A typical experiment out of four is shown.

IL-18, 1 μg/ml LPS plus 10 ng/ml IL-18, 2 μg/ml plate-bound anti-CD3, 10 μg/ml SEB or 1 μg/ml Con A. IL-18 was neutralized by addition of increasing concentrations of recombinant human IL-18 BP (3–300 ng/ml). After 24 h culture supernatants were harvested and IFN-γ production was assessed by ELISA. These experiments were repeated at least 3 times.

In vivo IL-18 neutralization assay

To assess the contribution of IL-18 in IFN-γ induction *in vivo*, C56BL/6 mice were either pretreated with PBS (control) or with different concentrations of IL-18 BP. After 20 min mice were then injected with 25 mg/kg body wt LPS or 500 μg/kg body wt anti-CD3. Serum was harvested after 6 h and IFN-γ levels were assessed by ELISA. Experiments were done with at least three mice per group.

Mixed leukocyte reaction (MLR)

B6D2F1 spleen cells were irradiated with 2500 rad. Cells (1×10^6) were then co-cultured with 0.4×10^6 C57BL/6 responder spleen cells in 200 μl/well in 96-well flat-bottom plates. IL-18 BP was added in increasing concentrations (3–300 ng/ml). As a positive control, 200 ng/ml cyclosporin A was used to block T cell activation. After 72 h, culture supernatants were harvested for IFN-γ measurement by ELISA or 1 μCi/well [^{3}H]thymidine was added for 16 h and [^{3}H]thymidine incorporation was measured upon harvesting total DNA on glass fibers. Stimulation index = incorporation of experimental value/incorporation of responders only. These experiments were repeated at least 3 times.

Cytokine ELISA

IFN-γ, IL-12 (p40) and IL-18 in serum or culture supernatant were assessed using commercially available ELISA kits (R & D Systems) or matched antibody pairs (PharMingen) according to manufacturer's recommendation.

Results

Kinetics of experimental acute GvHD

The transfer of parental C57Bl/6 spleen cells into B6D2F1 hosts leads to the development of acute GvHD (4,24). We

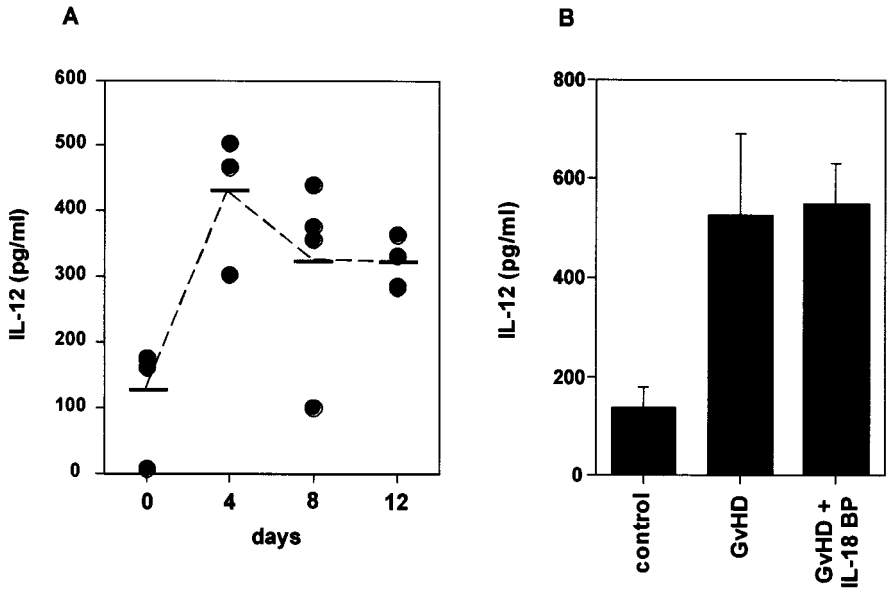

Fig. 3. Kinetic of IL-12 production during acute GvHD. (A) Serum samples of mice undergoing GvHD were collected at different time points after disease induction and IL-12 levels were measured by ELISA. Symbols indicate serum IL-12 levels of individual mice, bars indicate mean values of three to four sera per time point. (B) Effect of IL-18 BP on IL-12 synthesis during acute GvHD. Sera of control mice, or mice undergoing acute GvHD, with or without daily treatment with IL-18 BP, were collected after 8 days and IL-12 levels were measured by ELISA. Mean values ± SD are shown (n = 4).

and others have previously shown the importance of $CD8^+$ T cell-mediated cytotoxicity, in particular the relative contribution of perforin versus FasL, in the tissue destruction observed during the pathogenesis of this disease (4–9,24). In this study, we aimed at assessing the role of IL-18 in regulating disease-promoting host T cells and their effector functions. Upon transfer donor T cell infiltration was readily observed in both spleen and small intestinal epithelium, already 4 days post cell transfer. These cells then further expanded and represented the majority of the IEL in the small intestine by day 12 (Fig. 1A and B). Consequently, we observed a progressive decrease in the resident $H2^{d+}$ $TCR\gamma\delta^+$ and $TCR\alpha\beta^+$ IEL (data not shown). This expansion of donor T cells was paralleled by an increase in serum IFN-γ levels. IFN-γ was detectable at day 4, peaked at day 8 and declined thereafter (Fig. 1C).

To assess whether this increase in serum IFN-γ levels is paralleled by IL-18 and FasL induction, we examined next IL-18 and FasL expression in the spleen by RT-PCR. Figure 1(D) shows that low IL-18 mRNA expression was detectable in the spleen of control mice. However, IL-18 mRNA expression was strongly induced during acute GvHD. Similarly, FasL expression was barely detectable in the spleen of control animals, but gradually increased during the course of the disease.

Neutralization of IL-18 does not inhibit the pathogenesis of acute GvHD

IL-18 BP is an endogenous soluble decoy receptor with potent IL-18-neutralizing activity (26,27). We have used recombinant IL-18 BP isoform a, which binds also mIL-18, to assess the role of IL-18 as a disease-promoting factor in the pathogenesis of acute GvHD. Recombinant IL-18 BP efficiently neutralized the IFN-γ-inducing activity of IL-18 *in vitro* (data not shown and Fig. 4A) and *in vivo* (data not shown and Fig. 4D). Optimal IL-18-neutralizing concentrations of recombinant IL-18 BP *in vivo* were assessed by injecting 5 mg/kg body wt LPS into C57Bl/6 mice and monitoring IFN-γ levels in the serum (26). IL-18 BP strongly blocked LPS-induced IFN-γ production over a range of 4–12 mg IL-18 BP/kg body wt (data not shown). IL-18 BP (6 mg/kg body wt) was thus injected daily into GvHD-induced animals. After 8 days, at the peak of serum IFN-γ levels (Fig. 1C), mice were sacrificed and analyzed for different aspects of acute GvHD. In both spleen as well as intestinal epithelium, a significant infiltration of $H2^{d-}$ donor T cells was observed at this stage of the disease. However, no obvious alterations in donor T cell distribution were observed upon treatment with IL-18 BP in both organs (Fig. 2A). Similar results were obtained at later stages of the disease, i.e. 14 days post disease induction (data not shown). Serum IFN-γ levels were also strongly increased after 8 days post GvHD induction, but not reduced in IL-18 BP-treated animals (Fig. 2B).

IL-18 not only affects T_h1 cell differentiation and IFN-γ production in T cells, but also enhances cell-mediated cytotoxicity and FasL expression [reviewed in (11–13)]. We have thus further assessed the expression of FasL and IFN-γ in spleen and intestine of GvHD animals. Figure 2(C) shows that GvHD caused a significant increase in FasL and IFN-γ mRNA levels in the spleen cells of GvHD-affected animals. Similarly, IFN-γ was strongly induced in the intestinal mucosa. Yet, IL-18 BP treatment had no effect on GvHD-induced IFN-γ mRNA expression in both target organs and only limited effects on FasL expression in the small intestine. Cytotoxic donor T cells recognizing host antigen on tissue cells are believed to contribute significantly to the tissue destruction in the target organs observed during acute GvHD. We have thus also analyzed anti-host cytotoxicity by splenic donor T cells. Figure

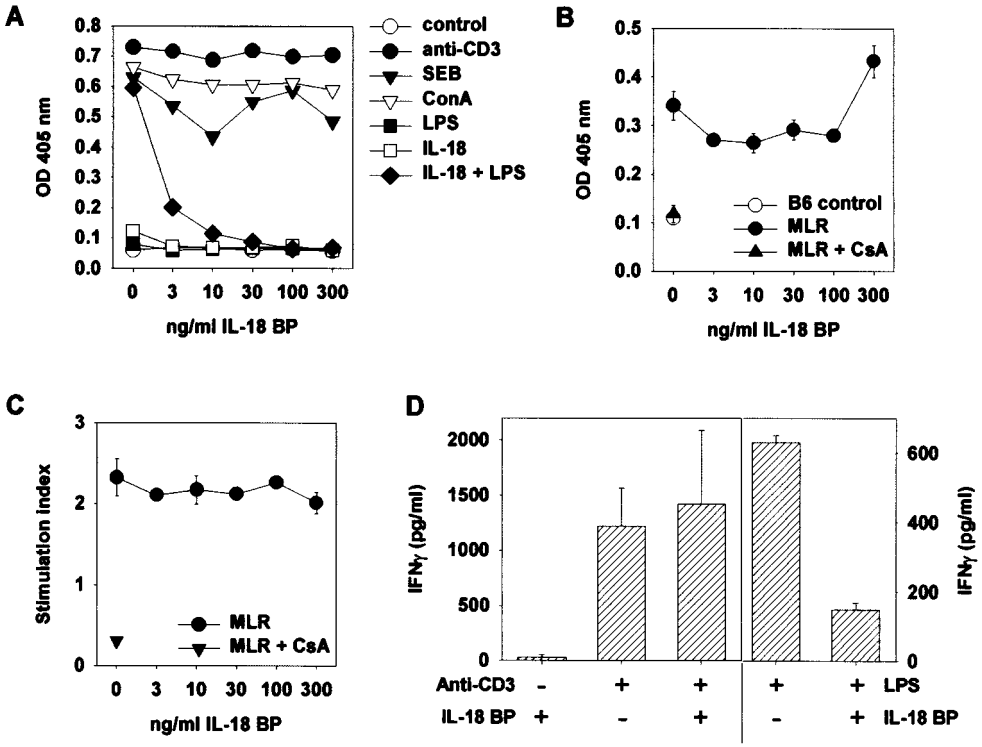

Fig. 4. Role for IL-18 in LPS-, but not mitogen-induced IFN-γ production by T cells. (A) Spleen cells were stimulated with anti-CD3, SEB, Con A, LPS, IL-18 or IL-18 plus LPS. IL-18 was neutralized by addition of increasing concentrations of IL-18 BP. IFN-γ released into the supernatant was measured by ELISA. A typical experiment out of three is shown. (B and C) MLR was induced as described in Methods. IL-18 was neutralized by increasing concentrations of IL-18 BP, as positive control 200 ng/ml cyclosporin A was added. IFN-γ secreted was measured by ELISA (B), proliferation was measured by [^{3}H]thymidine incorporation (C). Experiments were repeated 3 times with identical results. A typical experiment is shown. (D) IL-18 neutralizing effect of IL-18 BP *in vivo*. Mice were treated with 4 mg IL-18 BP/kg body wt or PBS prior to injection with anti-CD3 or LPS. Sera were collected 6 h later and IFN-γ levels were analyzed by ELISA. Three mice per groups were analyzed.

2(D) shows that during acute GvHD H2^d-specific alloreactive donor T cells were present in the spleen, resulting in potent killing of target cells. Yet, this anti-host cytotoxicity was not inhibited upon *in vivo* neutralization of IL-18 by daily administration of 6 mg IL-18 BP/kg body wt (Fig. 2D).

IL-12 production during GvHD

Another important pro-inflammatory IFN-γ-inducing cytokine is IL-12. Serum IL-12 levels were monitored in parallel during the course of acute GvHD. Figure 3(A) shows that some IL-12 was already detectable in control mice, but levels rapidly increased after induction of the disease and remained at elevated levels during the observation period. We also analyzed whether neutralization of IL-18 by IL-18 BP would affect the levels of IL-12 in the serum. Figure 3(B) shows that IL-12 is observed at increased levels at day 8; however, daily treatment with IL-18 BP had no obvious effect on IL-12 production.

Differential effects of IL-18 neutralization on LPS- and T cell activation-induced IFN-γ production

Our experimental data obtained suggest a non-essential role for IL-18 in the CTL and IFN-γ induction during the initiation phase of experimental acute GvHD. We have thus assessed how the presence or absence of bioactive IL-18 affects IFN-γ production upon TCR-mediated activation. Spleen cells were stimulated with typical T cell agonists, such as anti-CD3, SEB or Con A, or with LPS in the absence or presence of IL-18, and IFN-γ production was analyzed. In spleen cell cultures both LPS and IL-18 alone were insufficient in inducing IFN-γ expression. In contrast, simultaneous treatment with LPS and IL-18 induced high levels of IFN-γ (Fig. 4A). This synergistic activity most likely depends on the LPS-induced IL-12 production in monocytes/macrophages and IL-12-induced up-regulation of IL-18 receptors on T cells (33). IL-18-induced IFN-γ production was inhibited by the addition of as low as 3 ng/ml IL-18 BP. In contrast, anti-CD3-, Con A- and SEB-induced IFN-γ production was not affected by the neutralization of IL-18 (Fig. 4A). Thus, IFN-γ expression upon direct T cell stimulation *in vitro* does not appear to require IL-18.

We next analyzed the role of IL-18 in the induction of IFN-γ in an *in vitro* activation model that resembles acute GvHD more closely. In a MLR, responder T cells (C57Bl/6) recognize alloantigen on irradiated stimulator spleen cells (B6D2F1). As a result, alloreactive responder T cells get activated, proliferate and produce cytokines, such as IFN-γ. In this experimental set-up, we have assessed the role of IL-18 on T cell proliferation and IFN-γ production. Whereas low concentrations (3–100 ng/ml) of IL-18 BP slightly reduced MLR-induced IFN-γ production, higher concentrations even enhanced

cytokine secretion (Fig. 4B). Similarly, proliferation of alloreactive T cells was not affected by neutralization of IL-18 with IL-18 BP, but completely blocked by cyclosporin A (Fig. 4C).

These data support the notion that during the onset of acute GvHD donor T cells do not require IL-18 for the development of cytotoxic effector functions and IFN-γ production. They further suggest that IFN-γ production upon direct T cell activation, *in vitro* and *in vivo*, does not require IL-18-mediated amplification. We have thus tested this idea by analyzing the inhibitory effect of IL-18 on LPS- or anti-CD3-induced IFN-γ production *in vivo*. Mice were thus treated with PBS or 4 mg IL-18 BP/kg body wt, prior to injection of LPS or anti-CD3. Serum was collected after 6 h and analyzed for IFN-γ levels by ELISA. Figure 4(D) shows that injection of anti-CD3 and LPS induced high levels of serum IFN-γ; however, only LPS-induced IFN-γ production was blocked by IL-18 BP treatment. These data confirm that IL-18 BP can efficiently neutralize IL-18 *in vivo* and can thus block LPS-induced IL-18-mediated IFN-γ production, but also confirm that CTL induction and IFN-γ production during the initiation phase of GvHD and upon TCR-specific activation occurs independently of IL-18.

Discussion

IL-12 and IL-18 possess potent synergistic IFN-γ- and CTL-inducing activity [reviewed in (11–13)]. Thus, interfering with IL-12 or IL-18 activity may affect IFN-γ production and could provide a potentially attractive therapeutic treatment of destructive immune responses, including acute GvHD. Previous reports have demonstrated elevated serum levels of IL-18 in patients undergoing acute GvHD upon allogeneic bone marrow transplantation (21,22). Intriguingly, IL-18 levels correlated with the severity of the disease and were reduced after effective disease treatment (22). Similarly, increased IL-18 expression was observed in experimental models of acute GvHD, and its expression correlated with IFN-γ production and disease progression [(23) and our own results]. Thus, it has been suggested that IL-18 may represent a disease-promoting factor and therefore a potential therapeutic target in the pathogenesis of acute GvHD (34).

In this study, we have used recombinant IL-18 BP to neutralize IL-18 and assess a potential pathogenic role for IL-18 in acute GvHD. *In vitro* and *in vivo* experiments have substantiated the potent IL-18-neutralizing activity of this cytokine antagonist. It was thus rather surprising to find no alterations in any disease parameter measured upon treatment of diseased mice with IL-18 BP. Most importantly, neutralization of IL-18 did not inhibit IFN-γ production and CTL activity during the initiation phase of acute GvHD. Our additional *in vitro* and *in vivo* experiments may at least in part explain this observation, and may allow us to draw some principle conclusions about T cell-mediated IFN-γ production. Whereas LPS-induced IFN-γ expression *in vitro* and *in vivo* was efficiently blocked by IL-18 BP, thus confirming a potent role for IL-18 in T cell activation and IFN-γ induction, T cell mitogen- or alloantigen-induced IFN-γ secretion was unaffected by IL-18 neutralization. Therefore, direct T cell activation by superantigen, lectin or anti-CD3, but also by alloantigen, as in the case of acute GvHD and MLR, does not appear to require IL-18-mediated amplification. A similar observation has been recently reported by Fantuzzi *et al*. (35), who found that IL-1β-converting enzyme activity, which processes pro-IL-1β and pro-IL-18 to their mature form, is required for LPS- but not Con A-induced IFN-γ production in murine spleen cells. While this has been only indirect evidence for a non-essential role of IL-18 in lectin-induced IFN-γ production, we here now demonstrate more specifically a redundant or non-essential role for IL-18 in IFN-γ induction upon direct TCR stimulation *in vitro* and *in vivo*. This observation may reflect what has been recently discussed as 'innate' and 'acquired' T cell activation pathways (36), where IL-18-induced and TCR-induced IFN-γ production would represent the 'innate' and 'acquired' pathways respectively.

The lack of a functional role for IL-18 in the pathogenesis of acute GvHD is particularly intriguing since IL-18 has been suggested to play a crucial role in the amplification of T_h1 immune responses and CTL induction. For example, IL-18 promotes perforin- and FasL-mediated cytotoxicity (14,37). Both cytotoxic effector mechanisms have been previously shown to represent important elements in the pathogenesis of acute GvHD (4–9). Similarly, administration of recombinant IL-18 to mice undergoing chronic GvHD ameliorates the disease by promoting the development of regulatory alloreactive donor CTL (25). Thus, IL-18 is capable of promoting CTL induction and IFN-γ expression in T cells, yet appears to be non-essential for donor T cell activation during acute GvHD.

It is likely that IL-18 synthesis during acute GvHD is not a cause but a consequence of tissue destruction. A recent study has shown that donor T cell-expressed FasL causes IL-18 production and release by liver Kupffer cells during the pathogenesis of experimental acute GvHD (38). Interestingly, elevated serum IL-18 levels were not observed if GvHD was induced by injecting FasL-defective (*gld/gld*) donor T cells or if GvHD was induced in caspase-1-deficient recipient mice. Caspase-1 is a cysteine protease, required for the maturation of pro-IL-18 to its mature form [reviewed in (12)]. Thus, IL-18 is produced and released by recipient cells in response to donor T cell activation (i.e. FasL expression). A similar mechanism may lead to the recently described IL-18 production in MLR (39). IL-18 production during acute GvHD may thus represent a regulatory event rather than an amplification loop of the inflammatory response. Very recently, Reddy *et al*. (40) have found that neutralization of IL-18 may cause an exacerbation of GvHD induced by allogeneic bone marrow transplantation, whereas administration of recombinant IL-18 ameliorated the disease due to sensitization of the alloreactive T cells to Fas-induced apoptosis. While we also observed rather an increase in anti-host cytotoxicity and donor T cell expansion, the disease-promoting effect of IL-18 neutralization was not as obvious as observed by Reddy *et al*. In part, this may be explained by the absence of a functional recipient immune system in the bone marrow transplantation model versus the transfer of parental spleen cells into F_1 recipients, as used in this study.

Our data also confirm previous observations that IL-12 is elevated during acute GvHD (21–23). We have found that elevated IL-12 levels are already detectable 4 days after disease induction and remained elevated during the course of the disease. Interestingly, IL-12 induction preceded the induction of IFN-γ. Although detectable already after 4 days,

peak levels of IFN-γ were only observed after 8 days. Thus, its possible that IL-12, rather than IL-18, participates in IFN-γ induction during acute GvHD. The role of IL-12 in the pathogenesis of acute GvHD is rather unclear. Whereas it has been found that early injection of neutralizing anti-IL-12 induced long-term protection and a shift towards a T_h2-like cytokine response (41,42), Sykes *et al.* have observed that a single injection of recombinant IL-12 causes the induction of regulatory anti-donor CTL and reduced pathology (43,44). Thus, pro-inflammatory cytokines, such as IL-12, IL-18 and IFN-γ, may have both beneficial as well as disease-promoting activities during the pathogenesis of GvHD.

These findings provide important insight into the mechanisms that regulate T cell responses to alloantigen and subsequently the development of acute GvHD, and may provide a basis for potential future therapeutic approaches.

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Abbreviations

B6D2F1	C57Bl/6 × DBA2 F_1
Con A	concanavalin A
FasL	Fas ligand
GvHD	graft-versus-host disease
HBS	HEPES-buffered saline
IEL	intraepithelial lymphocyte
IL-18 BP	IL-18-binding protein
LPS	lipopolysaccharide
MLR	mixed leukocyte reaction
SEB	staphylococcal enterotoxin B

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