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Early Intestinal Th1 Inflammation and Mucosal T Cell Recruitment During Acute Graft-Versus-Host Reaction

Denis Snider and Hong Liang

Little is understood about the earliest cytokine responses and the role(s) of donor CD4 T cells in the intestine during the induced graft-vs-host reaction (GVHR). We investigated the activation and mucosal homing phenotype of the donor CD4 cells and the kinetics of cytokine responses within the intestine and associated lymphoid tissues during early GVHR. Significant frequencies of donor CD4 cells accumulated within recipient Peyer’s patches (PP), mesenteric lymph nodes (MLN), lamina propria (LP), and spleen (SP), during the first 9 days of GVHR. Many donor CD4 cells in SP, MLN, and LP expressed CD44 and also expressed de novo the mucosal homing integrin α4β7 (LPAM-1). A large IFN-γ response occurred by day 3 in cells from PP and MLN, but much later (day 9) in SP and LP cells. IL-10 production by SP and MLN cells was elevated initially but declined substantially by day 9. IL-4 production by SP, MLN, and PP cells was low on day 3 and showed gradual decline in LP by day 9. IL-5 production by LP cells gradually increased in direct contrast to IL-5 production by MLN cells. The MLN CD4 cells showed the most dynamic changes, with high numbers of activated/effector donor CD4 cells and altered cytokine production consistent with a developing Th1 response. The IFN-γ responses in PP and MLN preceded that of the SP, suggesting an intestinal origin for some Th1 effector cells in GVHR. Donor CD4 T cells apparently acquire the ability to home to the LP during early GVHR. The Journal of Immunology, 2001, 166:5991–5999.

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Thus, there are open questions regarding the function of donor CD4 Th1 cells in the intestine during GVHR and whether the donor CD4 T cells develop within the intestinal environment and produce a local Th1 inflammation in intestinal GVHR.

We decided to approach these questions by examining intestinal tissues and associated draining lymphoid structures at very early time points after induction of the GVHR in the mouse model. The cytokine profile displayed by these tissues indicate that early Th1 microenvironment does develop in the intestine, and that donor CD4 T cells express the mucosal homing integrin αβ7, within these tissues. These results indicate that intestinal GVHR can be initiated and/or propagated by the response of naive donor CD4 T cells within the intestinal environment, and that the intestine undergoes a shift to Th1 very early in GVHR.

Materials and Methods

Mice

Male 6- to 7-wk-old DBA/2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) for breeding as required. Female and male 6- to 8-wk-old B10.BR mice were purchased (The Jackson Laboratory) initially and then bred within the Central Animal Facility at McMaster, under specific pathogen-free conditions. Female B10.BR were bred with male DBA/2 to generate F1 (B10.BR × DBA/2) mice. For GVHR, male mice were used exclusively as donor (B10.BR or F1) and recipient F1. All mice were housed in autoclaved cages with filter tops and given autoclaved food and water ad libitum, in the central animal facility at McMaster.

Generation of GVHR

Spleen cells were prepared sterile (see below) within 1 h of injection from 10- to 14-wk-old male B10.BR mice or F1 control male splees. Spleens (30-50 × 10^6) were suspended in 0.4 ml sterile PBS at room temperature and injected via tail vein into nonirradiated male F1 mice. Mice receiving <0.3 ml cells due to failure of injection were excluded from study. Usually, groups of three to five mice were given injections. Control mice receiving F1, spleen cells were included in all experiments.

Histological techniques and IEL counts

Pieces of jejunal tissue (0.5 cm) were taken from mouse intestine and fixed in formalin before embedding in paraffin. The tissue was cut in transverse sections that were mounted on slides, processed to remove paraffin, and rehydrated before staining with hematoxylin and eosin. Sections were examined with a 20× objective for elongation of crypts and disruption of villi. The number of IEL were then counted using full length villi, but villus-crypt ratio of lengths was considered memory/effector cells of nonmucosal origin. Preliminary work involved three to four mice per group were used to generate culture supernatants from cells taken at various days after induction of various cell preparations. The frequencies of donor (B10.BR) or recipient F1 CD4 T cells and CD4 T cells that express high levels of CD44 were determined using a three-color staining protocol. This involved incubation of 10^6 viable cells with mAb 2-A2G2 before incubation with biotin-anti-H-2K^D^d as a first step. The second step included FITC-anti-CD4, PE-anti-CD44, and SA-PerCP. All Abs were pretreated for maximal binding and lowest nonspecific signal. Control tubes were stained with FITC-CD4, biotin-H-2K^D^d, SA-PerCP, and a control rat IgG labeled with PE (PharMingen) to provide appropriate background fluorescence signals for donor (H-2K^D^d) or recipient (H-2K^D^d) CD4 cells. A minimum of 40,000 events were collected based on the lymphocyte (forward scatter (FSC) × side scatter (SSC)) gate, using a FACScan instrument, equipped with CellQuest acquisition and analysis software (Becton Dickinson). For analysis, events were first gated on lymphocyte FSC × SSC and positive CD4 signal. Then two-color plots were prepared from the gated events showing H-2K^D^d+ and H-2K^D^d- cells expressing CD44. Cells expressing high (bright) levels of CD44 were considered to have the activation/effector phenotype. A four-color protocol was used to determine the proportions of αβ7-positive CD4 T cells and the subsets expressing CD44. The first step was blocking with 2.4G2 mAb and labeling with the biotin-H-2K^D^d, and second step involved APC-anti-CD4, FITC-anti-CD44, PE-anti-αβ7, and SA-PerCP. Up to 30–100,000 events were acquired on a FACScanCalibur, using the lymphocyte FSC × SSC gate. For analysis, events were gated on lymphocyte scatter, positive signal for CD4, and those gated events were then additionally gated as positive or negative based on H-2K^D^d expression. Finally, the resulting data of donor and recipient CD4 cells was analyzed for CD44 and αβ7 expression, using analysis regions similar to that of Williams and Butcher (31). Cells expressing high levels of αβ7 and CD44 were considered to be mucosal memory T cells; those with no αβ7 but high levels of CD44 were considered memory/effector cells of nonmucosal origin. Preliminary work involving collagenase digest of spleen and MLN indicated no adverse effects of this enzyme treatment on the expression of CD44 or αβ7 molecules (data not shown).

Cell culture and anti-CD3 stimulation for cytokine expression

Mononuclear cells were counted in preparations of spleen, MLN, LP, and LP using a hemacytometer and then distributed in 96-well plates at a density of 4 × 10^5/well in 200 μl RPMI 1640 containing 5% FCS, penicillin, streptomycin, and added t-glutamine. Some wells were previously coated with anti-CD3 (clone 145 2C11 (32) at a concentration of 5 μg/ml in bicarbonate buffer, pH 8.5. Cells were incubated for 48 h, and the supernatants were then removed and stored at −20°C until use in ELISA. At least six individual cultures with stimulation of either anti-CD3 or control medium. Supernatants of each culture were pooled to determine the mean picograms per milliliter of cytokine produced by various cell preparations.

ELISA for measurement of cytokines in culture supernatants

ELISA kits for specific detection of mouse IL-4, IL-5, IL-10, and IFN-γ were purchased from R&D Systems (Minneapolis, MN) and used according to the manufacturer’s specifications. The minimum dilution of cell culture supernatants was 1/2, and some samples needed dilution of up to 1/200 due to high concentrations of some cytokines. The culture supernatants derived from cells taken at all time points (day 3, 6, or 9) were measured at the same time, to avoid variation between ELISA measurements. The results from at least two separate GVHR experiments were pooled to determine the mean picograms per milliliter of cytokine produced by various cell preparations.
Statistical methods

Comparisons of percentages of CD44 or αββ7+ cells based on flow cytometric data were examined using the Kruskal-Wallis nonparametric rank test, where differences were considered significant if p < 0.05. Comparisons among groups of data for IEL numbers, spleen and body weights, or cytokine concentrations were performed using the two-sided Student t test.

Results

Characterization of acute systemic and intestinal GVHR using the B10.BR F1 (B10.BR × DBA/2) strain combination

We first examined our unique mouse strain combination for typical histological and cellular manifestations of acute GVHR. To induce GVHR, 30 or 50 × 10⁶ B10.BR SP cells were transferred to 8-wk-old (B10.BR × DBA/2)F₁ mice, and both the intestine and spleen were examined 6, 9, and in some experiments 20 days later. SP were macroscopically enlarged and weighed significantly more than control F₁ mice (given F₁ splenocytes) by day 9, in all F₁ mice that received B10.BR cells (Table I). Increased SP weights or spleen-body weight ratios (spleen index) are typically observed in systemic GVHR (4). Both dosages of 30 and 50 × 10⁶ spleen cells induced significant spleen enlargement. Body weight does not begin to decline in GVHR mice until after day 14 (23), and we observed weight loss in GVHR mice by day 20 (Table I).

Pieces of jejunum were taken on day 9 from F₁ mice that received B10.BR SP cells or control F₁ SP cells. Sections were prepared and stained for histological examination. We observed increased crypt length as well as some altered villus structure in mice that received B10.BR cells. Villus lengths were not altered significantly on day 9 in GVHR mice but decreased slightly by day 20. Villus lengths were not altered significantly on day 9 in GVHR mice but decreased slightly by day 20 along with a more significant decrease in crypt length, resulting in a slightly higher villus-crypt ratio (Table I). Moderate alterations in villus length have been previously found for the unirradiated F₁ model of GVHR (3). Some villi showed edema, increased cell numbers in the LP, and occasional disruption of the epithelium by day 9 (data not shown). In addition, there were increased numbers of IEL along the length of the villi by day 9 (Table I). These increased IEL count and the villus-crypt ratio changes are typical manifestations of acute GVHR, as described previously by others (2, 3).

Localization of donor CD4 T cells and their expression of CD44 in intestine and associated lymphoid tissues during early GVHR

We isolated cells from SP, LP, Peyer’s patches (PP), and mesenteric lymph nodes (MLN) on day 9 of GVHR to determine the frequency of donor CD4 T cells that could contribute to inflammation in intestinal tissues during GVHR. The cells were stained with Abs against H-2KdD and CD4 molecules and analyzed by flow cytometry. The results indicated clearly the presence of significant numbers of donor T cells in all of the tissues by day 9 (Fig. 1A). Although at lower frequency than in the SP, the percentages of donor CD4 T cells in the MLN, PP, and LP ranged from 8 to 13%

CD4 T cells express high levels of CD44 within 24–48 h of activation (33, 34), and CD44 is considered a marker of memory CD4 T cells. In addition, effector CD4 T cells constitutively express CD44 in inflamed tissue sites, where CD44 interacts with extracellular matrix proteins and augments T cell activation (35–37). We examined CD44 expression on both donor and recipient CD4 T cells in intestinal and systemic tissues on day 9 of GVHR induction. Data in Fig. 1, A–E, show that a large fraction of donor cells expressed CD44 in all tissues. In cell preparations isolated from both the MLN and SP, a 2- to 3-fold higher fraction of donor CD4 T cells expressed CD44 compared with recipient CD4 T cells or CD4 T cells from F₁ controls (Fig. 1, B and C). The proportion of donor and recipient CD4 T cells expressing CD44 in the PP of GVHR mice was 3-fold higher than that of control F₁ mice (Fig. 1D). Both donor and recipient CD4 T cells from GVHR mice showed the highest proportion of CD44 expression in the LP (near-identical proportions ≈ 84%). However, this was only marginally higher than the expression of CD44 on CD4 cells of control mice given F₁ spleen cells (Fig. 1E). Thus, donor CD4 T cells had a higher degree of activation than recipient CD4 T cells within MLN and SP by day 9 of GVHR.

Detection of mucosal homing integrin αββ7+ on activated/effector donor CD4 T cells early in acute GVHR

The mucosal homing integrin αββ7+ is expressed by B and T cells that normally circulate from blood to mucosal sites and is frequently found on T or B cells that enter the intestinal LP (38–41). Recent studies have indicated a selective trafficking of T cells that express high amounts of αββ7+ and CD44 through Peyer’s patch, MLN, and LP (31). Because we observed many donor cells located in MLN, PP, and LP that expressed CD44 during early GVHR, we decided to determine what proportion of these cells also expressed the mucosal homing integrin. Thus, coincident expression of αββ7+ would indicate direct trafficking of donor CD4 T cells into intestinal tissues. Fig. 2A illustrates typical flow cytometry data distinguishing donor and recipient CD4 T cells obtained from MLN at day 9 of GVHR. Between 6 and 10% of donor CD4 T cells expressed both CD44 and high levels of αββ7+, a frequency that was

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Donor Strain</th>
<th>Day</th>
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<th>Body Weight</th>
<th>IEL Count</th>
<th>villus/Crypt Ratio</th>
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<td></td>
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<td>Mean (g) SEM</td>
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* Cells (50 × 10⁶) from donor in experiments 1 and 2. A third experiment with 30 × 10⁶ cells showed similar results.

* Day tissue sampled post-injection of cells.

* Significant, p < 0.01 for B10.BR cells vs F₁ cells or no cells.

* Significant, p < 0.05 for B10.BR cells vs F₁ cells or no cells.
3- to 4-fold higher than that found among recipient CD4 T cells (Fig. 2B). Interestingly, similar high proportions of \( \alpha_4 \beta_7^+ \) cells were found among SP cells, but both PP and LP showed equivalent fractions of \( \alpha_4 \beta_7^+ \) CD44+ donor and recipient cells (Fig. 2B). It was possible that the donor \( \alpha_4 \beta_7^+ \) CD4 T cells found in tissues from GVHR mice represented expansion of a small pool of donor CD4 T cells that had previously expressed \( \alpha_4 \beta_7^+ \) within the original spleen cell inoculum. To test whether the donor \( \alpha_4 \beta_7^+ \) was expressed de novo on donor CD4 T cells as a result of the GVHR, we depleted \( \alpha_4 \beta_7^+ \) cells from the donor inoculum by FACS before injection and then examined tissues on day 9 of GVHR. Comparative data in Fig. 2B show that even with prior depletion of donor \( \alpha_4 \beta_7^+ \) cells, a substantial fraction of donor CD4 T cells did express \( \alpha_4 \beta_7^+ \) in the MLN, SP, PP, and LP, by day 9. In fact, donor \( \alpha_4 \beta_7^+ \) CD4 cells were at higher frequency than recipient cells of the same phenotype in MLN and SP. Thus, donor CD4 T cells with a memory/effector phenotype and that express the mucosal homing integrin are generated during early GVHR.

IFN-\( \gamma \) production in systemic, intestinal, and intestine-associated lymphoid tissues during the early inductive phase of GVHR

We measured the production of the Th1-type cytokine IFN-\( \gamma \) by cells from MLN, PP, LP, and SP taken from mice at days 3, 6, and 9 after induction of acute GVHR. Cell suspensions were prepared and cultured for 48 h in the presence or absence of anti-CD3 Ab to determine the spontaneous and T cell-mediated cytokine production. The SP cells showed initial (days 3–6) production of IFN-\( \gamma \) that was not significantly different from SP cells taken from F1 control mice (Fig. 3). However, by day 9 both spontaneous and anti-CD3 production were greatly elevated (5- to 10-fold) relative to control. This kinetic of SP cell IFN-\( \gamma \) production has been observed previously using other combinations of mouse strains and is indicative of the systemic, Th1-dominated acute GVHR (5, 6, 42). The expression of IFN-\( \gamma \) in the intestine and intestinal-associated lymphoid tissues during early GVHR.
lymphoid tissues was more complex. MLN and PP cells taken from GVHR mice had a substantial early (day 3) elevation of spontaneous and anti-CD3-induced IFN-γ production. The IFN-γ production changed by day 9, when MLN cells produced even higher amounts of IFN-γ, but PP cells produced normal levels of IFN-γ. LP cells from GVHR mice did not show any increase in IFN-γ above that of control until day 9, paralleling the response of SP. Thus, MLN and PP are sites of increased production of IFN-γ very early in GVHR and the LP responds with increased IFN-γ only later, when responses are high in systemic tissues.

**IL-4 and IL-5 production from cells in systemic, intestinal, and intestine-associated lymphoid tissues during the early inductive phase of GVHR**

The murine intestine is a highly regulated immune environment and typically produces large amounts of regulatory cytokines, with immune responses often dominated by Th2 type cytokines (34, 44). IL-4 is a signature cytokine produced by Th2 cells and also is able to direct developing Th cells to differentiate into Th2 cells (45). We therefore examined IL-4 production by intestinal associated tissues during acute GVHR, presuming that if IL-4 were reduced in production, this would correlate with a dominant Th1 response, or if increased in production an opposing effect on Th1 cytokines would be evident. There was no spontaneous IL-4 production by any tissues from either GVHR mice or controls (Fig. 4). Anti-CD3-stimulated IL-4 production by MLN, PP, and SP cells was depressed 3- to 5-fold 3–6 days after induction of GVHR. However, the IL-4 production by SP and PP cells returned to near normal levels by day 9 of GVHR. In contrast, the IL-4 produced from MLN cell cultures remained depressed throughout days 3–9. LP cells showed an opposite pattern to those of PP and spleen, with normal production of IL-4 early on, and a subsequent 3-fold drop in production by day 9, compared with control tissues. Thus, the early high IFN-γ production in PP and MLN as well as the later high production in LP correlated with depressed IL-4, indicative of a Th1-dominated response at those times in those tissues.

IL-5 is an important intestinal cytokine with regard to its role in the differentiation of IgA-secreting plasma cells (46, 47). IL-5 is often considered a Th2-type cytokine (48, 49). However, its exact regulation in relation to IL-4 production in the intestine is not clearly understood, and to this point it has not been studied in intestinal GVHR. Spontaneous IL-5 production like that of IL-4 was very low or insignificant in all cell preparations (Fig. 5). In contrast, stimulation of cell cultures with anti-CD3 resulted in significant IL-5 production, but with different patterns of kinetics in the different tissues. SP cells from GVHR mice produced moderate amounts of IL-5 in the normal control range at all time points. Thus, IL-5 production by SP T cells was not altered by induction of GVHR. Surprisingly, PP cells showed normal or low production of IL-5 similar to SP cells during early GVHR. MLN cells had a distinct IL-5 production kinetic that began with high IL-5 production on day 3 and a return to normal low production by day 9. LP cells from GVHR mice provided the most dramatic results in regard to IL-5 production. On day 3, production of IL-5 was the
same as control LP cells, but this increased to 20-fold higher production by day 9. Thus, production of IL-5 by LPL cells from GVHR mice was opposite that of IL-4 production, showing a divergence of production of these Th2 types in the context of an intestinal Th1-type inflammation.

IL-10 production from cells in systemic, but not intestinal tissues during the early inductive phase of GVHR

Spontaneous and anti-CD3-mediated production of the immunoregulatory cytokine IL-10 was also examined. IL-10 is frequently referred to as a Th2-type cytokine and has many features in common with IL-4, particularly its ability to limit macrophage activation, reduce Th1 cell differentiation, and limit inflammation in mucosal tissues (50–52). A regulatory T cell (so-called Tr1 cell) has been described that produces large quantities of IL-10 and can modulate Th1-type inflammatory responses, including those of intestinal tissues during the early inductive phase of GVHR. Cell suspensions were isolated from SP, MLN, LP, and PP on days 3, 6, or 9 after GVHR induction and were cultured for 48 h with or without anti-CD3 Ab (see Materials and Methods). Data show mean (±SEM) picograms per milliliter IL-10 (y-axis) in culture supernatants as determined by ELISA (see Materials and Methods). Data are pooled from three separate experiments (n = 4–6). ○, ■. Response by cells taken from control F1 mice, compared with cells taken from GVHR mice (○, ■). *, Significant differences of IL-10 production between GVHR cells and control cells (p < 0.05).

Figures 5 and 6. Production of IL-10 by cell suspensions from systemic, intestinal, and intestine-associated tissues during early GVHR. Cell suspensions were isolated from SP, MLN, LP, and PP on days 3, 6, or 9 after GVHR induction and were cultured for 48 h with or without anti-CD3 Ab (see Materials and Methods). Data show mean (±SEM) picograms per milliliter IL-10 (y-axis) in culture supernatants as determined by ELISA (see Materials and Methods). Data are pooled from three separate experiments (n = 4–6). ○, ■. Response by cells taken from control F1 mice, compared with cells taken from GVHR mice (○, ■). *, Significant differences of IL-10 production between GVHR cells and control cells (p < 0.05).

Discussion

The experimental results reported here clearly indicate that donor CD4 T cells localize in the intestine and associated lymphoid tissues early during acute GVHR. Further, these cells express the CD44 molecule indicating previous activation and effector or memory cell function. A significant fraction of these cells found in the LP express the α4β7 mucosal homing integrin, and this expression can occur de novo during early acute GVHR. This early phenotypic shift among CD44+ donor CD4 T cells is completely consistent with the generation of effector CD4 T cells within the intestinal microenvironment and their subsequent homing to the LP. Thus, there is a clear potential for an intestinal circuit of donor CD4 T cells that can contribute to early GVHR and perhaps perpetuate the Th1 disease in the intestine. The donor CD4 cells may be alloreactive (anti-H-2b) or may have specificity for intestinal Ags, responded to early in GVHR. Our work was primarily directed at examination of CD4 cells in the initiation of intestinal GVHR but does not exclude a role for CD8 cells. Additional experiments must address the function and specificity of these donor CD4 cells and any CD8 cells or NK cells that participate early in intestinal GVHR.
These observations raise the possibility that blockade of cells bearing the \( \alpha_4\beta_7 \) integrin may prevent or reduce the early development of intestinal GVHR. Tanaka et al. (54) published data that showed a combination of anti-\( \alpha_4 \) and anti-\( \beta_7 \) Abs would block development of intestinal GVHD. This was done using an irradiated parent to the F1 GVHD model, in which independent effects of the \( \alpha_4 \) and \( \beta_7 \) Abs on separate cell populations could occur. The effects of intestinal damage by irradiation may alter the number and nature of cells infiltrating the intestine. Hence, combinations of anti-\( \alpha_4 \) and anti-\( \beta_7 \) Abs may alter other cell functions beyond those cells that express the \( \alpha_4\beta_7 \) heterodimer. It appears that the \( \alpha_4\beta_7^+ \) cells in the donor population are critical for rapid intestinal destruction seen in the irradiation model. Their observations are consistent with ours in that \( \alpha_4 \) and \( \beta_7 \)-expressing cells are part of the graft-vs-host response in the intestine. However, our work indicates that donor \( \alpha_4\beta_7^+ \) cells are not required at the initiation of GVHR in the nonirradiation model, because if \( \alpha_4\beta_7^+ \) cells are removed from the inoculum, GVHR is still induced. In addition, donor cells can later (de novo) express \( \alpha_4\beta_7 \). It is tempting to speculate that some naive donor CD4 T cells circulate through the PP and MLN and acquire \( \alpha_4\beta_7 \) expression, allowing them to better home to the LP.

Our observations of cytokine production in the spleen during GVHR are largely consistent with previous reports and provide some novel observations. Cytokines have been investigated by several groups using both RT-PCR and protein assay analyses (5, 6, 8, 42). All of these studies point to the early (day 8 –10 peak) destruction seen in the irradiation model. Their observations are consistent with ours in that \( \alpha_4 \) and \( \beta_7 \)-expressing cells are part of the graft-vs-host response in the intestine. However, our work indicates that donor \( \alpha_4\beta_7^+ \) cells are not required at the initiation of GVHR in the nonirradiation model, because if \( \alpha_4\beta_7^+ \) cells are removed from the inoculum, GVHR is still induced. In addition, donor cells can later (de novo) express \( \alpha_4\beta_7 \). It is tempting to speculate that some naive donor CD4 T cells circulate through the PP and MLN and acquire \( \alpha_4\beta_7 \) expression, allowing them to better home to the LP.

Our observations of cytokine production in the spleen during GVHR are largely consistent with previous reports and provide some novel observations. Cytokines have been investigated by several groups using both RT-PCR and protein assay analyses (5, 6, 8, 42). All of these studies point to the early (day 8 –10 peak) production of IFN-\( \gamma \), in both spontaneous and stimulated (anti-CD3 or T cell mitogen) cell cultures. Our results in spleen are in complete agreement with those earlier findings. Rus et al. (6) examined kinetics of IL-4 and IL-10 mRNA and protein during acute GVHR. Their results indicate an early increase in mRNA for both cytokines but a decline in T cell-stimulated IL-4 protein production during the early stages of acute GVHR, with no changes in spontaneous production. This is consistent with our observations of low anti-CD3-induced IL-4 production by spleen cells. The IL-10 results also indicated an elevated spontaneous IL-10 production by spleen cells consistent with the mRNA results of Rus et al. In addition, the data clearly showed that anti-CD3-stimulated IL-10 starts high but declines quickly during 9 days to levels indistinguishable from normal spontaneous production. Both the early, suppressed IL-4 and the decline in IL-10 were coincident with the increased IFN-\( \gamma \) and point to a deviation of the splenic cytokine environment from a Th2- toward a Th1-dominated microenvironment. Previous work by Williamson et al. (10) indicated no detectable splenic IL-5 in response to Con A by day 2 of GVHR, but enhanced IL-5 production much later (day 70) if GVHR mice were treated with anti-IL-12. We stimulated splenic cells with anti-CD3 and did observe some production of IL-5. However, our data show that the amount of IL-5 produced from GVHR spleen cells was not different from that for control spleens and that there was no change in splenic IL-5 production between days 3 and 9. This indicated the independence of IL-5 production from IL-4 and IL-10 during early systemic (splenic) GVHR.

A main objective in our studies was to characterize the early cytokine profile in intestinal-associated lymphoid tissues, because the intestine is a primary target of GVHR but has distinct immunoregulatory controls compared with systemic tissues. Our results provide the first analyses of cytokine production in the intestinal-associated tissues during acute GVHR in the mouse. These results indicate clear contrasts between intestinal tissues and spleen with regard to cytokine expression early in GVHR. The overall picture is one in which the MLN and small intestinal LP show the greatest changes reflective of a developing Th1-dominant response, contrasting the PP that at best is involved only in the initiation of a Th1 response at the earliest time points. A summary of the dynamics of cytokine changes in the intestine and associated lymphoid tissues compared with the spleen is illustrated in Fig. 8.

The LP appeared to respond primarily as target organ for the effector phase of the developing Th1 response in GVHR. For instance, both spontaneous and anti-CD3-induced IFN-\( \gamma \) production was elevated and anti-CD3-induced IL-4 was reduced by day 9, paralleling the responses in spleen. The modest amounts of spontaneous IL-10 production by LP cells decayed to undetectable levels by day 9, presumably indicating lack of local control of the developing Th1 response. Increased T cell-derived IL-5 production parallels IFN-\( \gamma \) and was reciprocal to the IL-4, showing that IL-5 is independent of the regulatory balance between Th1 or Th2 cytokines in this tissue. IL-5 is a differentiation factor for IgA-producing B cell blasts (55, 56), and this may help explain the increased number of plasma cells found in the intestine during acute GVHR (2). The coincidence of IL-5 and IFN-\( \gamma \) can be explained by the peculiar nature of intestinal T cells (both LP and intraepithelial) that are known to secrete large quantities of both cytokines after in vitro stimulation with anti-CD3 Abs (57). The high IL-5 production was significant in LP only by day 9, and this occurs several days after abnormally high levels of IL-5 are produced in the MLN. This suggests that the earliest wave of reactive T cells in the MLN could provide the precursors for effector cells in the LP that secrete high levels of IL-5 (and IFN-\( \gamma \)) by day 9.

The PP demonstrated a restricted, brief, but substantial change in cytokine production during early GVHR. The day 3, anti-CD3 responses indicated high production of IFN-\( \gamma \) and coincident low production of IL-4. Both spontaneous and anti-CD3 production of

![FIGURE 7. Summary of kinetics of cytokine responses in SP, MLN, PP, and LP early in GVHR. This composite chart illustrates the dynamics of the IL-4, IL-10, IFN-\( \gamma \), and IL-5 production in various tissues between day 0 and day 9 of GVHR. For simplicity, the symbols represent trends, reflective of both spontaneous (if changes occurred) and anti-CD3 responses.](http://www.jimmunol.org/)
IFN-γ was elevated earlier in PP than in the spleen. The production of IFN-γ and IL-4 returned to normal by day 9. A clear interpretation of this finding is that the PP provide an early differentiation environment for Th1 cells that could then travel to the draining MLN, where they continue their differentiation before eventual homing to the LP. IL-10 production in the PP does not change over this time (in contrast to SP and MLN where it declines); therefore, regulatory control by the activated T cells may remain dominant in this tissue, despite the early bias toward IFN-γ production. In fact, the day 3 spontaneous IFN-γ production in the PP is the highest among all tissues tested and 20 times above normal control levels. This might be explained by an increased presence of activated NK cells secreting IFN-γ. NK cells have been described in systemic tissues as early as day 3 (58–60) and among intestinal IEL during GVHR (61). NK cell functions among cells from PP, MLN, and LP have not been reported for the acute GVHR model. Investigation of NK cell activity very early (days 1–3) in the PP would be a reasonable future approach to explaining this observation.

The MLN in GVHR mice showed the greatest and most complex alterations in cytokine production among the intestinal-associated lymphoid tissues. As discussed above, donor CD4 T cells expressing CD44 were in high frequency in the MLN (similar to SP), inferring a large presence of allogeneic donor CD4 T cells by day 9 of GVHR. The IL-4 and IL-10 responses in the MLN paralleled the same trends of response displayed by the spleen, with overall depressed IL-4 and early elevated IL-10 that declines by day 9. IFN-γ production is substantially different in MLN, however. Like PP, both spontaneous and anti-CD3-induced IFN-γ was very high, beginning early on day 3. However, unlike PP (but similar to spleen) the IFN-γ production continued to rise to 30- to 100-fold above normal levels by day 9. The reciprocal expressions of low IL-4 and IL-10 vs high IFN-γ indicate that MLN was a site of early and continuous Th1 response during the early days of developing intestinal GVHR. The early, high, and spontaneous production of IFN-γ could have resulted from active NK cells in this tissue, just as in the PP. There was also a high expression of IL-5 early in the MLN that decays to normal levels by day 9. It is unclear why this should occur. One possibility is that the high initial IL-5 plus IFN-γ responses reflect the involvement of migrating intestinal memory T cells that express both cytokines (57).

Overall, it appears that the MLN shifts its phenotype to a Th1-grafting intestinal memory T cells that express both cytokines (57). The MLN in GVHR mice showed the greatest and most complex alterations in cytokine production among the intestinal-associated lymphoid tissues. As discussed above, donor CD4 T cells expressing CD44 were in high frequency in the MLN (similar to SP), inferring a large presence of allogeneic donor CD4 T cells by day 9 of GVHR. The IL-4 and IL-10 responses in the MLN paralleled the same trends of response displayed by the spleen, with overall depressed IL-4 and early elevated IL-10 that declines by day 9. IFN-γ production is substantially different in MLN, however. Like PP, both spontaneous and anti-CD3-induced IFN-γ was very high, beginning early on day 3. However, unlike PP (but similar to spleen) the IFN-γ production continued to rise to 30- to 100-fold above normal levels by day 9. The reciprocal expressions of low IL-4 and IL-10 vs high IFN-γ indicate that MLN was a site of early and continuous Th1 dominated environment early in GVHR response, even earlier than systemic tissues such as the SP. The Th1 response then continues to increase with accumulation of donor CD4 T cells.

There are some provocative implications to the early cytokine response during intestinal GVHR. First, it has long been suspected that intestinal GVHR may be driven in part by intestinal Ags (digestive, bacterial, etc.), along with the initial stimulation of donor anti-recipients of MLN. Because we have observed an early and substantial IFN-γ response in the intestinal-associated tissues, it is possible that large numbers of recipient Th1 cells that are specific for intestinal Ags could participate in the local intestinal pathology. IFN-γ can induce increased epithelial permeability as well as crypt cell hyperplasia (9, 62, 63). Increased permeability early in the intestine could allow early access to large amounts of intestinal to drive responses by Ag-specific recipient cells. In this regard, the higher proportions of recipient CD44+CD4 T cells in the MLN and LP that we observed in early GVHR could reflect many of the recipient memory T cells engaged by intestinal Ags. It will now be necessary to investigate the Ag specificities of the donor and recipient CD44+ cells as well as the cytokine response to those Ags to fully appreciate the nature of the developing intestinal GVHR and to allow new approaches to control this response.


