Murine models

A two-phase pathogenesis of graft-versus-host disease in mice

L van Leeuwen¹, A Guiffre¹, K Atkinson^{1,4}, SP Rainer² and WA Sewell³

¹Department of Haematology, St Vincent's Hospital and University of New South Wales, Sydney, NSW, Australia; ²Department of Anatomical Pathology, St Vincent's Hospital and University of New South Wales, Sydney, NSW, Australia; and ³Centre for Immunology, St Vincent's Hospital and University of New South Wales, Sydney, NSW, Australia

Summary:

Activation of donor T cells is required for the development of graft-versus-host disease (GVHD), a major complication of bone marrow transplantation. We investigated a murine model of GVHD across major and minor histocompatibility barriers. BALB/c recipients were lethally irradiated and transplanted with 10^7 bone marrow and 5×10^6 spleen cells from C57BL/6 donors. There were two separate phases of clinical disease. The first phase was most severe on day 7 after transplant. Weight and condition improved until day 12 and then a second phase of clinical GVHD commenced, which persisted until euthanasia. IL-2 mRNA expression, as a measure of T cell activation, was determined by quantitative PCR. The two phases of clinical GVHD were preceded by two separate peaks of IL-2 mRNA in the spleen. Host MHC class II⁺ cells became undetectable by flow cytometry 7 days after transplantation, whereas donor MHC class II⁺ cells increased during the first 9 days after transplantation. Removal of donor MHC class II⁺ cells from the graft had no effect on the first phase. Possible roles for host and donor antigen-presenting cells (APC) in the two phases of the disease are discussed.

Bone Marrow Transplantation (2002) **29**, 151–158. DOI: 10.1038/sj/bmt/1703328

Keywords: graft-versus-host; MHC; antigen presentation; transplantation; cytokines

host alloantigens is a prerequisite for disease induction³ and T cell depletion is a highly effective means of preventing GVHD.⁴ Although widespread clinical trials of T cell depletion achieved substantial reductions in the incidence of GVHD, the benefits were counterbalanced by increases in the rates of graft failure and recurrence of malignancy.^{5–7} Thus there appears to be a relationship between GVHD and the processes that facilitate engraftment, as well as with those that exert a graft-versus-leukemia effect. The reasons for this correlation are unclear and thus further elucidation of the underlying mechanisms of GVHD remains an important objective of transplantation research.

In clinical BMT, GVHD may be caused by mismatches at either MHC or minor histocompatibility loci. When BMT is performed between MHC-matched siblings, GVHD still arises, because of mismatching at one or more minor histocompatibility loci.⁸ Minor histocompatibility mismatching occurs because differences in cellular proteins give rise to peptides which are presented by self MHC and recognized as foreign by donor T cells.⁹ In BMT, antigen may be presented to donor T cells by two different types of antigen-presenting cells, derived from either the host or the donor.¹⁰

We have previously reported the use of a murine model of severe acute GVHD involving bone marrow transplantation across both major and minor histocompatibility barriers.¹¹ We noted two distinct episodes of disease in allogeneic transplant recipients manifested by changes in weight and visible condition. The cytokine interleukin-2 (IL-2) is regarded as an excellent marker of T cell activation.¹² We have measured IL-2 by quantitative PCR to investigate T cell activation in this murine model.

Materials and methods

Animal procedures

BALB/c and C57BL/6 mice were obtained from the Animal Resources Centre (Perth, Australia) and maintained at the Biological Testing Facility of the Garvan Institute of Medical Research at St Vincent's Hospital Sydney as previously described.¹¹ Experiments were performed on allogeneic (C57BL/6 to BALB/c) transplanted animals, syngeneic (BALB/c to BALB/c) transplanted animals, and irradiated and untransplanted BALB/c mice. For bone marrow transplantation, recipient BALB/c mice received a lethal dose

Acute graft-versus-host disease (GVHD) remains the most significant obstacle to successful allogeneic BMT or peripheral blood stem cell transplantation for leukemia and other conditions. In MHC-matched unrelated donor transplants, acute GVHD of grade II or above occurs in up to two-thirds of cases.¹ Acute GVHD has a complex pathophysiology which can involve wasting, diarrhea, skin and gut lesions and liver damage.² T cell activation in response to

Correspondence: Associate Professor WA Sewell, Centre for Immunology, St Vincent's Hospital Sydney, Darlinghurst, NSW 2010, Australia

⁴Present address: Center for Cellular Therapy, American Red Cross, Rosslyn, VA, USA

Received 26 June 2001; accepted 24 October 2001

of irradiation (10 Gy over 20 min) from a particle accelerator. On the following day, defined as day 0, C57BL/6 donors were killed, bone marrow cells and splenocytes were extracted from femurs and whole spleens, and single cell suspensions were prepared in RPMI 1640 media supplemented with 0.1% (v/v) heat-inactivated FCS, antibiotics, glutamine, 0.024 M NaHCO₃ and 0.01 M Hepes (Trace Biosciences, Sydney, Australia). Recipient BALB/c mice then received 10⁷ bone marrow cells and 5 × 10⁶ splenocytes via tail vein injection and were monitored daily for weight and condition. Animals were humanely killed if they lost more than 40% of their original body mass, if they lost weight rapidly (more than 1 g per day for 2 consecutive days) or if they became morbid. Experimental protocols used in the study were approved by the Garvan Institute/St Vincent's Hospital Animal Experimentation Ethics Committee and complied with the code of practice on experimentation in animals of the National Health and Medical Research Council of Australia.

Preparation of cDNA

Samples were taken for RNA extraction at 2 day intervals beginning on day 2 and continuing until day 16 post transplant after which significant GVHD-related mortality was observed. Five mice were sampled from each group at each time point. Total cytoplasmic RNA was extracted from whole tissues (skin or spleen) using the RNeasy kit (Qiagen, Hilden, Germany) with a modified protocol. Tissues (half spleen and approximately 1 cm² of abdominal skin) were taken from animals that had been killed and placed immediately in liquid nitrogen. Samples were ground in a mortar and pestle under liquid nitrogen and the powder transferred to 4 M guanidinium thiocyanate, 0.04 M sodium citrate, 0.72% (v/v) β -mercaptoethanol and 0.8% (v/v) sarkosyl (Solution D).¹³ The mixture was thoroughly disrupted by passing through a 25G needle to shear genomic DNA and two phenol/chloroform extractions were performed (500 μ l lysis mix: 50 μ l 3 M sodium acetate: 500 μ l water saturated phenol: 100 μ l 49:1 chloroform/isoamyl alcohol) to facilitate removal of collagen and DNA. The final aqueous phase was then transferred to the RNeasy columns for extraction according to the manufacturer's instructions. RNA was eluted from the columns in DEPC treated H₂O, the yield determined by spectrophotometry, and RNA integrity confirmed by denaturing formaldehyde agarose gel electrophoresis. cDNA was synthesised from 0.5 μ g total RNA using Superscript RT (Gibco BRL, Gaithersburg, MD, USA) in a final volume of 10 μ l.¹⁴ Template-negative and enzyme-negative controls were conducted alongside the sample tubes.

β -actin PCR

To verify the quality of the cDNA, it was amplified for the housekeeping gene β -actin. PCR primers (forward: 5'-TTACCAACTGGGACGACATG-3'; backward: 5'-ATACAGGGACAGCACAGCCT-3')¹⁵ were synthesized commercially (Beckman, Sydney, Australia). Two μ l of 1:4 diluted cDNA were added to a PCR reaction containing: 200 μ M each dNTP (Pharmacia, Uppsala, Sweden); 1 ×

PCR buffer; 1.5 mM MgCl₂; 1 μ M each primer; 1.1 U *Tth* DNA polymerase (Biotech International, Perth, WA, Australia), for a final volume of 10 μ l and amplified using cycles of 94°C, 55°C and 72°C for 1 min each in a Perkin-Elmer (Norwalk, CT, USA) 9600 thermocycler. Products were visualized with ethidium bromide using 1% (w/v) agarose gel electrophoresis in TBE buffer. Exponential amplification was found to occur between 15 and 20 PCR cycles (data not shown) and subsequent reactions were performed using 18 cycles. All cDNA samples were amplified for β -actin and resulting band strengths compared by densitometry. Any samples with low or no signal were resynthesized and tested again for β -actin to ensure uniformity of cDNA synthesis. One syngeneic skin RNA consistently failed to produce a β -actin product and was discarded.

Interleukin-2 quantitative PCR (Q-PCR)

IL-2 PCR primers (IL-2A: 5'-AACAGCGCACCACTCAA-3'; and IL-2B: 5'-TTGAGATGATGCTTTGACA-3')¹⁶ were used for PCR in 10 μ l volumes consisting of cDNA corresponding to 25 ng of total RNA, and reaction mix as above. Reactions employed 30 cycles of 94°C, 62°C and 72°C for 1 min each with a 10 min 72°C final extension step. All samples were assayed in parallel using this system as a semi-quantitative guide to IL-2 expression. A competitive DNA standard was made by excising a short piece of DNA from IL-2 PCR product and religating the ends.¹⁴ The competitor gave a product of 327 bp while the wild-type product was 442 bp, allowing separation of the two by agarose gel electrophoresis. The competitor and wild-type target molecules had similar amplification efficiencies and the quantitation method worked consistently over a range of target starting concentrations.¹⁴

For Q-PCR, five-fold serial dilutions of the competitor were added with a constant volume of sample cDNA to five consecutive tubes. Template-negative and enzyme-negative cDNA controls were added to separate tubes. The system was optimized with cDNA from BALB/c spleen cells that were activated with PMA (4 ng/ml) and ionomycin (1 μ g/ml) for 24 h in RPMI 1640 medium as above except with 10% (v/v) FCS. Results were assessed with ethidium bromide and samples which were undetectable by this method were subjected to Southern blotting with a vacuum system and Zeta-Probe membranes (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Hybridization was conducted using oligonucleotides, end-labelled with ³²P and homologous for a binding site on the PCR product of both wild type and competitor. Bands were imaged by video camera documentation of ethidium bromide stained gels (Gel Doc 1000; BioRad) or densitometer readings of X-ray films (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA, USA). Band intensities were scored using NIH Image, the data entered into Cricket Graph and the ratio of competitor to target determined for each dilution. A line of best fit was generated and the resulting formula used to determine a numerical value for the point where competitor and target concentrations were equal. Final results were expressed as molecules per μ g of starting total RNA.

Flow cytometric analysis

FITC-labelled antibodies (mouse anti-mouse I-A^d, clone 39–10–8 and mouse anti-mouse I-A^b, clone AF6–120.1) and isotype controls (mouse IgG₃κ and mouse IgG_{2a}κ) (Pharmingen, San Diego, CA, USA) were diluted 1:100 in PBS with 1% (w/v) BSA (PBS-BSA). Bone marrow and spleen cells were harvested as described above, subjected to red cell lysis for 5 min in 0.83% (w/v) NH₄Cl at 4°C and washed twice in PBS-BSA. Cells were then labelled with antibody for 30 min on ice and either analyzed immediately on a Coulter XL flow cytometer, or fixed in PBS with 1% sucrose and 0.5% paraformaldehyde for analysis up to 1 week later.

In vitro depletion experiments

To deplete MHC Class II⁺ cells from the transplant, cells from spleen and bone marrow were labelled with anti-mouse I-A^b antibody (Pharmingen) for 30 min at 4°C. Cells were washed twice and MACS beads conjugated to anti-mouse IgG_{2a+b} antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) were added for 15 min at 4°C. Cells were then washed twice and added to the MACS column. The cell suspension was passed three times through a magnetized column and with each pass cells that bound to the column were discarded. The negative fraction remaining was used in the transplant and the cell dose was adjusted so that all mice received the same number of T cells. In each experiment, a small number of cells were kept for flow cytometric analysis of depletion efficiency.

Histological analysis

Samples of ear were excised immediately post mortem, fixed in 10% formalin, and processed for histologic examination with hematoxylin and eosin. Samples were examined blind by an experienced pathologist (SPR) and were scored for four features (Civatte bodies, lymphocytic infiltrates, hydropic degeneration and hyperkeratosis) on a 0–3 scale adapted from one used for grading human GVHD.¹⁷

Statistical operations

Comparisons of weights were performed using the repeated measures ANOVA while comparisons of Q-PCR data were made with the independent-samples *t*-test (where two samples were to be compared) or with one-way ANOVA with *post hoc* testing where more than two samples were to be compared. Transformations were used to correct cases where the data violated assumptions such as normality (judged graphically by normal Q–Q plots) and equality of variance (judged by Levene's test). All operations were carried out using SPSS for Windows 7.5.1 (SPSS Inc, Chicago, IL, USA). *P* values <0.05 were considered significant.

Results

Allogeneic BMT recipients undergo two periods of weight loss

Lethally irradiated animals given syngeneic (BALB/c to BALB/c) transplants lost weight immediately after irradiation, reaching a nadir at day 5 post transplant of 4 g below original body mass. They then recovered, regained normal body mass by day 16, and remained healthy thereafter (Figure 1). BALB/c mice lethally irradiated on day –1 without receiving a transplant lost weight until day 5 by which time they had shed an average of 4.5 g. They then began to regain weight and condition for the next 4 days, after which they rapidly wasted, apparently from hemopoietic failure, and were killed within 2 weeks post irradiation (Figure 1). Until day 9, the syngeneic recipients and irradiated but untransplanted animals were not significantly different in weight.

Lethally irradiated animals given allogeneic transplants (C57BL/6 to BALB/c) also lost weight rapidly after transplantation, but their weight loss was more prolonged and severe. They reached a nadir at day 7 of 7 g below starting mass, when their condition was poor with ruffled fur, and small numbers were killed according to Ethics Committee guidelines. Animals that died before day 12 were excluded from the determination of mean weights such as shown in Figure 1. The wasting experienced by allogeneic BMT recipients over the first 7 days was more severe and more prolonged than either control group. Excess weight loss became significantly greater than both syngeneic recipients (*P* < 0.001) and irradiated but untransplanted animals (*P* < 0.02) at day 6. Therefore, the excess weight loss experienced by allogeneic recipients to day 7 must be associated with the presence of allogeneic cells. The weight and visible condition of allogeneic recipients improved markedly after day 7 to a maximum at approximately day 12 of only

Figure 1 Weight change in transplant recipients. BALB/c recipients were given total body irradiation on day –1 followed by 1×10^7 bone marrow and 5×10^6 spleen cells from C57BL/6 donors (allogeneic) or the same numbers of cells from BALB/c donors (syngeneic) or no treatment (irradiated but untransplanted). Mice were weighed daily and data are expressed as mean and standard deviation. In this experiment all recipients in the allogeneic group survived until at least day 12, and the longest survivor was killed on day 28. Group sizes were *n* = 12 (irradiated but untransplanted), *n* = 10 (syngeneic), *n* = 15 (allogeneic). The data are representative of three separate experiments.

4.5 g below starting mass. Weight at day 7 was significantly lower than at all days prior to day 5 ($P < 0.001$) and between days 11 and 13 ($P < 0.005$). After day 12 the weight and visible condition of allogeneic recipients again began to deteriorate (Figure 1) until animals were killed at a median time of day 18, by which time they showed signs of acute GVHD such as hair loss, thickening of the skin and perianal irritation. Thus there are two distinct periods of weight loss in the allogeneic recipients: an early phase which is partially resolved between days 7 and 12, and a late stage which commences after day 12 and continues until death.

IL-2 mRNA in spleen and skin of GVHD affected mice

A quantitative IL-2 RT-PCR was developed with an IL-2 competitor fragment that was shorter than the wild-type sequence. Serial dilutions of the competitor were added to each sample (Figure 2a). The band intensities were quantitated and the ratio of competitor to target was determined for each dilution (Figure 2b). To investigate T cell activation after transplantation, two tissues were chosen for IL-2 mRNA analysis: spleen, which can be regarded as an effector organ of GVHD, and skin, which is an example of a target organ. cDNA samples were subjected to competitive Q-PCR for IL-2. This clearly demonstrated that IL-2 expression in the spleen underwent two episodes (Figure 3a), one very high peak at day 2 followed by a trough and then a smaller peak from days 8 to 12. Expression at each

Figure 2 The Q-PCR system. (a) Ethidium bromide stained gel shows IL-2 PCR amplification of a constant quantity of cDNA (lanes 2–10) and a series of two-fold dilutions of competitor DNA starting with 1.46×10^7 competitor molecules in lane 2. Molecular size markers are shown in lane 1 and relevant marker sizes (bp) are indicated to the left. The sizes of wild-type and competitor IL-2 products are shown to the right. (b) Densitometry data from (a) are expressed as the ratio of the band intensity of wild-type to construct (T_n/C_n) against the known concentration of construct (C_0). The line of best fit and its formula are shown.

Figure 3 Expression of IL-2 mRNA in spleens and skins of transplant recipients. Spleen (a) and skin (b) samples were taken from groups of $n = 5$ mice at 2 day intervals after lethal irradiation and transplantation. Samples were also taken post mortem from five unmanipulated BALB/c mice. RNA was extracted, cDNA synthesised and competitive Q-PCR for IL-2 was performed. Results are expressed as mean and standard deviation.

peak was significantly stronger than at other times ($P < 0.005$). Thus T cell activation took place in the spleen on two separate occasions and preceded overt illness on each occasion. By contrast, IL-2 expression in the spleens of syngeneic BMT recipients was much lower, and these differences were significant on day 2 ($P < 0.001$) and on day 10 ($P < 0.004$). These data imply that the conditioning regimen alone had little effect on IL-2 expression. There was very little IL-2 mRNA in spleens of healthy unmanipulated mice.

In allogeneic skin there was a peak in IL-2 expression on day 6, followed by a trough on day 10 and a rise at later time points (Figure 3b). Expression in syngeneic skin was relatively constant at a level similar to that detected from day 12 onwards in allogeneic skin. At day 6 the allogeneic samples were significantly higher than the syngeneic samples ($P < 0.02$) at a time coinciding with the first phase of the disease. At days 12 and 14 the allogeneic samples were not significantly greater than the syngeneic ones. Samples taken from untreated mice showed a similar level of IL-2 expression to the syngeneic mice.

Histological analysis

Specimens were taken for histology and examined for evidence of acute GVHD. In cutaneous samples from allogeneic BMT recipients, there were clear signs of moderately intense acute GVHD on day 7. Most animals exhibited most features including Civatte bodies, hyperkeratosis, lymphocytic infiltrates and hydropic degeneration (Figure 4). In allogeneic recipients, similar results were obtained on days 12 and 16, although hyperkeratosis was less prominent on day 16, while Civatte bodies and lymphocytic infiltrates were more prominent on day 16 (Table 1). Neither irradiated and untransplanted nor syngeneic recipients showed any signs of acute GVHD with the exception of a consistent low scoring for Civatte bodies at days 12 and 16 and slight lymphocytic infiltration in two out of five syngeneic animals on day 16 (Table 1). Untreated animals were negative for all features (Table 1). In allogeneic recipients, the similar histological findings on days 7, 12 and 16 contrast with the data on weight and condition, which demonstrated marked improvement from day 7 to day 12 and then deterioration by day 16 (Figure 1). Thus the microscopic features of GVHD in the skin did not correlate with overall clinical severity of GVHD.

Figure 4 Histological features of cutaneous GVHD. The sections are taken from mice that received (a) syngeneic or (b) allogeneic transplants 7 days previously. The sections are of ears and consist of surface keratin, epidermis, dermis and cartilage. In the allogeneic recipient there are several features that are absent in the syngeneic recipient. These include hyperkeratosis and an infiltrate of lymphocytes within the epidermis affecting predominantly the basal layer. The infiltrate is seen in association with hydropic degeneration (left hand arrow) and scattered Civatte bodies (right hand arrow). The underlying dermis also contains a lymphocytic infiltrate and shows thickening and oedema. The cartilage is unaffected. Original magnification $\times 200$.

Host and donor MHCII-positive cells in spleen and bone marrow

To investigate the presence of MHCII⁺ cells following allogeneic bone marrow transplantation, flow cytometric analysis was carried out on splenocytes or bone marrow cells during the early phase of the disease. Samples were taken on days 2, 5, 7 and 9, and analyzed for host (H-2^d) and donor (H-2^b) MHC class II. Figure 5a shows the relative levels of host MHCII⁺ cells over time, indicating that these cells undergo a rapid and sustained decline, reaching levels undetectable by flow cytometry on day 5 in the spleen and day 7 in the bone marrow. Thus host MHCII⁺ cells are undetectable when the overt GVHD features of the first phase diminish after day 7. A sharp and significant ($P < 0.001$) rise in host MHCII⁺ cells was observed on day 5 in the bone marrow only and although consistent in all five animals sampled, was transitory. Donor MHCII⁺ cells by contrast rose throughout the period of observation (Figure 5b), a result which tallies closely with engraftment in this model as evidenced by increased numbers of blood neutrophils (data not shown). Donor MHCII⁺ numbers are plentiful by day 9 before commencement of the second episode of GVHD.

Depletion of donor MHCII-positive cells from the graft

To determine whether donor (H-2^b) MHCII⁺ cells in the graft were required for the first phase of wasting, these cells were depleted from the donor bone marrow and spleen suspensions by magnetic bead separation. More than 98% depletion of positive cells was obtained as determined by flow cytometry, and transplantation was performed as usual with depleted cells (Figure 6). In this experiment, which was performed at a different time from the one shown in Figure 1, the onset of the second stage of clinical GVHD was slower and the median time to death in MHCII⁺-depleted and control groups was 22 days. Nevertheless, the two phases of clinical disease, separated by recovery, were readily apparent, and all animals were killed by day 27. The depletion procedure did not affect engraftment or the development of the first stage of disease as evidenced by the pattern of weight loss between days 5 and 7 (Figure 6, $P = 0.4$). This experiment could not be used to investigate the second stage of GVHD because of the rapid increase in donor MHCII⁺ cells after transplantation (Figure 5) and therefore the second stage data are not shown.

Discussion

We describe a murine model of bone marrow transplantation, in which GVHD follows a two-phase pathogenesis, with a short-lived and non-fatal first phase and a lethal second phase separated by a period of recovery. The pattern of two phases of weight loss is not unique to our model, but can be discerned in the weight charts of a number of other models of murine GVHD.^{18–22} Irradiation contributed to the first phase of weight loss, because both syngeneic recipients and irradiated untransplanted mice exhibited substantial weight loss for the first 5 days (Figure 1).

Table 1 Histological analysis of cutaneous GVHD

Treatment	Day ^a	No. of mice	GVHD feature			
			Hyperkeratosis	Civatte bodies	Lymphocytic infiltrates	Hydropic degeneration
Allogeneic recipient	7	5	2.0 ^b	1.8	1.6	1.8
	12	5	2.0	2.2	1.8	2.2
	16	5	1.0	2.6	2.6	2.0
Syngeneic recipient	7	3	0	0	0	0
	12	3	0	0.7	0	0
	16	5	0	1.0	0.4	0
Irradiated/untransplanted	7	3	0	0	0	0
	12	3	0	0.3	0	0
Untreated	—	5	0	0	0	0

^aSections of skin were prepared from mice at various days after transplant.

^bThe slides were scored blind for GVHD features on a scale of 0–3, with higher numbers indicating greater severity. The mean score is shown for each feature.

Figure 5 Host and donor-derived MHCII⁺ cells in allogeneic BMT recipients. Irradiated BALB/c hosts were given C57BL/6 transplants and euthanased in groups of $n = 5$. Splenocytes and bone marrow cells were stained with anti-Ia^d (host) (a) or anti-Ia^b (donor) (b) antibodies and analyzed by flow cytometry. Points represent the mean with standard deviations marked.

Irradiation is well recognized to be associated with initial morbidity after allogeneic transplantation.²¹ However, in our model, the first phase of weight loss cannot simply be attributed to the effects of irradiation. In the allogeneic recipients, weight loss became significantly more severe than in the syngeneic group from day 6 onwards (Figure 1). Furthermore, there was clear histological evidence of GVHD in allogeneic recipients on day 7 (Figure 4). There-

Figure 6 Effect of *in vitro* removal of MHCII⁺ cells on allogeneic transplants. C57BL/6 donor spleen and bone marrow cells were divided into two groups and one was depleted of MHCII⁺ cells by negative selection using magnetic beads. Control mice were transplanted as usual. There were three recipients of depleted cells and six recipients of control cells. The mean and standard deviation are shown. The data are representative of two experiments.

fore in the first phase of clinical disease, the presence of allogeneic cells was associated with greater and more prolonged weight loss and histological evidence of GVHD.

We have assessed mRNA expression of IL-2, which plays a complex role in GVHD. On the one hand, neutralization of IL-2 was followed by amelioration of GVHD,²³ but on the other hand, administration of high doses of IL-2 inhibited GVHD.²⁴ Regardless of these contradictory findings, IL-2 is widely recognized as a marker of T cell activation.¹² In our model there are two separate peaks of T cell activation in the spleen, as measured by IL-2 mRNA expression, which precede each phase of clinical disease by approximately 4 days. Separate phases of IL-2 expression have not previously been reported, to our knowledge, in a model of GVHD. Other reports have investigated cytokine gene expression in GVHD,^{25–28} but did not identify separate peaks of cytokine expression, possibly because tissue sampling was not sufficiently frequent. In

the skin, the IL-2 peak on day 6 coincides with the first phase of clinical disease (Figures 1 and 3). The onset of the second phase of clinical disease is associated with detectable skin IL-2 expression on days 12–16.

The two phases of GVHD are not easily explained by current concepts on the induction and pathogenesis of this condition. The cytokine storm hypothesis^{29,30} suggests a cascade of inflammatory cytokines initiated by the conditioning regimen and alloantigen recognition, but separate episodes of T cell activation are not predicted. It seems unlikely that effects of cell cycling could be associated with the dramatic clinical improvement in the recipients from day 7 to day 12. The time delay between peak IL-2 expression in the spleen on day 2 and in the skin on day 6 is reminiscent of the observation that allogeneic cells were retained in the spleen and lymph nodes for a few days after intravenous administration, and then re-entered the circulation.^{31,32} This phenomenon may account for the first peak of IL-2 expression in the spleen 2 days after transplant, and in the skin 4 days later. These authors did not report a second round of retention of allogeneic cells in lymphoid tissue. It is possible that in our experiments the second peak of IL-2 mRNA in the spleen may result from IL-2-producing T cells trafficking to the spleen between days 6 and 10.

An alternative possibility is that the separate episodes of GVHD may be caused by different populations of APC. In MHC mismatched BMT, the donor T cells are activated either directly in response to host APC, or indirectly in response to APC derived from the donor. It is possible that the first phase of GVHD in our model may be initiated by direct recognition of host APC by donor T cells, and that the second phase may arise from indirect presentation of host antigens by donor-derived APC to donor T cells. We propose that clinical improvement between days 7 and 12 may be a consequence of the marked reduction in host MHCII cell numbers by day 7 after transplant (Figure 5). We found that *in vitro* depletion of MHCII cells from the graft had no effect on the first stage (Figure 6). This result suggests that donor APC are not required for the first stage, and is consistent with the possibility that host APC are responsible for the first stage.

There are a number of limitations of this proposal. Firstly, although host MHCII cells became undetectable by flow cytometry, this method cannot exclude the possibility that low numbers of host MHCII cells persist. Indeed, persistence of host Langerhans cells has been observed for 39–120 days after human allogeneic bone marrow transplantation.³³ Persistence of host APC would be a mechanism whereby donor CD8⁺ T cells could be involved in the second stage. Furthermore, it has been reported that host APC were required for the development of GVHD in a model of MHC matched, minor histocompatibility mismatched BMT.¹⁰ Secondly, most of the MHCII positive cells were B cells, whereas dendritic cells, the most important APC, were not specifically identified. Thirdly, the *in vitro* depletion of MHCII cells cannot be used to interpret the second stage, because of the marked expansion of donor MHCII positive cells from day 7 onwards (Figure 5). Finally, one possibility not excluded by the current experiments is that residual host T cells may contribute to the first phase of IL-2 mRNA expression in the spleen.

In conclusion, we present data on a fully mismatched MHC model in which there are two clinical episodes of GVHD. Evidence is provided that weight loss in the first phase is not only caused by irradiation, but also by GVHD. The findings on IL-2 expression indicate separate episodes of T cell activation, and we propose the hypothesis that the distinct episodes of disease may be associated with direct followed by indirect antigen presentation to donor T cells.

Acknowledgements

We thank Dr Phil Hodgkin for helpful discussion. This work was supported by the Arrow Foundation, the Kanematsu Memorial Award, an Australian Postgraduate Award to LvL, and an infrastructure grant from the NSW Health Department.

References

- 1 Atkinson K. Unrelated donor hematopoietic stem cell transplantation. In: Atkinson K (ed.). *Clinical Bone Marrow and Blood Stem Cell Transplantation*, 2nd edn. Cambridge University Press: Cambridge, 2000, pp 619–646.
- 2 Tabbara IA. Allogeneic bone marrow transplantation: acute and late complications. *Anticancer Res* 1996; **16**: 1019–1026.
- 3 Korngold R, Sprent J. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. *J Exp Med* 1978; **148**: 1687–1698.
- 4 Poynton CH. T cell depletion in bone marrow transplantation. *Bone Marrow Transplant* 1988; **3**: 265–279.
- 5 Butturini A, Gale RP. T cell depletion in bone marrow transplantation for leukemia: current results and future directions. *Bone Marrow Transplant* 1988; **3**: 185–192.
- 6 Ash RC, Horowitz MM, Gale RP *et al*. Bone marrow transplantation from related donors other than HLA-identical siblings: effect of T cell depletion. *Bone Marrow Transplant* 1991; **7**: 443–452.
- 7 Aschan J, Ringden O, Sundberg B *et al*. Increased risk of relapse in patients with chronic myelogenous leukemia given T-cell depleted marrow compared to methotrexate combined with cyclosporin or monotherapy for the prevention of graft-versus-host disease. *Eur J Haematol* 1993; **50**: 269–274.
- 8 Goulmy E, Schipper R, Pool J *et al*. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *New Engl J Med* 1996; **334**: 281–285.
- 9 Goulmy E. Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy. *Immunol Rev* 1997; **157**: 125–140.
- 10 Shlomchik WD, Couzens MS, Tang CB *et al*. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* 1999; **285**: 412–415.
- 11 van Leeuwen L, Guiffre AK, Sewell WA *et al*. Administration of mycophenolate mofetil in a murine model of acute graft-versus-host disease after bone marrow transplantation. *Transplantation* 1997; **64**: 1097–1101.
- 12 Smith KA. Interleukin-2: inception, impact, and implications. *Science* 1988; **240**: 1169–1176.
- 13 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–159.
- 14 van Leeuwen L, Guiffre AK, Sewell WA, Atkinson K. Rapid

- synthesis of a DNA competitor for use in quantitative polymerase chain reaction analysis using simple molecular techniques. *Anal Biochem* 1996; **243**: 196–198.
- 15 Rolfe FG, Hughes JM, Armour CL, Sewell WA. Inhibition of interleukin-5 gene expression by dexamethasone. *Immunology* 1992; **77**: 494–499.
 - 16 Montgomery RA, Dallman MJ. Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction. *J Immunol* 1991; **147**: 554–560.
 - 17 Field A, Rainer S, Turner J, Munro V. Histopathology of hematopoietic stem cell transplantation. In: Atkinson K (ed.). *Clinical Bone Marrow and Blood Stem Cell Transplantation*, 2nd edn. Cambridge University Press: Cambridge, 2000, pp 1093–1116.
 - 18 Blazar BR, Taylor PA, Smith S, Valleria DA. Interleukin-10 administration decreases survival in murine recipients of major histocompatibility complex disparate donor bone marrow grafts. *Blood* 1995; **85**: 842–851.
 - 19 Sykes M, Szot GL, Nguyen PL, Pearson DA. Interleukin-12 inhibits murine graft-versus-host disease. *Blood* 1995; **86**: 2429–2438.
 - 20 Blazar BR, Sharpe AH, Taylor PA *et al*. Infusion of anti-B7.1 (CD80) and anti-B7.2 (CD86) monoclonal antibodies inhibits murine graft-versus-host disease lethality in part via direct effects on CD4+ and CD8+ T cells. *J Immunol* 1996; **157**: 3250–3259.
 - 21 Hill GR, Crawford JM, Cooke KR *et al*. Total body irradiation and acute graft-versus-host disease the role of gastrointestinal damage and inflammatory cytokines. *Blood* 1997; **90**: 3204–3213.
 - 22 Tsukada N, Kobata T, Aizawa Y *et al*. Graft-versus-leukemia effect and graft-versus-host disease can be differentiated by cytotoxic mechanisms in a murine model of allogeneic bone marrow transplantation. *Blood* 1999; **93**: 2738–2747.
 - 23 Via CS, Finkelman FD. Critical role of interleukin-2 in the development of acute graft-versus-host disease. *Int Immunol* 1993; **5**: 565–572.
 - 24 Sykes M, Romick ML, Hoyles KA, Sachs DH. *In vivo* administration of interleukin 2 plus T cell-depleted syngeneic marrow prevents graft-versus-host disease mortality and permits alloengraftment. *J Exp Med* 1990; **171**: 645–658.
 - 25 Allen RD, Staley TA, Sidman CL. Differential cytokine expression in acute and chronic murine graft-versus-host-disease. *Eur J Immunol* 1993; **23**: 333–337.
 - 26 Via CS. Kinetics of T cell activation in acute and chronic forms of murine graft-versus-host disease. *J Immunol* 1991; **146**: 2603–2609.
 - 27 Baker KS, Allen RD, Roths JB, Sidman CL. Kinetic and organ-specific patterns of cytokine expression in acute graft-versus-host disease. *Bone Marrow Transplant* 1995; **15**: 595–603.
 - 28 Pirenne J, D'Silva M, Degiovanni G *et al*. Relevance of tumor necrosis factor to graft-versus-host disease after small bowel transplantation. *Transplant Int* 1993; **6**: 258–262.
 - 29 Antin JH, Ferrara JL. Cytokine dysregulation and acute graft-versus-host disease. *Blood* 1992; **80**: 2964–2968.
 - 30 Hill GR, Ferrara JLM. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood* 2000; **95**: 2754–2759.
 - 31 Korngold R, Sprent J. Selection of cytotoxic T-cell precursors specific for minor histocompatibility determinants. I. Negative selection across H-2 barriers induced with disrupted cells but not with glutaraldehyde-treated cells: evidence for antigen processing. *J Exp Med* 1980; **151**: 314–327.
 - 32 Sprent J, Schaefer M, Lo D, Korngold R. Properties of purified T cell subsets. II. *In vivo* responses to class I vs. class II H-2 differences. *J Exp Med* 1986; **163**: 998–1011.
 - 33 Perreault C, Pelletier M, Belanger R *et al*. Persistence of host Langerhans cells following allogeneic bone marrow transplantation: possible relationship with acute graft-versus-host disease. *Br J Haematol* 1985; **60**: 253–260.