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The Parent-into-F1 Model of Graft-*vs*-Host Disease as a Model of *In Vivo* T Cell Function and Immunomodulation

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Abstract

Since its description roughly 30 years ago, the parent-into-F1 model of graft-*vs*.-host disease has provided insights into the mechanisms of *in vivo* T cell activation and the pathogenesis of autoimmune conditions. A new and emerging role for the P→F1 model is one of identifying agents with immunomodulatory activity and defining *in vivo* mechanisms that promote cell mediated or antibody mediated immune responses. Because F1 mice are not irradiated prior to donor cell transfer, the P→F1 model has in the past not been strictly analogous to human hematopoetic stem cell transplantation. However with the advent of newer non-myeloablative conditioning regimens, the model may assume more relevance. In this article, we first provide a review of relevant earlier fundamental observations followed by a summary of recent work from our laboratory in which acute and chronic GVHD in this model have been used not only to study normal T cell responses *in vivo* but also to define mechanisms important in the pathogenesis of autoimmunity and immunomodulation.

Keywords

Graft-*vs*.-host disease; lupus; cytotoxic T lymphocytes; interferon gamma; tumor necrosis factor alpha

A. INTRODUCTION

Prior to the identification of lymphocyte subsets, experiments in rats had demonstrated that the injection of immunogically competent cells into newborns, F1 hybrids, irradiated recipients or tolerized adults could induce a condition termed homologous disease characterized by features resembling human connective tissue disease [1–6]. It was subsequently shown in mice that the transfer of homozygous parental strain lymphocytes into normal F1 recipients induced a graft-*vs*.-host disease (GVHD), which resembled homologous disease in rats. This model, termed the parent-into F1 (P→F1) model of GVHD differs from other murine models of GVHD in that the recipient is not irradiated and the donor inoculum consists of splenic or lymph node lymphocytes without the addition of bone marrow cells. Nevertheless, as a model of alloantigen driven T cell attack on host tissues, the model has potential relevance to humans with GVHD

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following hematopoetic stem cell (HSC) transplantation. The model has also provided insights into the pathogenesis of human lupus and into normal *in vivo* T cell effector responses (reviewed in [7]. This review will focus primarily on recent results from our laboratory and their implications in the pathogenesis of immune mediated conditions.

B. IMMUNOPATHOGENESIS AND CLINICAL PHENOTYPE OF GVHD IN THE P→F1 MODEL

Many of the critical immunopathological mechanisms involved in GVHD development in the P→F1 model were elucidated by several laboratories in the 1980's (reviewed in [8–11], the salient features of which are discussed. Briefly, following *in vivo* transfer, homozygous parental strain (donor) T cells recognize F1 (host) alloantigens of the opposite parent. Initially, a graft-*vs*.-host reaction is imitated by donor CD4+ T cell recognition of host allogeneic MHC II molecules. The development of clinical GVHD evolves over weeks to months and takes one of two forms depending on whether donor CD8+ T cells become secondarily activated.

Chronic GVHD

If donor T cell activation is limited to the CD4+ T subset following encounter with host alloantigens, an immunostimulatory GVHD ensues, which also been termed chronic, autoimmune or lupus-like GVHD. IgG production by B cells requires two signals, an antigen specific signal delivered through the B cell receptor (signal 1) and a CD4+ T helper cell dependent signal (signal 2). Chronic GVHD is a result of donor CD4+ T cell cognate help for potentially all host B cells (signal 2) through recognition of foreign MHC II. The resultant polyclonal B cell hyperactivity leads to autoantibody production by those autoreactive B cells that have also encountered self-antigen through the B cell receptor (signal 1). Immune complex formation and a lupus-like renal disease ensue resulting in death at approximately 4–6 months from renal insufficiency. Chronic GVHD occurs in P→F1 combinations which have in common the selective activation of donor CD4+ T cells either by depletion of donor CD8+ T cells prior to injection (purified donor CD4+ T cells) into a fully allogeneic F1 e.g., C57Bl/6 → (C57Bl/6 × DBA/2)F1 (B6→>BDF1); or the injection of unfractionated donor T cells into an MHC II only disparate F1 (e.g., B6→B6 × $B6^{bm12}$). In the absence of an MHC I disparity, donor CD8+ T cells do not become activated. Moreover, it is not necessary for the recipient to be an F1, but merely MHC II disparate e.g., B6→$B6^{bm12}$ as murine CD4+ T cells are MHC II negative and a host-*vs*.-graft response does not occur [12]. Surprisingly, chronic GVHD occurs following the transfer of DBA/2 splenocytes into B6D2F1 recipients despite the transfer of both CD4+ and CD8+ donor T cells into a fully allogeneic recipient. This outcome reflects a defect in DBA/2 CD8+T cells that is not completely understood and discussed in more detail in Section D.

Acute GVHD

In contrast to the above CD4+ T cell driven scenario, a different form of GVHD occurs when both donor CD4+ and CD8 + T cells are injected into a fully MHC I + II disparate F1 recipient. The initial step, activation of donor CD4+ T cells, is similar to the initial step in chronic GVHD, i.e. MHC II specific donor CD4+ T cells promote host B cell expansion and autoantibody production. In acute GVHD, donor CD4+ T cells also provide help for the activation of donor CD8+ T cells specific for host allogeneic MHC I. An unresolved question is whether CD4+ T cell provide help for donor CD8+ T cells through a direct effect or whether donor CD4+ T cells act indirectly by "licensing" host APC [13–15] which, in turn induce maturation of CD8 + pCTL.

In both acute and chronic GVHD, the initial 7–10 days after donor cell transfer are characterized by donor CD4+ T cell expansion, host lymphoproliferation and host B cell expansion, however

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in acute GVHD donor CD8+ T cells also engraft, expand and mature into effector cytotoxic T lymphocytes (CTL) which eliminate host lymphocytes from days 10–14 after transfer [16]. With the loss of immunocompetent host cells, a profound immunodeficiency is present by day 14 [17–20] termed acute, lethal or suppressive GVHD. Lethality is high at 2–3 weeks of disease when the source of donor T cells is lymph nodes whereas the use of unfractionated splenocytes includes stem cells which allow repopulation of the host immune system and result in improved and prolonged survival [21,22].

C. CLINICAL PHENOTYPE OF ACUTE AND CHRONIC GVHD CAN BE PREDICATED BY EARLIER SURROGATE MARKERS

In vitro CTL Function Differentiates Acute From Chronic GVHD at Two Weeks of Disease

Initially acute and chronic GVHD phenotypes were defined using clinical endpoints such as mortality, hair loss, hunched posture and weight loss (acute GVHD) or the development of autoimmune parameters such as serum autoantibody levels and immune complex glomerulonephritis (chronic GVHD). Work by the Shearer laboratory (reviewed in [17,18, 20,23] demonstrated that an immunological phenotype could be discerned as early as 14 days after donor cell transfer using *in vitro* CTL generation to either hapten modified self (TNP-self) or allogeneic targets. At 14 days of disease, acute GVHD mice exhibit a complete loss of CTL responses to both TNP-self and to alloantigen whereas GVHD induced across an MHC II difference only (B6→B6 × $B6^{bm12}$) was associated with a selective loss of CTL function to TNP self and preservation of CTL responses to alloantigen. Although initially puzzling the interpretation of these findings was facilitated by *in vitro* studies of Singer *et al*. [24] that examined the T helper pathways involved in the maturation of murine *in vitro* CD8+ CTL. These workers demonstrated that for TNP-specific responses, antigen presentation by self-APC to CD4+ T cells was the sole pathway of Th cell IL-2 production. In contrast, allo-specific CTL exhibited redundancy in T helper pathways and three pathways were identified: an indirect pathway in which IL-2 production was induced by alloantigens presented to CD4+ T cells on self APC; and two direct pathways in which IL-2 production was induced by CD4+ T cells or CD8+ T cells in response to alloantigens presented allogeneic APC. These three pathways of alloantigenic T cell stimulation were subsequently shown to be operative also in human *in vitro* allogeneic CTL responses of PBL also [25]. The loss of CTL responses to TNP-self and to alloantigen in acute GVHD mice, although associated with a defect in IL-2 production *in vitro*, could not be corrected with exogenous helper factors thereby indicating a loss of functional precursor T cells and consistent with the splenic lymphopenia and immunodeficiency present at two weeks. In chronic GVHD mice, the selective loss of TNP-self CTL response and its correction with exogenous helper factors did not implicate a loss of T cell precursors but instead indicated a defect in the indirect pathway of Th cell IL-2 production, possibly due to cytokine dysreguation. A similar pattern of impaired TNP-self and preserved allogeneic *in vitro* CTL responses was subsequently observed in another chronic GVHD combination, DBA→F1 [20] which had been shown to result in lupus like disease [8]. These results raised the possibility that the selective loss of self-TNP CTL response was a marker of on-going autoimmunity and a common feature of chronic GVHD. This idea was subsequently confirmed in other P→F1 MHC II disparate combinations [10,18,23,26] and in the MRL/lpr model of spontaneous lupus [27]. Although young MRL/lpr mice exhibit relatively intact *in vivo* CTL responses to TNP-self and to alloantigens, with age there is a selective loss of the TNP-self CTL response with relative preservation of allogeneic CTL responses. Moreover, depletion and add back studies indicated that loss of TNP-self CTL responses were due to a CD4+ T cell population which could suppress the normal TNP-self CTL responses of age matched control MRL +/+ splenocytes. These studies supported the idea that the selective loss of *in vitro* TNP-self CTL function was a common feature of conditions characterized by T cell driven B cell hyperactivity and likely reflected the production of

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cytokines by CD4+ T cells which downregulate MHC self restricted CTL responses through the indirect pathway. A similar pattern of selective loss of indirect pathway function coupled with intact direct pathway function has been reported in humans with other causes of B cell hyperactivity to include early asymptomatic HIV patients [28] and patients with systemic lupus erythematosus [29] . Although the precise mechanism underlying the selective loss of the indirect Th pathway has not been fully elucidated, the diversity of conditions where it is seen underscores the limited repertoire of immune responses and supports the idea that immune mechanisms that are fully defined in the P→F1 model will not only have relevance to human immune mediated conditions may also identify new therapeutic targets.

Additional Early Surrogate Markers of Acute and Chronic GVHD

Based on the foregoing, it is clear that as early as two weeks after parental cell transfer, acute and chronic GVHD mice exhibit differential *in vitro* CTL responsiveness which allows the distinction of these two forms of GVHD without the use of clinical phenotypic markers. Subsequent work demonstrated that additional markers were present at two weeks after parental cell transfer that could reliably distinguish acute and chronic GVHD mice [16,30,31]. Such surrogate markers obviate the need for longer term studies performed solely for the purpose of phenotype determination. Long term studies however are still important for evaluating the effects of a given manipulation on the severity of acute or chronic disease. The major surrogate markers summarized in Table 1 represent the two distinct underlying pathological processes involved. Acute GVHD requires the engraftment of both donor T cell subsets and is mediated by a strong antihost CD8+ CTL response yielding host lymphocyte elimination. Thus, at two weeks, acute GVHD mice exhibit engraftment of both donor T cell subsets, demonstrable anti-host CTL activity *ex vivo* (best seen at day 10), and a profound reduction in host lymphocytes. This strong cell mediated immune response is accompanied by striking elevations of the Th1 cytokine IFN-g which peaks at day 7 but remains strongly elevated through day 14 [16,32]. Fas and Fas ligand (FasL) are significantly upregulated on both donor and host T cells and is IFN-g dependent [30]. From a functional standpoint, the upregulation of FasL on donor T cells improves donor CD8+ anti-host CTL activity whereas the upregulation of Fas on host B cells enhances their recognition by FasL positive donor CTL. The role, if any, of upregulated Fas on donor T cells and FasL on host T cells is under investigation. During the third week of acute GVHD donor T cells become difficult to detect in the spleens of recipient mice [33] which likely reflects a number of down regulatory events typical of *in vivo* CTL responses to include activation induced cell death. However, as discussed later, mature donor CTL effectors may also emigrate from the spleen and attack other non-lymphoid organs such as lung and kidney [34].

Because chronic GVHD results from selective activation of donor CD4+ T cells, B cell expansion proceeds unchecked in the absence of donor CD8+T CTL. As shown in Table 1, chronic GVHD mice exhibit donor CD4+ T cell engraftment only, increased host B cells numbers compared to normal F1 mice and autoantibody production at two weeks of disease. An early and consistently detected autoantibody is IgG anti-single stranted (ss)DNA antibody. There appear to be no stochastic events involved in its production as typically 100% of mice exhibit elevated IgG anti-ssDNA response by 2 weeks of disease (C. Via, unpublished observations). In our experience, lack of an anti-ssDNA elevation at two weeks is indicative of an injection failure and/or technical problems that can be corroborated by a corresponding lack of donor CD4+ T cell engraftment. Although some anti-ssDNA ab is produced in acute GVHD, the levels are transient and decline as B cells are eliminated.

Compared to acute GVHD mice, chronic GVHD mice exhibit much less striking elevations of IFN-g production however levels are still elevated over controls as early as day 7 [31]. Similarly, upregulation of Fas and FasL on both donor and host lymphocytes in chronic GVHD

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is elevated compared to control F1 mice it is significantly lower than that observed for acute GVHD mice [30]. It has not been determined whether the low level increases in Fas and FasL in chronic GVHD mice have a functional role.

D. USE OF THE P→F1 MODEL FOR THE ELUCIDATION OF NORMAL T CELL FUNCTION *IN VIVO*

The understanding of antigen specific *in vivo* T cell responses is critical to our ability to devise therapeutic approaches for a wide variety of immune mediated conditions. Conditions as diverse as rheumatoid arthritis, inflammatory bowel disease, and lupus erythematosus are all immune mediated and likely share common mechanisms, particularly cytokine dysregulation. Unfortunately, the study of the antigen specific T cells in immune mediated conditions is difficult due not only to their low frequency but also in many cases to the fact that the inciting antigen is not known, precluding the use of tetramer technology. Adoptive transfer models using transgenic T cells specific for nominal antigen have been enormously helpful to our understanding of *in vivo* cellular immunology mechanisms. Based on the foregoing, it is clear that, despite the deleterious effects on the recipient F1, acute GVHD models a normal *in vivo* CMI CTL response and chronic GVHD models a normal T cell dependent antibody response. The antigen specific cells driving disease are of donor origin and can be identified by flow cytometry. Thus the GVHD model can thought of as an additional adoptive transfer model for the study of normal *in vivo* CMI or T-dependent antibody mediated responses.

Studies of Naïve T Cell Activation and Costimulation in GVHD

As described above, the initiation of acute and chronic GVHD is T cell dependent [8]. Therefore, the model is well suited for the study early events in T cell activation *in vivo*. While many such *in vivo* studies have confirmed principles based on *in vitro* studies there have been some striking exceptions which underscore the importance of *in vivo* models in the understanding of immune function. For example, a burst of IL-2 production by donor CD4+ T cells during the first days after donor cell transfer was readily detected *ex vivo* in both acute and chronic GVHD mice [35]. Given the well accepted role of IL-2 in naive T cell activation and expansion *in vitro*, these results are not unexpected and confirm the central role of donor T cells in GVHD initiation. Surprisingly, inhibition of IL-2 in acute GVHD mice did not block T cell activation or disease induction as might be predicted but instead converted disease phenotype from acute GVHD to chronic GVHD [36]. These results and their interpretation are discussed further in Section E. ii.

B7-CD28

The B7-CD28 family of costimulatory molecules provides a critical second signal necessary for naïve T cell activation upon engagement of the T cell receptor (reviewed in [37]. In the absence of this costimulatory signal, T cell receptor engagement by naïve T cells does not result in maturation into effector cells but instead results in anergy. B7-CD28 costimulatory blockade at the time of donor cell transfer would therefore be predicted to block development of both forms of GVHD and such is in fact that case. Complete *in vivo* blockade with CTLA4Ig (a fusion protein that binds to both CD80 and CD86), blocks both acute and chronic GVHD development when administered at the time of donor cell transfer [38,39]. Interestingly, delayed (day 7) administration of CTLA4 Ig reverses chronic GVHD parameters although it requires several weeks, implying that autoantibody production in chronic GVHD requires continuous T cell help for B cells. In contrast, acute GVHD could not be reversed with delayed CTLA4Ig administration supporting the idea that mature CTL effectors are not responsive to B7 mediated control mechanisms [39]. Similarly, treatment of GVHD mice with combined anti-CD80 and anti-CD86 mAb beginning at donor cell transfer reproduces the effect seen with CTLA4 Ig e.g., complete inhibition of either acute or chronic GVHD [40]. These results

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underscore the critical role of the B7 family of costimulatory molecules and indicate that despite the increasing number of costimulatory molecules described in recent years, the B7- CD28/CTLA4 family is critical for naïve T cell activation *in vivo*.

CTL Effector Generation

The presence of anti host CTL in acute GVHD is a long-standing observation [41]. Recent work has demonstrated that in the spleens of B6→F1 acute GVHD mice, anti host CTL are CD8+, MHC I restricted and kill host cells by both the perforin and Fas/FasL pathway [30, 33]. This conclusion is based on *ex vivo* studies demonstrating that in a four hour chromium release assay, all of the anti host CTL activity can be accounted for by perforin and Fas pathways. It is possible however that other killing pathways (e.g., TNF-a mediated) contribute to the host attack *in vivo* that are not detected in *ex vivo*. There appears to be nothing unusual about effector CTL generation in acute GVHD mice in that they follow an expected pattern of expansion, maturation and downregulation typical of *in vivo* CTL generation. By 7 days after donor cell transfer, donor CD8+ T cells exhibit significant expansion and *ex vivo* activity is first detectable [16]. Maximal CD8+ T cell numbers and anti-host killing occur during the 9–11 day time period after which both decline. After day 14, *ex vivo* anti-host CTL activity approaches baseline and donor CD8+ T cells are difficult to detect in the spleens of acute GVHD mice likely reflecting the presence of downregulatory mechanisms such as activation induced cell death and possibly others. It is also possible that effector anti host CTL migrate out of the spleen by two weeks and attack other host tissues.

Although it is possible that other killing pathways in addition to perforin or Fas (e.g., TNF-a mediated) contribute to the host attack *in vivo* which are not detected in an *ex vivo* 4 hour Cr release assay, recent work does not support this idea [31]. We have recently demonstrated that a sensitive indicator of anti-host killing activity in acute GVHD is host splenic B cell numbers. Following the initial donor CD4+ T cell driven expansion of host B cells (days 3–10), host B cell numbers decline precipitously from day 10 onward and are barely detectable at day 14. This drop occurs coincident with maturation of CTL effector function and increased *ex vivo* killing activity. Of note, a mild impairment of anti host CTL activity could not be detected as a measurable reduction in *ex vivo* killing but was readily be observed as impaired elimination of host B cells. Moreover, when both the perforin and FasL pathway of anti-host CTL are blocked *in vivo*, host B cells are not reduced compared to control F1 mice and instead are significantly increased indicative of chronic GVHD [31]. These results support the idea that FasL and perforin are the major effector molecules involved in the host B cell elimination characteristic of acute GVHD.

E. ROLE OF CTL IN THE ACUTE GVHD PATHOGENESIS: THE DBA→F1 PARADOX

Although it was initially unclear as to whether donor CD8+ T cells mediated acute GVHD through suppressor activity [8] or through cytotoxic activity [20], it is now generally agreed that anti host cytotoxicity is a prominent if not exclusive factor in mediating host lypmphopenia [42]. An unexplained paradox has been the observance of chronic GVHD in DBA →F1 mice. As previously discussed, donor inocula containing both CD4+ and CD8+ parental T cell subsets injected into fully allogeneic (MHC I + II disparate) F1 induce acute GVHD as exemplified by the injection of unfractionated B6 splenocytes into B6D2F1 mice. Surprisingly, the injection of unfractionated splenocytes from the other parent (DBA/2) into B6D2F1 mice results in chronic GVHD.

Studies using irradiated recipients were unable to demonstrate differences in the anti-F1 CTL capacity of B6 *vs.* DBA/2 donor cells [43], however the non rate limiting conditions of the

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experimental protocol used complicated interpretation. Using a limiting dilution analysis, it was demonstrated that the anti-F1 precursor CTL frequency of B6 mice was approximately 9–10 fold greater than that of DBA/2 mice [20]. Additionally, unfractionated B6 spleen cells contain almost 2-fold more CD8+T cells than do DBA/2 spleens [11]. Thus, DBA mice exhibit a quantitative and possibly a qualitative defect in CD8+ T cells. Acute GVHD (as measured by host B cell elimination at two weeks of disease) can be induced in DBA→F1 mice by significantly increasing the number of donor splenocytes in the donor inoculum. Typically 50 × 10^6 unfractionated B6 splenocytes →BDF1 mice induces acute GVHD whereas 80–100 × 10^6 DBA/2 splenocytes →BDF1 mice results in chronic GVHD. Increasing the DBA/2 donor inoculum 100–180 × 10^6 donor cells converted disease phenotype from chronic to acute consistent with the reduced anti-F1 precursor CTL frequency [44]. The differential *in vitro* anti-F1 precursor CTL frequency of B6 mice compared to DBA/2 mice was confirmed *in vivo* by Rus *et al*. [16] who demonstrated that supplementation of the DBA/2 donor inoculum with CD8+ T cells from H-2^d mice of the B6 background (B10.D2) was much more effective in converting chronic GVHD to acute GVHD than was supplementation with an equal number of additional DBA/2 CD8+T cells. The exact nature of the defect in DBA/2 CD8+ T cells is still under investigation and it is not clear whether the defect is intrinsic to CD8+ T cells or, as has been suggested, is secondary to defective CD4+ T cell help for CTL due to a skewing towards a Th2 response [45].

F. GVHD AS A MODEL FOR THE STUDY OF *IN VIVO* IMMUNOMODULATION

i. Agents which Promote CTL and CMI *In vivo* Convert Chronic GVHD to Acute GVHD in the DBA→F1 Model

Regardless of the exact nature of the defect in DBA/2 CD8+T cells, the DBA→F1 model of GVHD has proven useful in the study of agents with potential immunomodulatory activity, particularly CTL promoting properties. CD8+T cells are transferred in the DBA/2 donor inoculum when unfractionated splenocytes are used, however they fail to mature into sufficient numbers of CTL effectors to induce acute GVHD. It is possible that administration of agents which promote CTL effector development and/or function (either directly or indirectly) will induce anti-host CTL in DBA→F1 mice and convert disease phenotype from chronic to acute GVHD.

rIL-12 Administration—rIL-12 is a strong Th1 cytokine inducer and promoter of CTL. Administration of rIL-12 early (days 0–4) after parental cell transfer in DBA→F1 mice induced an acute GVHD phenotype consisting of anti-host CTL activity *ex vivo*, host B cell elimination and a reduction of serum autoantibodies [46]. These results demonstrate that regardless of the exact nature of the defect in DBA/2 CD8+T cells, rIL-12 and possibly other agents can either correct or compensate for the defect and induce maturation of CD8+ T cells into CTL effectors converting chronic GVHD to acute GVHD. Because this effect can be determined using surrogate markers at two weeks, the DBA→F1 model is useful for rapid screening agents with CTL promoting properties.

CD80 Blockade—As discussed above, complete B7-CD28 costimulatory blockade using either CTLA4Ig or combined anti CD80/CD86 mAb prevents the development of acute GVHD in B6→F1 mice and chronic GVHD in DBA→F1 mice [38,39]. Because the B7 ligands, CD28 and CTLA4 promote T cell stimulation and inhibition respectively [37], B7 blockade could potentially result in either inhibitory and stimulatory patterns depending on the timing of blockade and the ligand which is interrupted. Such an outcome was observed with selective CD80 blockade. Whereas selective CD86 blockade was almost as effective as combined CD80/CD86 blockade in inhibiting both acute and chronic GVHD onset, selective CD80 blockade surprisingly promoted acute GVHD development and was best seen in the DBA→F1 model

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of chronic GVHD [40]. These data support the hypothesis that CD28 mediated activation of donor CD4+ T cells is preferentially dependent on CD86 ligand binding. In contrast, CTLA4 mediated down regulation of donor T cells, particularly CD8+ T cells is preferentially mediated by CD80 ligand binding. Moreover, the conversion by CD80 blockade of chronic GVHD to acute GVHD in the DBA→F1 model indicates that donor CD8+ T cells are potentially functional and can be enhanced by removing downregulatory influences.

ii. Agents that Selectively Inhibit CTL and CMI *In vivo* Convert Acute GVHD to Chronic GVHD in the B6→F1 Model

The foregoing results in DBA→F1 mice raise the possibility that the converse might also be true, i.e. that selective blockade of cytokines or molecules critical for CTL induction but not involved in CD4+ T cell help for B cells will convert acute GVHD to chronic GVHD. Such results would be best seen in a robust model of anti-host CTL development e.g. B6→F1 mice.

IL-2 Blockade—The first support for this idea was seen with IL-2 blockade in B6→F1 mice [36]. Given the critical role of IL-2 in naïve T cell activation and the observation that elevated IL-2 levels are present at the onset of both acute and chronic GVHD, it could be hypothesized that IL-2 blockade would act similar to that seen with complete costimulatory blockade and prevent the development of both acute and chronic GHVD. Surprisingly, acute GVHD was converted to chronic GVHD with anti-IL-2 mAb treatment as evidenced by a complete block in anti host CTL activity *in vitro*, an increase in host B cell numbers, and an increase in serum anti-ssDNA ab and IL-4 dependent Ig isotypes. Despite the complete elimination of anti-host CTL activity and expansion of B cells, donor CD8+ T cell engraftment was comparable to control acute GVHD mice suggesting that donor CD8 maturation was skewed towards a non-CTL effector function (e.g. Tc1 or Tc2). We can not exclude the possibility that IL-2 neutralization *in vivo* was incomplete, however these results indicate that maturation of CD8 + T cells into CTL effectors is more sensitive to IL-2 reductions than is initial CD4+ and CD8 + T cell activation and expansion. Importantly, CD4+ T cell help for B cells and subsequent autoantibody production appears to be relatively IL-2 independent. Taken together with the defective *in vitro* TNP self-responses both CTL and IL-2 production) in chronic GVHD, these results support the idea that autoimmune chronic GVHD does not require IL-2 and instead is characterized by a skewing away from IL-2 dependent responses.

TNF-a Blockade in Acute GVHD Mice—Therapeutic inhibition of TNF-a has proven to be a major advance in the treatment of inflammatory arthritic conditions such as rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis [47]. Although generally well tolerated, TNF-a blockade is associated with some adverse events that have not been entirely explained mechanistically. These include the reactivation of tuberculosis and induction of humoral autoimmunity which in some cases is associated with clinical features of lupus. Our studies of TNF-a blockade in acute GVHD mice provide a mechanism for the untoward effects seen in humans receiving TNF-a blockade. Specifically, we have observed that TNF-a blockade profoundly inhibits the induction and maturation of CTL from naïve T cells but has no detectable effect on CTL effector function once they have developed [32]. Using the B6→F1 model of acute GVHD, we observed that a single injection of anti-TNF-a mAb at the time of donor cell transfer converted acute GVHD to chronic GVHD whereas no effect was seen if mAb administration was delayed until day 7 after parental cell transfer. These results were associated with a selective cytokine blockade and skewing of the immune response away from CTL and towards antibody production. That is, TNF-a blockade profoundly inhibited the striking elevations of IFN-g characteristic of acute GVHD but did not alter antibody promoting, non-Th1 cytokines such as IL-4, IL-10 and IL-6. Thus, in immune mediated conditions characterized by a mixed cytokine production pattern, TNF-a blockade may shift the balance away from CMI and, in predisposed individuals, could potentate autoantibody responses.

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Moreover, IFN-g is an important cytokine in mounting a protective response to tuberculosis infection [48]. Our results demonstrating that TNF-a blockade selectively inhibits IFN-g production indicate that a similar inhibition of IFN-g in humans could contribute to the tuberculosis exacerbation reported in patients receiving therapeutic TNF-a blockade. Because of the profound effect of TNF-a blockade on de novo CTL development in acute GVHD mice, our studies raise concern regarding the ability of patients receiving TNF-a blocking agents to generate effective CTL responses from naïve T cells. Immunity to pathogens is complex and often redundant, however patients receiving TNF-a blockers might be at risk for those conditions in which CTL play important protective roles e.g., tumor surveillance and immunity to viral/ intracellular pathogens. Of particular concern are Epstein-Barr virus, smallpox and possibly HIV.

IFN- g Blockade in Acute GVHD Mice—The selective activation of donor CD8+ T cells in acute GVHD is associated with a massive increase in IFN-g compared to the low level increase in IFN-g seen in chronic GVHD [31]. Moreover, treatment of acute GVHD mice with neutralizing doses of anti-IFN-g mAb reduces upregulation of Fas and FasL to levels seen in chronic GVHD mice [30]. These results suggest that IFN-g is a critical cytokine in acute GVHD development, particularly FasL medicated killing of host cells. However recent studies have revealed the role of IFN-g to be complex, reflecting the pleiotropic nature of this cytokine. The large amount of IFN-g produced in acute GVHD is made predominantly by donor cells, especially CD8+ T cells [16,31] however the recipient F1 also produces a small amount. To ensure that IFN-g was completely blocked in acute GVHD, we used donor cells unable to produce IFN-g (IFN-g KO) and anti-IFN-g mAb to neutralize the host contribution. Based on our results with other cytokines critical for CTL development (IL-2 and TNF-a), it might be expected that IFN-g blockade would also block anti host CTL development and convert disease phenotype to chronic GVHD. Surprisingly, we observed an intermediate phenotype in acute GVHD mice with IFN-g blockade in which host B cells are neither completely eliminated at two weeks (as seen in acute GVHD) nor increased over normal levels (as seen in chronic GVHD). *Ex vivo* CTL analysis revealed that IFN-g blockade completely blocks Fas mediated CTL killing by donor cells. Perforin mediated CTL killing, while impaired, was nevertheless detectably elevated over control levels. The complete elimination of Fas mediated killing prevents the complete elimination of host B cells however residual perforin activity is sufficient to prevent B cell expansion and the conversion to chronic GVHD. Follow up studies using donor T cells lacking a functional IFN-g receptor indicated that the ability of IFN-g to promote Fas killing and optimize perforin killing is not primarily due to a direct effect on donor T cells as IFN-gR KO→F1 mice exhibited no significant differences from WT →F1 in donor cell FasL upregulation or host B cell Fas upregulation. Host B cells were significantly reduced in IFN-gR KO→F1 acute GVHD mice compared to normal F1 however not quite to the levels of WT→F1 suggesting that signaling through the IFN-gR on donor T cells, while not essential for host killing, nevertheless optimizes killing.

G. RECENT STUDIES IN CHRONIC GVHD MICE THAT RELATE TO HUMAN LUPUS

It should be emphasized that chronic GVHD in the P→F1 model does not mimic human chronic GVHD in the HSC transplant setting but instead strongly resembles human lupus (reviewed in [8] and is therefore useful not only in the elucidation of mechanisms involved in lupus pathogenesis but also in the screening of agents with therapeutic potential. Human lupus is a highly heterogeneous disease and no single animal model of lupus mimics the diversity of human disease expression. Although the NZB/W and MRL/lpr are long standing and well accepted lupus models, they have not been shown to be superior to the P→F1 model by virtue of a clinical disease phenotype that more closely resembles human lupus. Moreover, the

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underlying initiating factors in spontaneous murine lupus, although unknown, likely differ among murine models. However once begun, disease pathogenesis may be mediated by common effector mechanisms. Thus, the demonstration of immune mechanisms operative in more than one murine model of lupus would appear to have a high probability of identifying similar mechanisms in humans. The clinical phenotype of P→F1 chronic GVHD mimics that subset of human lupus patients with predominately immune complex mediated renal disease; The model exhibits the following investigational advantages over spontaneous murine lupus models such as the NZBxW and MRL/lpr; 1) F1 mice are normal prior to disease induction, thus pathologic mechanisms identified are likely be indicative of common pathogenic mechanisms and not due to an isolated single gene defect; 2) the exact time of disease onset is known which allows not only allows better definition of disease kinetics but also permits therapeutic studies aimed at either preventing disease development (treatment prior to parental cell transfer) or reversing active disease (treatment given at specified time points after parental cell transfer; 3) the initiating antigen and the pathogenic T cells are known which allows their identification and study separate from the remainder of the T cell pool; and 4) acute and chronic GVHD can be reliably differentiated at two weeks of disease allowing a rapid turn around time for studies aimed at elucidating mechanisms important in naïve T cell activation or the influence of potential immunomodulatory compounds on naïve T cell activation.

Incomplete Host B Cell Elimination by Anti-Host CTL in Acute GVHD Permits the Evolution to Chronic GVHD and Lupus-Like Disease

Despite the development of early surrogate markers, long-term studies are still necessary for the testing of agents with potential therapeutic activity in lupus. In particular, it is necessary to confirm that any alteration in surrogate markers at two weeks is accompanied by improvement in disease severity, especially in those instances where an agent or manipulation results in an intermediate phenotype or in a small but statistically significant change in one or a few immunological markers at two weeks. An important example is the induction of acute GVHD using perforin deficient donor cells (pfp KO→F1). At two weeks of disease, pfp KO→F1 mice exhibit many of the features of acute GVHD such as significant donor CD4+ and CD8+ engraftment, strong IFN-g production, upregulation of Fas and FasL and FasL mediated anti host CTL activity [33]. Nevertheless, at both 2 weeks and 4 weeks, host B cells elimination is not as complete in pfp KO→F1 mice compared to wild type (WT) →F1 mice. Incomplete B cell elimination is observed in pfp KO→F1 mice even when then number of donor cells are increased such that twice as many pfp KO CD8+ T cells are engrafted compared to WT donor cells. The consequences of incomplete elimination of host B cells are seen over the course of subsequent months. As anti-host CTL downmodulate, the remaining host splenic B cells continue to stimulate donor CD4+T cells and pfp KO→F1 mice develop autoantibodies and immune complex glomerulonephritis. Thus, a partial defect in CD8+ CTL effector function results in a failure to eliminate host B cells prior to CTL downregulation that is associated with the evolution from acute GVHD to chronic GVHD phenotype over the long term.

The evolution of acute GVHD to chronic GVHD is not restricted to pfp KO donor cells but has also been reported in B6→ (BALB/c × B6)F1 mice (CB6F1) (49]. B6 mice make a strong anti-host CTL response in this P→F1 combination however the kinetics of B cell elimination are delayed compared to that seen in B6→BDF1 mice and B cell elimination is not sustained, rebounding as early as 4 weeks of disease. Similar to pfp KO→F1 mice, acute GVHD in B6→CB6 mice changes to a lupus-like phenotype by 8–12 weeks. Limiting dilution analysis demonstrated no difference between the B6 anti-Balb/c *vs* B6 anti-DBA/2 precursor CTL frequency. However the Th precursor frequency for B6 anti- DBA/2 was 3-fold higher than that for B6 anti-BALB/c responses. Taken together, these results indicate that regardless of whether defective anti-host CTL effector function is a primary or a secondary defect, a common pattern emerges when host B cells are not completely eliminated during the window of maximal

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CTL activity, i.e., the evolution of acute GVHD to a chronic GVHD with lupus-like disease and autoimmunity.

Intracellular Signaling in Pathogenic Lupus T Cells

In the P→F1 model, the antigen specific T cells driving disease are of donor origin and can be analyzed separately from non specifically activated (host) T cells, making this model well suited for the study of signaling pathways active in lupus pathogenic T cells. We have recently reported [50] that purified pathogenic T cells (i.e. donor CD4+ T cells) driving lupus-like disease in chronic GVHD exhibit activation in the form of spontaneous cytoplasmic signaling pathway activity that can be detected without *in vitro* restimulation and involves a T cell–specific pathway, phosphoinositol 3-kinase (PI-3), and a nonspecific stress/cytokine pathway (JNK-1) but does not involve the Raf-1, p38 MAPK, or ERK-1 pathways. This pattern was not seen in isolated host, CD4+ T cells supporting the pathogenic specificity of these pathways. The increased PI-3 kinase and JNK-1 activity in donor CD4+ T cells was associated with evidence of functional T cell activation in the form of increased T cell receptor membrane signaling activation (increased Lck and Fyn phosphorylation) and increased transcription activation (phosphorylation of inhibitor of nuclear factor kB). These results support the idea that the pathogenic T cells in immune mediated diseases exhibit characteristic signaling pathway activation and that selective therapeutic blockade of such pathways might be allow disease improvement without suppressing T cell immunity to other antigens.

Sex Differences in T Cell Activation

Human lupus exhibits a striking female predominance [51]. Similarly, lupus like renal disease in long term chronic GVHD mice is more severe using female donor and hosts (f→F) than that seen using males (m→M). Recent work [51] indicates that long term sex-associated differences in disease severity are predicted by surrogate markers at two weeks of disease. For example f→F chronic GVHD mice exhibited a 2 to 3-fold greater engraftment of donor CD4+ T cells at two weeks that persisted throughout the disease course. Enhanced engraftment of donor CD4 + T cells was due to a longer initial proliferation period during week 2 of disease compared to m→M donor cells. Crossover studies (m→F, f→>M) demonstrated that the two week immunological phenotype segregates with the sex of the host. The host factors that influence the expansion of pathogenic T cells are currently under investigation. These results demonstrate that seemingly small and brief differences in initial naïve T cell activation can result in a greater long-term burden of autore-active T cells resulting in greater disease severity.

Inorganic Mercury (iHg) Accelerates Lupus Like Disease

iHg is well known for its ability to induce autoimmune disease in susceptible mice and to exacerbate disease in spontaneous models of murine lupus. We tested the idea that low-level iHg exposure can interact with disease triggers to enhance disease expression [52]. Using murine strains that are resistant to iHg induced autoimmunity, we exposed normal DBA/2 donor mice and B6D2F1 recipient mice to a brief (2-week) and low dose (20 or 200 μg/kg every other day) course of iHg. Following a one week wash out period, chronic DBA→F1 GVHD was induced and long term studies performed. iHg pretreatment accelerated chronic GVHD mortality and worsened disease parameters suggesting that low-level, nontoxic iHg preexposure may interact with other risk factors to enhance subsequent autoimmune disease development.

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H. RECENT STUDIES IN ACUTE GVHD MICE THAT RELATE TO HUMAN HSC TRANSPLANTION

Long Term Survivors of Acute GVHD in the P→F1 Model Exhibit Similarities to Human Acute GVHD

We have recently observed that histopathology in long term (3 month and 6 month) survivors of acute GVHD in the P→F1 model bears many similarities to that of patients with acute GVHD, particularly the lesions in the skin, liver and lung [34]. Using in situ apoptosis staining in conjunction with histopathology as a measure of organ damage, the earliest and most intense attack in acute GVHD mice is observed in the spleen, peaking at approximately two weeks whereas apoptotic organ damage in the liver, lung and skin peaked at three months. Although the kidney is not one of the principle targets in human acute GVHD, kidneys in GVHD mice exhibited increased apoptotic cell numbers but not until 6 months. Histologically, the kidneys in acute GVHD mice exhibited cellular infiltrates and tubular damage with sparing of the glomeruli giving a histological picture bearing some similarities to that seen in allograft rejection. Complement activation (C5b-9 deposition) and apoptosis indices in acute GVHD mice paralleled the extent of tissue damage by H & E in general, indicating the involvement of both processes. Increased apoptosis is consistent with a donor CTL effector attack however increased deposition of terminal membrane attack complex in the absence of a conditioning regimen suggests not only a failure of normal C3b mediated complement clearance mechanisms but also that C5b-9 terminal complement complex might perpetuate tissue damage. These results suggest a role for the P→F1 model in elucidating as yet unrecognized T cell specific mechanisms of tissue injury in the non-myeloablative, non-cytoreductive transplant setting.

I. CONCLUSIONS

The mechanisms underlying GVHD in the P→F1 model discussed above have provided valuable insights into both normal *in vivo* T cell responses and pathogenic T cells mediating diseases such as lupus. The identification of immune pathways common to both the P→F1 model and human immune mediated diseases supports the idea that the repertoire of immune responses is limited and that therapeutic interventions useful in murine GVHD may also be beneficial in human immune mediated diseases.

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Table 1

Summary of Surrogate Markers of Acute GVHD at Two Weeks of Disease

Parameter	Acute GVHD	Chronic GVHD
Donor Engraftment	CD4+, CD8+	CD4+ only
Serum IFN-g	strong elevation (>100 fold)	mild elevation (≤ 10 fold)
Fas/FasL upregulation	strong	low level
Anti-host CTL	present	absent
Host B cells	reduced (10% of normal)	increased (150% of normal)
Autoantibodies	transient	sustained