In vivo IL-4 prevents allo-antigen driven CD8⁺ CTL development

Charles S. Via*, Kateryna Soloviova *, Maksym Puliaiev*, Roman Puliaev*, Irina Puliaeva*, Suzanne C. Morris[§], Fred D. Finkelman[§].

*Department of Pathology, Uniformed Services University of Health Sciences, Bethesda MD 20814; and [§]Division of Immunology, University of Cincinnati College of Medicine, Cincinnati, OH 45267; Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229; and Department of Medicine, Cincinnati Veterans Affairs Medical Center, Cincinnati, OH 45220.

Key words: graft-vs.-host disease, T cells, IL-4, CTL, Mouse, Cytokine

Address correspondence to: Charles S. Via M.D., Department of Pathology, Room 3B100, 4301 Jones Bridge Road, Uniformed Services University of Health Sciences, Bethesda, MD 20814. Phone: 301 295 3801; Fax 301 295 1640

Abbreviations used: BDF1, B6D2F1; B6, C57Bl/6; *GrB*, *granzyme B*; HVG, host-vs.-graft; KO, knock out; p→F1, parent-into-F1; pfp, perforin; Tfh, *T follicular helper*; WT, wild type.

ABSTRACT

IL-4 has been shown to suppress acute graft vs. host disease (GVHD) in irradiated hosts. Here we evaluated whether IL-4 suppresses acute GVHD in the un-irradiated parent-into-F1 GVHD model with relevance to renal allograft rejection. IL-4 completely suppressed CD8 CTL when administered with donor cells however this effect was lost if its administration was delayed 3 days. IL-4 did not inhibit donor CD8⁺ T cell homing to the host spleen but rather prevented donor CD8⁺ T cell differentiation into CTLs. Studies with IL-4R α -deficient donor cells or recipient mice demonstrated that IL-4 effects on the host, rather than, or in addition to IL-4 effects on donor cells, were critical for suppression of CTL. Because IL-4 decreased all splenic dendritic cell populations and increased neutrophil and CD8⁺ T cells, IL-4 may suppress donor CD8⁺ CTL by decreasing Ag presentation and/or increasing host myeloid and CD8⁺ T cell suppression of donor T cells.

1. Introduction

The parent-into-F1 model of acute graft-vs.-host disease (GVHD) is useful for the study of in vivo CD8 CTL development. The injection of homozygous parental T cells into fully MHC I + II disparate heterozygous F1 mice induces the activation of donor CD4⁺ and CD8⁺ T cells specific for host MHC Ags. Donor CD8⁺ T cell activation and maturation into CTL effectors is initiated by a strong IL-2 response by the donor CD4⁺ T cells (1). This process is accompanied by a burst of IFN- γ , upregulation of Fas/FasL expression, and acquisition of CTL effector markers, including perforin. By day 14, spleens from recipient F1 mice exhibit a typical “cytotoxic” or acute GVHD phenotype characterized by profound elimination of host B and T cells accompanied by engraftment of donor CD4⁺ and CD8⁺ T cells. Acute GVHD in this model can be blocked by costimulatory blockade (CTLA4-Ig or combined anti-CD80/anti-CD86 mAb) (2) or IL-2 blockade (3) at the time of donor cell transfer.

The cytokines produced during GVHD have a profound effect on the type of GVHD that develops. Blocking TNF prevents donor CTL development and converts disease phenotype at two weeks from acute cytotoxic to a chronic, stimulatory autoimmune phenotype (B cell expansion, autoantibody production and engraftment of donor CD4⁺ T cells but not CD8⁺ T cells) (4). Blocking IFN- γ partially suppresses acute GVHD development by decreasing Fas/FasL expression but only partially decreasing perforin expression (5); this only partial suppression of CTL function probably explains the failure of chronic GVHD to develop in IFN- γ -suppressed mice. Treatment with IL-12 has the opposite effect

of IFN- γ and TNF suppression: it strongly stimulates donor CD8⁺ CTL development and converts chronic GVHD to acute GVHD (6).

Because the type 2 cytokine IL-4 generally antagonizes the effects of IFN- γ and IL-12 and can inhibit CTL differentiation (7), it might be expected that IL-4 would inhibit the development of acute GVHD and promote chronic GVHD development. Indeed, a relatively strong IL-4 response in irradiated human cancer patient recipients of allogeneic bone marrow is associated with less severe GVHD than develops in patients who have less of an IL-4 response (8).

In addition, IL-4 produced by basophils, NKT cells, and conventional CD4⁺ T cells has been associated with suppression of GVHD in mouse models (9-12) and treatment with exogenous IL-4 has some suppressive effect on acute GVHD (9). However, no formal studies have investigated the timing or dose requirements of the IL-4 effects or have determined whether acute GVHD suppression is mediated predominantly through direct effects on donor T cells or through effects on host cells; issues that have important implications for the use of IL-4 or IL-4-secreting cells to prevent or ameliorate GVHD.

To investigate these issues, we used a model in which acute GVHD is induced by injecting C57BL/6 (B6) CD4⁺ and CD8⁺ T cells into (C57BL/6 x DBA/2)F1 (BDF1) mice (B6 $\rightarrow$ DBF1) (13) and treated these mice with complexes prepared by mixing IL-4 with a neutralizing anti-IL-4 mAb. These complexes (IL-4C) prevent the excretion and catabolism of IL-4 and slowly dissociate in vivo to greatly extend IL-4 half-life (14). We observed that IL-4C can completely inhibit acute GVHD without skewing towards a chronic GVHD phenotype at two weeks.

This effect is due to a complete suppression of donor CD8⁺ CTL maturation, requires administration of IL-4C close to the time of donor cell transfer and appears to be mediated predominantly through IL-4 effects on host rather than donor T cells.

2. Materials and Methods

2.1 Mice. 6-8 week old male C57BL/6 J(H-2^b) donor mice and B6D2F1J (BDF1) (H-2^{b/d}) recipient host mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-4Ra-deficient mice on BALB/c and C57BL/6 backgrounds were a gift of Dr. Frank Brombacher, University of Capetown, South Africa, and were bred to each other to generate (BALB/c x C57BL/6)F1 IL-4Ra-deficient mice. All animal procedures were pre-approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of Health Sciences.

2.2 Induction of GVHD. Single cell suspensions of donor strain splenocytes were prepared as described (15) and transferred into age matched BDF1 hosts by tail vein injection. The percentages of donor CD4⁺ and CD8⁺ T cells populations were analyzed by flow cytometry and donor inoculum adjusted prior to transfer such that all F1 mice received $\sim 5 \times 10^6$ donor CD8 T cells. Typically, this required $55\text{--}65 \times 10^6$ unfractionated donor splenocytes.

2.3 IL-4C preparation. IL-4C were composed of IL-4 (Peprotech, Rocky Hills, NJ) and anti-IL-4 mAb (BVD4-1D11.2) purified from mouse ascites. IL-4 was mixed with anti-IL-4 mAb at a 1:5 (e.g. 1 μg IL-4 with 5 μg anti-IL-4 mAb) for 5 minutes at 4°C and the resulting IL-4C were then diluting to the desired concentration with 1% autologous mouse serum in PBS. It has been previously shown that this method saturates the anti IL-4 mAb with IL-4 and the IL-4C have strong and prolonged IL-4 agonist activity (11).

2.4 Flow cytometric analysis. Spleen cells were first incubated with anti-murine Fc γ receptor II/III mAb, 2.4G2 for 10 min and then stained with saturating

concentrations of Alexa Fluor 488-conjugated, APC-conjugated, biotin-conjugated, PE-conjugated, FITC-conjugated, PerCPCy5.5-conjugated, Alexa Fluor 647-conjugated and Pacific Blue-conjugated mAb against CD3, CD4, CD8, CD19, B220, H2-K^b, I-A^b, H-2K^d, I-A^d, CD11b and CD11c, CD44, CD62L, CXCR5, ICOS, PD1, CCR7, KLRG-1, and mPDCA purchased from either BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), eBioscience (San Diego, CA), or Invitrogen (Carlsbad, CA). Biotinylated primary mAb were detected using PE-Texas Red-streptavidin (BD Biosciences, San Jose, CA). Cells were fixed in 1% paraformaldehyde before reading.

Ex vivo intracellular staining for perforin (pfp), granzyme B (GrB), IFN- γ , TNF and IL-2 were performed using antibodies and reagents purchased from BD Biosciences (San -Jose, CA) or Biolegend (San Diego, CA) and staining performed according to the manufacturer's instructions. Importantly, as previously described (16) there was no in vitro re-stimulation or use of Golgi blocking agents. Following completion of the staining protocol, cells were analyzed by flow cytometry immediately.

Multi-color flow cytometric analyses were performed using a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). Gating strategy: lymphocytes were gated by forward and side scatter and fluorescence data were collected for a minimum of 10,000 gated cells. Studies of donor T cells were performed on a minimum of 5,000 cells collected using a lymphocyte gate that was positive for CD4 or CD8 and negative for MHC class I of the uninjected parent (H-2K^d negative). B cells were gated as positive for B220 and either positive (host origin)

or negative (donor origin) for MHC Class II of the uninjected parent (I-A^d). Short lived effector CD8⁺ CTL were assessed as KRLG-1 positive, CCR7 negative gated donor CD8⁺ T cells. Host DC and macrophages were identified as I-A^d positive and CD11c or CD11b positive respectively using a broad forward and side scatter gate. In Fig. 7, DC were further gated as CD3/CD19 negative, CD11c positive and either CD8a positive or mPDCA positive.

2.5 Cytokine Expression by Real Time PCR. Real time PCR was performed as described (15). Briefly, splenocytes (1×10^7) were homogenized in 1 ml of RNA-STAT-60 (Tel-Test, Friendswood, TX). cDNA was synthesized from mRNA using TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using pre-made primers and probes from TaqMan® Gene Expression Assays and TaqMan® Universal PCR Master Mix (Applied Biosystems) for the following targets: IFN- γ , IL-10, IL-21, IL-4, OAS, MX-1 with 18s rRNA as an internal control. The calculation of relative gene expression differences was done by comparative $2^{-\Delta\Delta CT}$ method. The result was expressed as fold change in the experimental groups compared to uninjected B6D2F1 control.

2.6 Statistical Analysis. Statistical comparisons were performed using Prism 5.0 (Graphpad Software, San Diego, CA). Mice were tested individually and data are expressed as group mean $\pm$ SEM. Statistical significance between two groups was analyzed using Student's t-test. For multiple comparisons, two-way ANOVA with an additional Sidak-Bonferroni post-test was used. p-values < 0.05 were considered as statistically significant.

3. Results

3.1 IL-4 C treatment prevents acute GVHD and inhibits donor T cell survival. To determine whether skewing the cytokine milieu with IL-4 alters outcome in B6→F1 acute GVHD, we administered a broad dose range of IL-4C at the time of donor cell transfer and 3 and 6 days later and assessed spleens at day 14. Typical features of acute GVHD were seen in B6→ BDF1 mice that did not receive IL-4C, including both donor CD4⁺ and CD8⁺ T cell engraftment (Figs. 1A, 1B, bar 1) along with profound, significant elimination of host B cells and host CD4⁺ T cells and to a lesser extent, host CD8⁺ T cells (Figs. 1C-1E, bars 2 vs. 1 respectively). In the p→F1 model, host B cells are the most sensitive cell type to elimination by donor CD8⁺ CTLs, followed by host CD4⁺ T cells, host DC and lastly, host CD8⁺ T cells, which along with host NK cells mediate the counter regulatory host-vs.-graft (HVG) response and limit donor T cell engraftment (17). IL-4C treatment significantly altered the acute GVHD phenotype. At doses of 10 or 3 µg (IL-4 content of IL-4C), donor CD4⁺ and CD8⁺ T cell numbers were profoundly decreased (Figs. 1A, 1B, bars 2 and 3 vs. 1) and elimination of host B cells, CD4⁺ T cells and CD8⁺ T cells was prevented (Figs. 1C-1E, bars 3 and 4 vs. 2, p<0.05; bars 3 and 4 vs. 1, p=ns). At a 1 µg dose of IL-4C, GVHD mice showed no significant decrease in the number of donor CD4⁺ T cells and ~40% of typical donor CD8⁺ T cell number (Figs. 1A, 1B, bar 4), with preservation of host B cells and CD4⁺ T cells and a significant increase in host CD8⁺ T cells (Figs. 1C- 1E, bar 5). Most effects of IL-4C on acute GVHD were lost when the IL-4C dose was reduced to 0.3 µg. Similarly, the stimulatory effect of GVHD on

IFN- γ secretion 7 days after donor cell transfer was suppressed in a dose-dependent manner by IL-4C (Fig. 1F). Taken together, these data suggest that high dose IL-4 leads to the loss of donor T cells from the host spleen, while a lower dose permits some donor CD8⁺ T cell survival but inhibits their ability to kill host cells.

3.2 IL-4 must act early after donor cell transfer to suppress acute GVHD. To determine if there is a critical time for IL-4C suppression of acute GVHD, we compared the effects of a single, vs. every 3 day 1 μ g dose of IL-4C. Mice received a single IL-4C dose on day -3, 0, 3 or 6 relative to donor cell transfer and spleens were assessed on day 14. Untreated (control) acute GVHD mice exhibit engraftment of donor CD4⁺ and CD8⁺ T cells (Figs. 2A, 2B, bar 1) along with significant elimination of host B cells, CD4⁺ T cells and CD8⁺ T cells compared to normal, uninjected F1 mice (Figs 2C -2E, bars 2 vs. 1 respectively). Mice treated with IL-4C every 3 days (positive control) exhibited donor CD4⁺ and CD8⁺ T cell engraftment comparable to mice that received donor cells without IL-4C (Figs 2A, 2B, bars 6 vs. 1 respectively), yet not only had no elimination of host cells but also significantly more host B and CD8⁺ T cells than even mice that had received neither donor cells nor IL-4C (Figs. 2C- 2E, bars 7 vs. 1); they also had partial suppression of the GVHD-induced IFN- γ response (Figs. 2F, bars 7 vs. 2). These observations strengthen the conclusion drawn from similar treatment with IL-4C in Fig.1 that a relatively low dose of IL-4C inhibits donor CD8⁺ T cell cytotoxic function without killing these cells. Similarly, a single 1 μ g dose of IL-4C, administered on the day of donor cell transfer (Day 0), did not

significantly alter donor T cells number (Fig. 2A, 2B, bars 1 vs. 3), but significantly impaired the IFN- γ response and host B cell and CD4⁺ and CD8⁺ T cell elimination (Figs. 2C – 2F, bars 2 vs 4). Administration of IL-4C on any of the other time points boosted donor T cell engraftment without altering host B cell elimination, although there was some protective effect on host CD4⁺ and possibly CD8⁺ T cells, which are more resistant to elimination than B cells. Administration of a single IL-4C dose at all time points studied decreased the IFN- γ response to GVHD by approximately 25-30%, while multiple doses of IL-4C decreased this response by approximately 60% (Fig. 2F). Taken together, these results suggest that IL-4 acts predominantly during the initial activation and proliferation of donor T cells to prevent their differentiation into host MHC-specific CTLs and that IFN- γ is not sufficient for full CTL differentiation.

3.3 IL-4 impairs donor CTL marker expression and host Tfh expansion. To further evaluate whether the reduced number of engrafted donor T cells matures normally when mice are treated with a relatively high IL-4C dose, we examined day 14 donor T cell CD44 expression in mice treated with 5 μ g of IL-4C on days 0, 3 and 6. As previously seen, IL-4C treatment completely blocked elimination of host B cells and CD4⁺ T cells (Figs. 3A, 3B) and CD8⁺ T cells (data not shown) on day 14, in association with significant reductions in donor CD4⁺ and CD8⁺ T cell numbers (Figs. 3C, 3D) and decreases in the percentages of both donor (Figs. 3E, 3F) and host (Figs. 3G, 3H) T cells that express high levels of the activation marker, CD44. These results suggest that IL-4C impairs both the GVH reaction and the reciprocal host T cell-mediated HVG response by preventing the

maturation of effector T cells. Analysis of cytokine gene expression at day 14 demonstrated that high dose IL-4C blocks acute GVHD-associated elevations in IFN- γ , OAS, MX-1, IL-21 and IL-10 gene expression, as well as the acute GVHD-associated suppression of IL-4 gene expression (Fig. 4).

We then addressed the maturational status of engrafted donor T cells at 10 days post-transfer, a time when donor CTL activity typically peaks in acute GVHD (17). When examined at this time point, IL-4C treatment (5 μ g on days 0, 3 and 6) had already reduced the number of donor CD4⁺ and CD8⁺ T cells by ~90% (Figs. 5A, 5B). A similar but less pronounced effect was seen with a single 5 μ g dose of IL-4 at day 0. Among surviving donor T cells, the percentage that expressed TNF was significantly suppressed, although the percentage that expressed other markers associated with CTL function (KLRG-1, IFN- γ , and perforin) was unchanged or increased (Figs. 5C-5F). The effects of IL-4C at this time were not restricted to donor CD8⁺ T cells; numbers of donor CD4⁺ Tfh were also strikingly reduced (Fig. 5G). By contrast, IL-4C in either dosing regimen significantly boosted the number of PMN vs. control acute GVHD mice (Fig. 5H) without significantly altering splenic macrophage number (data not shown).

3.4 IL-4C does not prevent donor T cell homing to the host spleen or the initial activation of donor T cells. Because treatment with a relatively high dose of IL-4C causes the loss of almost all donor CD8⁺ T cells 10 -14 days after cell transfer, it was possible that IL-4 suppresses donor T cell homing to the spleen. To evaluate this possibility, we determined donor T cell number and activation state 5 days after donor cell injection in mice that were or were not also treated with 5 μ g of

IL-4C on days 0 and 3. Although the number of donor CD4⁺ and CD8⁺ T cells recovered from the host spleen was significantly reduced by IL-4C treatment, large numbers of donor T cells were still detected in IL-4C-treated mice 5 days after donor cell transfer (Figs 6A, 6B). Furthermore, IL-4C did not reduce the percentage of donor T cells that expressed the activation marker CD25 (Figs. 6C, 6D). It also increased the percentage of donor cells expressing a second activation marker, CD69 (Figs. 6E, 6F) and had little effect on the percentage of proliferating (Ki-67⁺) cells among the surviving donor T cells. Thus, inhibition of donor cell homing and initial activation by IL-4C is at most partial and cannot account for the complete suppression of GVHD observed at day 14.

3.5 IL-4C blockade of acute GVHD is not mediated primarily by a direct effect on donor T cells. The long period of increased IL-4 concentration that is required to decrease donor T cell number and activity raised the possibility that the effect of IL-4 on donor T cells might be indirect. To determine whether IL-4C suppresses acute GVHD by acting directly on donor T cells, we compared the ability of 5 µg of IL-4C on days 0, 3 and 6 to block acute GVHD following transfer of either wild-type (WT) or IL-4R α -deficient C57BL/6 donor cells (Fig. 7). Fourteen days after donor cell transfer, typical features of acute GVHD were seen in WT $\rightarrow$ F1 mice, including engraftment of both donor CD4⁺ and CD8⁺ T cells (Figs. 7A, 7B, bar 1) and profound elimination of host B cells, CD4⁺ and CD8⁺ T cells vs. normal F1 (Figs 7C - 7E, bars 1 vs. 2). Although treatment with 5 µg of IL-4C on days 0, 3 and 6 did not block donor T cell engraftment in this experiment (Figs. 7A-7B, bar 2), elimination of host B cells and CD4⁺ T cells was completely abrogated (Figs.

7C-7D, bar 3) as in our previous experiments that treated mice with 1-10 μg of IL-4C (Figs. 1 - 3). Moreover, host CD8^+ T cell number was significantly increased, consistent with a direct stimulatory effect of IL-4C on host CD8^+ T cells (Fig. 7E, bar 3). IL-4R α -deficient $\rightarrow$ F1 mice also developed the typical acute GVHD phenotype, although donor T cell engraftment was significantly greater than with wild-type C57BL/6 donor T cells (Figs. 7A, 7B (bars 3 vs. 1), with striking elimination of host B cells and CD4^+ T cells (Figs 7C, 7D, bars 4 vs 1). Nevertheless, IL-4C treatment of mice receiving IL-4R α -deficient donor T cells significantly impaired donor T cell engraftment (Figs. 7A-7B, bars 3 vs. 4), completely abrogated host B cell and CD4^+ T cell elimination and significantly increased host CD8^+ T cell number (Figs. 7C-7E, bars 4 vs. 5). Thus, IL-4 suppression of acute GVHD does not require direct stimulation of donor T cells by this cytokine.

To determine if an IL-4 effect on host cells is required for IL-4 suppression of acute GVHD, we developed an acute GVHD model in which the host, rather than the donor, is IL-4-unresponsive. Because BALB/c and C57BL/6, but not DBA IL-4R α -deficient mice were available, we switched to a parent $\rightarrow$ F1 model that used C57BL/6 donor T cells and (BALB/c x C57BL/6)F1 (CB6F1) recipients. Preliminary experiments confirmed that an inoculum of $\sim 5 \times 10^6$ CD8^+ and $8\text{-}10 \times 10^6$ CD4^+ T cells could induce acute GVHD in this model. We then compared the ability of WT B6 donor cells to induce acute GVHD in CB6F1 WT or IL-4R α -deficient (KO) mice that had or had not been treated with 5 μg of IL-4C on days 0, 3 and 6 (Fig. 8). The ability to fully interpret the results of this study is limited

by significantly greater donor CD4⁺ and CD8⁺ T cell engraftment in wild-type vs. IL-4R α KO mice (despite transferring the same number of donor cells) in the absence of exogenous IL-4 (Figs. 8A, 8B, bars 1 vs. 2). These differences were associated with significantly greater elimination of host cells by the wild-type donor cells. Acute GVHD was associated with a loss of ~90% of host B cells in wild-type hosts and an ~75% loss of B cells in IL-4R α -deficient hosts with comparable effects seen for host CD4⁺ and CD8⁺ T cells (Figs. 8C-8E, bars 3 and 4). As expected, IL-4C treatment completely blocked B cell elimination when acute GVHD was induced in wild-type hosts and, in fact, increased host splenic B cell number to ~130% of control (45 x 10⁶/spleen) in these mice (Fig. 8C, bars 5 vs.1), while IL-4C only partially restored splenic B cell number in IL-4R α -deficient hosts to 32 x 10⁶/spleen (55% of the uninjected IL-4R α -deficient F1 control number) (Fig. 8C, bars 6 vs. 2). Similarly, IL-4C restored host CD4⁺ T cell numbers to supra-normal (175%) levels in wild-type hosts (Fig. 8D, bars 5 vs.1) yet only partially (78%) restored host CD4⁺ T cell numbers in IL-4R α -deficient hosts (Fig. 8D, bars 6 vs. 2). By contrast, IL-4C treatment significantly boosted host CD8⁺ T cell numbers over untreated F1 in both wild-type (~ 3-fold) and IL-4R α -deficient (2-fold) hosts (Fig. 8E, bars 2 vs. 6; 1 vs. 5), demonstrating that IL-4C stimulates host CD8⁺ T cell expansion even when these cells cannot directly respond to IL-4. Thus, although IL-4C blocks acute GVHD in both wild-type B6 $\rightarrow$ CB6F1 and wild-type B6 $\rightarrow$ BDF1, the restoration of host B cell and CD4⁺ T cell numbers is complete in wild-type hosts and incomplete in IL-4R α -deficient

hosts with levels significantly reduced as compared to untreated IL-4R α -deficient F1 controls (Figs 8C, 8D, bars 6 vs. 2).

The partial restoration of host B cell number in IL-4R α -deficient recipient mice by IL-4C suggests that IL-4 can suppress cytotoxic GVHD, to a limited extent, through direct effects on donor T cells. Taken together with the more complete suppression of acute GVHD by IL-4C when only host cells are IL-4-responsive (Fig. 7), our results indicate that IL-4 predominantly suppresses acute GVHD through effects on host cells, and to a lesser extent through effects on donor T cells.

3.6 IL-4C suppression of acute GVHD phenotype is not primarily NK cell-mediated. Both CD8⁺ T cells and NK cells contribute to the counter regulatory host-vs. graft (HVG) response that impairs donor T cell (particularly CD8⁺) engraftment and the ensuing GVHD (17, 18). Because IL-4 is known to activate NK cells (19), which can suppress CD8⁺ T cell activity and survival, it seemed possible that this cytokine could suppress acute GVHD by activating NK cells. To evaluate this possibility, we examined the ability of NK cell depletion to block the suppressive effect of IL-4C on acute GVHD. Treatment with anti-NK1.1 mAb depleted >90% of NK cells compared to untreated mice (data not shown) and significantly increased donor CD4⁺ T cell engraftment in both control acute GVHD and IL-4C treated acute GVHD (Fig. 9A, bars 1 vs. 2 and 3 vs. 4). A similar but milder (and non significant) effect was seen for donor CD8 T cell engraftment (Fig. 9B, bars 1 vs. 2 and 3 vs. 4). This increase in donor T cell numbers is consistent with a loss of the NK portion of the down-regulatory HVG

response. Nevertheless, IL-4C significantly impaired donor T cell engraftment for both control and NK-depleted GVHD mice (Figs. 9A, 9B, bars 1 vs. 3 and 2 vs. 4). IL-4C treatment also significantly impaired acute GVHD-mediated elimination of host B cells, CD4⁺ T cells and CD8⁺ T cells in both control acute GVHD (Figs. 9C-9E, bars 2 vs. 4) and NK-depleted acute GVHD (Figs. 9C-9E, bars 3 vs. 5). Thus, IL-4 does not suppress acute GVHD primarily by activating NK cells.

3.7 IL-4C promotes expansion of host CD8⁺ T cells. Because host CD8⁺ T cells can limit GVHD by either differentiating into Tc2 cells (20) or by participating in a HVG reaction (17, 18) and IL-4 can stimulate CD8⁺ T cell proliferation in the absence of GVHD (21), we evaluated whether IL-4C has a stimulatory effect on host CD8⁺ T cells in the context of acute GVHD. Using the mice described in Fig. 6 that had or had not been treated with 5 µg of IL-4C on days 0 and 3, we determined host T cell number and activation state 5 days after donor cell injection. Host CD4⁺ T cell numbers in untreated acute GVHD mice were modestly but not significantly increased over control F1 at day 5 whereas IL-4C treatment significantly boosted host CD4⁺ T cell numbers over untreated control F1 mice (Fig. 10A). In contrast, although the number of host CD8⁺ T cells was not significantly increased by GVHD at this time point; it was increased by IL-4C treatment regardless of the presence of GVHD (Fig. 10B), although IL-4C treatment did not affect the percentage of host T cells expressing CD25 (not shown). Although GVHD was accompanied by a significant, IL-4-independent increase in host CD4⁺ T cell expression of the early activation Ag, CD69, IL-4C treatment was required to significantly increase the percent of CD69⁺ host CD8⁺

T cells in mice undergoing acute GVHD (Fig. 10 C, D). Furthermore, although expression of Ki-67 was significantly increased by GVHD in the absence of IL-4C treatment, this treatment induced an additional, significant increase in the percentage of Ki-67⁺ host CD4⁺ and particularly CD8⁺ T cells (Fig. 10 E, F). Thus, IL-4C treatment could contribute to the suppression of acute GVHD by enhancing host CD8⁺ T cell activation.

3.8 IL-4C effects on host myeloid cells may contribute to suppression of GVHD.

One possible mechanism for the suppressive effect of IL-4C on acute GVHD is suppression of MHC class I presentation to donor T cells. Because this is dependent on host DCs and should occur relatively early after donor cell transfer, we evaluated the effect of IL-4C on DCs during acute GVHD 5 days after donor cell transfer, using the cohort described in Fig. 6. Consistent with this possibility, IL-4C treatment (5 µg on days 0 and 3) significantly blocked the GVHD-associated expansion of host pDC, CD8a⁺ DC, and myeloid DC seen 5 days after cell transfer (Fig. 11A-C). Additionally, IL-4C treatment significantly increased the total numbers of Foxp3⁺ host CD4⁺ and CD8⁺ T cells (Fig. 11D + E) and numbers of proliferating Tregs (Foxp3⁺, Ki-67⁺) for both CD4⁺ and CD8⁺ host T cells vs. control F1 mice (Fig. 11F,11G).

An additional possible mechanism for IL-4C suppression of acute GVHD could be stimulation of myeloid derived suppressor cells (MDSCs), which have a monocyte/macrophage or neutrophil phenotype and are known to inhibit CD8⁺ CTL function and survival. IL-4C treatment considerably inhibited the number of splenic CD11b⁺CD11c⁻ cells that had light scatter characteristics typical of

monocyte/macrophages and did not increase the PMN population 5 days after donor cell administration (Fig. 11H + I), but increased the PMN population 10 days after donor cell administration (Fig. 5H).

4. Discussion

Because type 1 and inflammatory cytokines are associated with acute GVHD pathogenesis and IL-4 can inhibit the production and effects of these cytokines, we evaluated whether treatment with exogenous IL-4 could suppress acute GVHD development. Our results establish that IL-4 can completely suppress acute GVHD, without inducing chronic (autoimmune) GVHD during the 2 week period after donor cell administration. This observation is consistent with previous reports that: (1) IL-4 produced by NKT cells or basophils can inhibit acute murine GVHD in models that differ from the one used in our studies (9-12); (2) administration of a relatively low dose of IL-4C prior to and at the time of cell transfer partially inhibits acute GVHD in one of these models (9); and (3) relatively large numbers of IL-4 secreting cells are associated with reduced severity of acute GVHD in people treated with irradiation followed by allogeneic bone marrow (8). We have extended these observations by determining the timing and doses required for optimal IL-4 suppression of acute GVHD and providing insight into the mechanisms responsible for IL-4's suppressive effects.

Our results show that this suppressive effect requires IL-4 administration close to the time of donor cell transfer, but is increased if IL-4 continues to be administered for 5-6 days after cell transfer. Although this raised the possibility that IL-4 works by limiting the homing of donor T cells to the host spleen, IL-4 has little effect on the numbers of these donor CD8⁺ T cells in the host spleen soon after cell transfer. Instead, relatively high doses of IL-4 interfere with donor CD8⁺

T cell survival while lower doses of IL-4 allow survival but inhibit differentiation into effector CD8⁺ CTLs. Importantly, the main inhibitory effect of IL-4 on acute GVHD results from an effect on host cells, rather than donor cells, even though CD4⁺ and CD8⁺ T cells express considerable IL-4R α and respond strongly to IL-4. This result was demonstrated by showing that the suppressive effect of IL-4C is not diminished when IL-4R α -deficient donor cells are used to induce GVHD and is consistent with our previous observation that the acute GVHD-enhancing effect of IFN- γ is actually increased when this disease is induced by transfer of IFN- γ R-deficient T cells. However, IL-4C treatment still has a partial suppressive effect on acute GVHD when parental IL-4R α -sufficient T cells are transferred into IL-4R α -deficient F1 hosts. This is consistent with previous observations that suggest a direct suppressive effect of IL-4 on CTL differentiation (22). Taken together, these observations indicate that IL-4 suppresses CD8⁺ CTL maturation and acute GVHD by more than one mechanism, although the effect on host cells appears to predominate.

This conclusion led us to try to identify the host cell and mechanism involved in the IL-4 suppressive effect. One possibility, that IL-4 inhibits donor CD8⁺ T cells by stimulating host NK cells, was consistent with previous reports of IL-4 stimulatory effects on NK cells and NK cell suppressive effects on GVHD, but was not borne out by our data; elimination of host NK cells failed to decrease the suppressive effect of IL-4 on acute GVHD. Another possibility; that IL-4 acts primarily by making host cells resistant to donor CTLs is also inconsistent with our data, because it doesn't explain IL-4 suppression of the differentiation of

donor cells into cells that have CTL markers or the loss of donor CD8⁺ T cells when mice are treated with high doses of IL-4.

The requirement for early administration of IL-4 to suppress acute GVHD, even though it takes ~10 days for the development of potent CTLs in our GVHD model, suggested that IL-4 acts through an early process in T cell activation and differentiation, such as Ag presentation. Consistent with this, IL-4 treatment had a strong suppressive effect on the number of all populations of splenic dendritic cells. Although we have not evaluated whether IL-4 treatment also affects the Ag presenting ability of these cells, a previous in vitro study demonstrated that IL-4 suppresses T cell proliferation in an allogeneic mixed lymphocyte reaction predominantly through an effect on Ag presenting cells rather than a direct effect on T cells (9). This possibility is also consistent with our previously observation that IFN- γ enhances acute GVHD through an effect on host cells (23), and the well established stimulatory effect of IFN- γ on Ag presenting cell function (24).

We do not, however, favor the view that IL-4 suppression of acute GVHD is achieved through a single mechanism. In addition to the previously mentioned evidence that direct IL-4 effects on donor T cells contribute, to some extent, to suppression of acute GVHD, and the possibility that IL-4 inhibits CD8⁺ CTL maturation and acute GVHD through an affect on host Ag presentation, we have observed in other in vivo models that IL-4 can suppress T cell proliferation and survival by inducing a large population of myeloid suppressor cells (Morris, SC et al., manuscript in preparation). This observation is consistent with the increase in splenic myeloid cells 10 days after donor cell transfer in mice that have been

treated with IL-4 and with the general observation that individual cytokines typically act in multiple ways to reinforce a specific biological effect. Our evidence that IL-4 activates and increases the number of host CD8⁺ T cells during acute GVHD is also consistent with the possibility that this cytokine acts through several parallel mechanisms to suppress acute GVHD, because activated host CD8⁺ T cells might amplify the HVG response (18) or differentiate into GVHD-limiting Tc2 (20). Taken together, these observations using normal, unirradiated hosts provide important mechanistic information about IL-4 effects on the alloantigen-specific CD8⁺ CTL response in the setting of an intact immune system and may have clinical relevance when a strong CD8⁺ CTL response is undesirable e.g., renal transplantation. Our results raise the possibility of administering IL-4 or IL-4-producing cells at the time of transplantation to suppress the development of host anti-graft CD8⁺ CTL and acute transplant rejection.

Figure Legends

Figure 1. IL-4C treatment prevents acute GVHD in a dose-dependent manner.

Acute GVHD was induced in BDF1 hosts as described in Methods. Mice either received no further treatment or received 10 µg, 3 µg, 1 µg or 0.3 µg of IL-4C on days 0, 3 and 6 after donor cell transfer. At day 14, F1 spleens were assessed by flow cytometry for numbers of engrafted donor (A) CD4⁺ and (B) CD8⁺ T cells and surviving host B cells (C), CD4⁺ T cells (D) and CD8⁺ T cells (E). Host spleens were also assessed for IFN-γ gene expression by RT-PCR (F) as described in Methods. Values represent group mean ± SE (n= 4-5/grp). For all figures, *p<0.05, **p<0.01, ***p<0.001. For clarity, only the relevant significant comparisons are marked.

Figure 2. IL-4C acts early after donor cell transfer to suppress acute GVHD.

Acute GVHD was induced as described in Fig. 1 and in Methods. Mice either received no further treatment or received 1 µg IL-4C on days 0, 3 and 6; or a single dose of 1 µg IL-4C on day -3, 0, 3 or 6. At day 14, spleens were analyzed for (A) donor CD4⁺ T cells, (B) donor CD8⁺ T cells, (C) host B cells, (D) host CD4⁺ T cells (E) host CD8⁺ T cells and (F) splenic IFN-γ gene expression as described for Fig. 1.

Figure 3. IL-4C impairs T cell upregulation of the maturation marker CD44.

Acute GVHD was induced as in Fig. 1. Mice either received no further treatment or were treated with 5 µg of IL-4C on days, 0, 3 and 6. At day 14 donor, F1 spleens were analyzed by flow cytometry for total numbers of: (A) host B cells, (B) host CD4⁺ T cells, (C) donor CD4⁺ T cells and (D) donor CD8⁺ T cells. The

percentage of CD44 high cells is shown for (E) donor CD4⁺ T cells, (F) donor CD8⁺ T cells, (G) host CD4⁺ T cells and (H) host CD8⁺ T cells. Values represent group mean $\pm$ SE (n= 4-5/gp).

Figure 4. IL-4C blocks cytokine genes associated with acute GVHD. Using the same cohort described in Fig. 3, F1 spleens were analyzed by RT-PCR at day 14 for expression of the following cytokine genes: (A) IFN- γ , (B) IL-21, (C) Mx1, (D) OAS, (E) IL-10 and (F) IL-4.

Figure 5. IL-4C treatment blocks donor T cell expression of effector CTL markers. Acute GVHD was induced as described in Fig. 1. Mice either received no further treatment or were treated with 5 μ g of IL-4C on days 0, 3 and 6 or 5 μ g of IL-4C only on day 0. At 10 days after donor cell transfer, F1 spleens were analyzed for total numbers of donor (A) CD4⁺ and (B) CD8⁺ T cells; the percentage of donor CD8⁺ T cells expressing intracellular (C) TNF, (D) IFN- γ , (E) perforin; the percentage of donor CD8⁺ T cells expressing (F) KLRG-1 by surface staining; and total numbers of (G) donor TFH cells and (H) PMNs. Values represent group mean $\pm$ SE (n= 4-5/gp).

Figure 6. IL-4C does not prevent donor T cell splenic homing. Acute GVHD was induced as in Fig. 1 and mice were either untreated or treated with 5 μ g of IL-4C on days 0 and 3. At day 5 after donor cell transfer, F1 spleens were analyzed for total numbers of donor (A) CD4⁺ and (B) CD8⁺ T cells; the percentages of donor (C) CD4⁺ or (D) CD8⁺ T cells that expressed CD25; the percentages of donor (E) CD4⁺ or (F) CD8⁺ T cells that expressed high levels of CD69; and the percentages of donor (G) CD4⁺ or (H) CD8⁺ T cells that expressed Ki67.

Figure 7. IL-4C blockade of acute GVHD does not require donor T cell IL-4R signaling. Acute GVHD was induced in WT F1 mice following the transfer of B6 WT or B6 IL-4Ra KO donor splenocytes. GVHD mice were either untreated or received 5 µg of IL-4C on days 0, 3 and 6. At day 14, F1 hosts were analyzed by flow cytometry for total numbers of (A) donor CD4⁺ T cells, (B) donor CD8⁺ T cells, (C) host B cells, (D) host CD4⁺ T cells, and (E) host CD8⁺ T cells.

Figure 8. IL-4C partially inhibits acute GVHD in the absence of host IL-4Ra. Acute GVHD was induced following the transfer of B6 WT splenocytes into either CB6F1 WT or CB6F1 IL-4Ra KO hosts. GVHD mice received no further treatment or received 5 µg of IL-4C on days 0, 3 and 6. On day 14, mice were assessed by flow cytometry for total numbers of (A) donor CD4⁺ T cells, (B) donor CD8⁺ T cells, (C) host B cells, (D) host CD4⁺ T cells, and (E) host CD8⁺ T cells.

Figure 9. Host NK cells are not required for IL-4C blockade of acute GVHD. Acute GVHD was induced as described in Fig. 1 using B6 WT donors and BDF1 WT hosts. Host NK cells were depleted as described in Methods. F1 mice were either untreated or received 5 µg of IL-4C on days 0, 3 and 6 and spleens analyzed by flow cytometry at day 14 for total numbers of: (A) donor CD4⁺ T cells, (B) donor CD8⁺ T cells, (C) host B cells, (D) host CD4⁺ T cells and (E) host CD8⁺ T cells.

Figure 10. IL-4C promotes expansion of host T cells, particularly CD8⁺ T cells. Flow cytometry was used to analyze the cohort described in Fig. 6 for: numbers of (A) host CD4⁺ T cells and (B) host CD8⁺ T cells; and the percentages of host

CD4⁺ and CD8⁺ T cells (C + D) that upregulated CD69 or (E + F) expressed Ki-67.

Figure 11. IL-4C blocks GVHD associated expansion of host dendritic cell subsets and macrophages. The cohort described in Fig. 6 was evaluated by flow cytometry for host DC subsets, Tregs, macrophages and PMN using parameters described in Methods for (A) pDC, (B) CD8a⁺ DC, (C) myeloid DC, (D) CD4⁺ Foxp3⁺ T cells (E) CD8⁺ Foxp3⁺ T cells, (F) Ki-67⁺ Foxp3⁺ CD4⁺ T cells, (G) Ki-67⁺ Foxp3⁺ CD8⁺ T cells, (H) macrophages (CD11b⁺, CD11c⁻) and (I) PMNs.

Footnotes

¹ This work was supported by National Institutes of Health RO1AI047466 (CSV) and R01AI070300 (FDF) and U. S. Dept of Veterans Affairs (FDF).

² E-mail: charles.via@usuhs.edu

³ The opinions expressed herein are those of the authors, and are not necessarily representative of those of the Uniformed Services University of the Health Sciences (USUHS), the Department of Defense (DOD); or, the United States Army, Navy, or Air Force.

REFERENCES

1. Soloviova, K., M. Puliaiev, M. Haas, C. L. Dalgard, B. C. Schaefer, and C. S. Via. 2015. Intrinsic Differences in Donor CD4 T Cell IL-2 Production Influence Severity of Parent-into-F1 Murine Lupus by Skewing the Immune Response Either toward Help for B Cells and a Sustained Autoantibody Response or toward Help for CD8 T Cells and a Downregulatory Th1 Response. *J Immunol* 195: 2985-3000.
2. Via, C. S., V. Rus, P. Nguyen, P. Linsley, and W. C. Gause. 1996. Differential effect of CTLA4Ig on murine graft-versus-host disease (GVHD) development: CTLA4Ig prevents both acute and chronic GVHD development but reverses only chronic GVHD. *J Immunol* 157: 4258-4267.
3. Via, C. S., and F. D. Finkelman. 1993. Critical role of interleukin-2 in the development of acute graft-versus-host disease. *Int Immunol* 5: 565-572.
4. Via, C. S., A. Shustov, V. Rus, T. Lang, P. Nguyen, and F. D. Finkelman. 2001. In vivo neutralization of TNF-alpha promotes humoral autoimmunity by preventing the induction of CTL. *J Immunol* 167: 6821-6826.
5. Shustov, A., P. Nguyen, F. Finkelman, K. B. Elkon, and C. S. Via. 1998. Differential expression of Fas and Fas ligand in acute and chronic graft-versus-host disease: up-regulation of Fas and Fas ligand requires CD8+ T cell activation and IFN-gamma production. *J Immunol* 161: 2848-2855.

6. Via, C. S., V. Rus, M. K. Gately, and F. D. Finkelman. 1994. IL-12 stimulates the development of acute graft-versus-host disease in mice that normally would develop chronic, autoimmune graft-versus-host disease. *J Immunol* 153: 4040-4047.
7. Raphael, I., S. Nalawade, T. N. Eagar, and T. G. Forsthuber. 2015. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine* 74: 5-17.
8. Takabayashi, M., H. Kanamori, H. Takasaki, S. Yamaji, H. Koharazawa, J. Taguchi, N. Tomita, K. Fujimaki, S. Fujisawa, A. Maruta, and Y. Ishigatsubo. 2005. A possible association between the presence of interleukin-4-secreting cells and a reduction in the risk of acute graft-versus-host disease. *Exp Hematol* 33: 251-257.
9. Hermann, F. J., M. Rodriguez Gomez, K. Doser, M. Edinger, P. Hoffmann, G. Schiechl, Y. Talke, N. Gobel, K. Schmidbauer, S. N. Syed, H. Bruhl, and M. Mack. 2015. Basophils inhibit proliferation of CD4(+) T cells in autologous and allogeneic mixed lymphocyte reactions and limit disease activity in a murine model of graft versus host disease. *Immunology* 145: 202-212.
10. Leveson-Gower, D. B., J. A. Olson, E. I. Segal, R. H. Luong, J. Baker, R. Zeiser, and R. S. Negrin. 2011. Low doses of natural killer T cells provide protection from acute graft-versus-host disease via an IL-4-dependent mechanism. *Blood* 117: 3220-3229.

11. Pillai, A. B., T. I. George, S. Dutt, and S. Strober. 2009. Host natural killer T cells induce an interleukin-4-dependent expansion of donor CD4⁺CD25⁺Foxp3⁺ T regulatory cells that protects against graft-versus-host disease. *Blood* 113: 4458-4467.
12. Foley, J. E., J. Mariotti, K. Ryan, M. Eckhaus, and D. H. Fowler. 2008. Th2 cell therapy of established acute graft-versus-host disease requires IL-4 and IL-10 and is abrogated by IL-2 or host-type antigen-presenting cells. *Biol Blood Marrow Transplant* 14: 959-972.
13. Via, C. S. 2010. Advances in lupus stemming from the parent-into-F1 model. *Trends Immunol.* 31: 236-245.
14. Finkelman, F. D., K. B. Madden, S. C. Morris, J. M. Holmes, N. Boiani, I. M. Katona, and C. R. Maliszewski. 1993. Anti-cytokine antibodies as carrier proteins. Prolongation of in vivo effects of exogenous cytokines by injection of cytokine-anti-cytokine antibody complexes. *J Immunol* 151: 1235-1244.
15. Soloviova, K., M. Puliaiev, A. Foster, and C. S. Via. 2012. The parent-into-F1 murine model in the study of lupus-like autoimmunity and CD8 cytotoxic T lymphocyte function. *Methods Mol Biol* 900: 253-270.
16. Soloviova, K., M. Puliaiev, M. Haas, and C. S. Via. 2013. In vivo maturation of allo-specific CD8 CTL and prevention of lupus-like graft-versus-host disease is critically dependent on T cell signaling through the TNF p75 receptor but not the TNF p55 receptor. *J Immunol* 190: 4562-4572.

17. Puliaev, R., I. Puliaeva, L. A. Welniak, A. E. Ryan, M. Haas, W. J. Murphy, and C. S. Via. 2008. CTL-promoting effects of CD40 stimulation outweigh B cell-stimulatory effects resulting in B cell elimination and disease improvement in a murine model of lupus. *J Immunol* 181: 47-61.
18. Foster, A. D., K. Soloviova, I. Puliaeva, M. Puliaiev, R. Puliaev, F. Finkelman, and C. S. Via. 2011. Donor CD8 T cells and IFN-gamma are critical for sex-based differences in donor CD4 T cell engraftment and lupus-like phenotype in short-term chronic graft-versus-host disease mice. *J Immunol* 186: 6238-6254.
19. Morris, S. C., T. Orekhova, M. J. Meadows, S. M. Heidorn, J. Yang, and F. D. Finkelman. 2006. IL-4 induces in vivo production of IFN-gamma by NK and NKT cells. *J Immunol* 176: 5299-5305.
20. Fowler, D. H., and R. E. Gress. 2000. Th2 and Tc2 cells in the regulation of GVHD, GVL, and graft rejection: considerations for the allogeneic transplantation therapy of leukemia and lymphoma. *Leuk Lymphoma* 38: 221-234.
21. Morris, S. C., S. M. Heidorn, D. R. Herbert, C. Perkins, D. A. Hildeman, M. V. Khodoun, and F. D. Finkelman. 2009. Endogenously produced IL-4 nonredundantly stimulates CD8+ T cell proliferation. *J Immunol* 182: 1429-1438.
22. Lee, K. Y., P. S. Goedegebuure, D. C. Linehan, and T. J. Eberlein. 1995. Immunoregulatory effects of CD4+ T helper subsets in human melanoma. *Surgery* 117: 365-372.

23. Puliaev, R., P. Nguyen, F. D. Finkelman, and C. S. Via. 2004. Differential requirement for IFN-gamma in CTL maturation in acute murine graft-versus-host disease. *J Immunol* 173: 910-919.
24. Virelizier, J. L., and F. Arenzana-Seisdedos. 1985. Immunological functions of macrophages and their regulation by interferons. *Med Biol* 63: 149-159.

Fig.1

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6

Donor CD4⁺ T cells

Donor CD8⁺ T cells

Fig.7

Fig.8**Donor CD4⁺ T cells** **Donor CD8⁺ T cells** **Host B cells** **Host CD4⁺ T cells** **Host CD8⁺ T cells**

Fig.9

Fig.10

Fig.11