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ORIGINAL ARTICLE

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Effector mechanisms in murine allograft rejection: comparison of skin and heart grafts in fully allogeneic and minor histocompatibility antigen-mismatched strain combinations

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Abstract Cytotoxic T lymphocytes (CTLs) and macrophage-mediated delayed-type hypersensitivity (DTH) responses may both mediate allograft rejection. Furthermore, although allograft rejection is classically considered a type [22, 23, 38, 50, 52] 1 cellular immune response, type-2 cytokines can support rejection. This study examines whether the immunogenicity of the transplanted tissue, as determined by type of tissue (skin versus heart) and degree of antigenic mismatch, influences recruitment of these effector mechanisms. Graft survival, histological appearance and intragraft gene expression (IL-2, IFN-γ, IL-12 p40, IL-4, IL-10, perforin, Fas ligand (Fas L), iNOS and TNF-α) were compared for fully allogeneic, minor histocompatibility (mHC) antigen-mismatched and syngeneic skin and heart grafts. We found mRNA characteristic of CTLs and DTH responses in fully allogeneic and mHC antigen-mismatched skin and heart grafts. Concomitant type-1 and type-2 cytokine gene transcription was seen. These findings demonstrate that the tissue grafted and degree of antigenic disparity between donor and recipient do not restrict the repertoire of cellular immune responses involved in graft rejection. This finding has implications in the design of new immunosuppressive strategies for clinical transplantation.

Keywords Allograft rejection · Effector mechanism · Delayed-type hypersensitivity · Cytotoxic T cell · Cytokine

Introduction

Multiple mechanisms can contribute to the acute rejection of an allograft. The speed of rejection is influenced by the tissue grafted and the degree of antigenic mismatch between the donor and recipient [22, 23, 31, 38, 50, 52]. However, it is not clear if this difference in the speed of rejection reflects recruitment of different effector mechanisms.

Numerous studies have concentrated on the relative importance of $CD4^+$ and $CD8^+$ T cells in allograft rejection. $CD4^+$ T cells are usually required for graft rejection, whilst grafts are often rejected in a near-normal time in the absence of $CD8^+$ T cells [3, 27] In some instances $CD8^+$ T cells alone may reject grafts. It is difficult to compare these studies, as a wide variety of models has been used, transplanting different tissues across varying degrees of antigenic mismatch using a number of different methods to 'eliminate' T-cell subsets. Furthermore, study of the relative contribution of $CD4^+$ and $CD8^+$ T cells provides an incomplete characterisation of effector mechanisms that may be operating, as in CD4-dependent strain combinations these cells may be supporting either $CD8^+$ cytotoxic T lymphocytes (CTLs) or macrophage-mediated delayed-type hypersensitivity (DTH) responses. It is not clear if the tissue transplanted or degree of antigenic mismatch influences recruitment of these effector mechanisms (CTLs or DTH).

Historically, DTH responses were assumed to be responsible for graft rejection, given the intense mononuclear cell infiltrate seen in rejecting allografts. However, syngeneic grafts also become infiltrated with mononuclear cells, and skin allografts may survive this infiltrate. Furthermore, the exquisite specificity of killing seen in the rejection of allophenic skin grafts [33, 41] and cellular transplants made of mixtures of cells of different major histocompatibility (MHC) haplotype [16, 45] points to a role for CTLs. Differences in effector mechanism recruitment determined by the nature of the graft have not been systematically studied. For classical immune responses, the nature of the antigenic stimulus is a critical determinant of the need for $CD4^+$ Th cells in $CD8^+$ CTL priming [15]. It is proposed that the 'strength' of the antigenic stimulus may similarly influence the recruitment of $CD8^+$ cells in allograft rejection: less-immunogenic grafts, as determined by the tissue transplanted and the degree of antigenic mismatch, activating CTLs less well, resulting in DTH responses becoming more important. The findings of Rosenberg and Singer (reviewed in [42]) are consistent with such a scheme, with $CD8^+$ T cells being able to reject skin allografts independently of exogenous T-cell help ('dual function' T cells) if the immunogenic stimulus is strong, $CD4^+$ T-cell help being required for weaker stimuli. This help may be due to cytokine release as initially proposed, or through conditioning of APC [40]. This work examines whether there is a threshold below which $CD8^+$ T-cell recruitment becomes ineffective, leaving DTH response as the only mechanism available for graft rejection.

Which cytokines may mediate graft rejection is also not clear, and of fundamental importance to development of novel immunomodulatory strategies. Allograft rejection is classically regarded as a type-1 cellular immune response, but a number of studies have suggested that type-2 cytokine-dependent immune responses may be capable of rejecting allografts (reviewed in [9]). In particular, eosinophil activation is dependent upon type-2 cytokines. Eosinophils can mediate rejection of murine MHC class-II mismatched skin grafts [28], and eosinophil infiltrates are frequently seen in rejecting kidney [47] and liver allografts [1]. In clinical kidney allograft rejection these eosinophilic infiltrates are a poor prognostic marker.

This work investigates whether the phenotype of the immune response and effector mechanisms recruited during graft rejection vary with the tissue grafted or the degree of antigenic mismatch between donor and recipient. A constant recipient strain has been used to control for the influence of background genes in recruitment of effector mechanisms [22, 23, 31, 38, 50, 52]. First, we documented graft survival and histological changes in heart and skin isografts (C57BL/6 to C57BL/6), fully allogeneic (major and minor histocompatibility antigen-mismatched) grafts (BALB/c to C57BL/6), and minor histocompatibility antigen-mismatched grafts (BALB.B to C57BL/6). We then used the reverse transcription polymerase chain reaction (RT-PCR) to define cytokine gene transcription and effector-mechanism recruitment in each of these situations [10, 11, 36, 37, 44]. We characterised transcription of type-1 cytokine genes (IL-2, IFN-γ, and IL-12 p40), type-2 cytokine genes (IL-4, IL-10), genes for CTL effector molecules [perforin and Fas ligand (Fas L)] and genes for macrophage effector molecules (iNOS and TNF-α) within the graft.

Materials and methods

Animals

Mice were either bred in the University of Bristol SPF animal facility (C57BL/6 and BALB/c) or purchased from Harlan, UK (BALB.B). Male animals of 6–10 weeks of age were used throughout. All experimental animals were housed in conventional conditions and fed water and standard animal chow ad libitum. All animal experiments complied with UK Home Office regulations, and the 'Principles of laboratory animal care' (NIH) were followed throughout.

Transplants

Tail skin was grafted onto the lateral thoracic wall according to the technique of Billingham and Medawar [2] and secured in place by paraffin gauze and a plaster of Paris cast. Casts were removed at day 7and grafts scored daily until rejected (defined as >90% necrosis). Cervical heterotopic heart grafts were performed as described by Chen [7]. The day of rejection was taken as the 1st day when no heart beat was palpable. Tissue for histology was fixed in 10% formaldehyde, embedded in paraffin and stained with haematoxylin and eosin.

RT-PCR

Day-0 samples represent normal tissue; heart and skin was harvested as for transplantation experiments, but not grafted. On subsequent days mice were killed, the graft excised and total RNA extracted according to the technique of Chomczynski and Sacchi [8]. Complementary DNA (cDNA) was generated by reverse transcription of total RNA using avian myeloblastosis virus reverse transcriptase (Promega, UK), and PCR was performed using a DNA Touch Down thermal cycler (Hybaid, UK). PCR products were separated by electrophoresis in 2% agarose gels and visualised by ethidium-bromide staining and inspection under UV light using a dual intensity transilluminator (UVP). The gel images were captured by an ImageStore 5000 gel documentation system (version7.22, Ultra-Violet Products, Cambridge, UK). Positive control cDNA and controls for genomic DNA contamination were included in all experiments. Sequence-specific primers were designed to have a GC content of at least 50% and to anneal to different exons, spanning intron exon boundaries where possible. Primer sequences and amplification conditions are documented in Table 1. Total cDNA was equalised by comparison of housekeeping gene (β-actin) band intensities. For each strain combination, at each time point, three individual animals were studied for skin grafts and two individual animals for heart grafts.

Table 1. Primer sequences

Gene	Primer sequences (5′–3′)	Base location	GenBank acc. no.	Ann. temp. (C)	No. of cycles
β-actin	F ATGCCATCCTGCGTCTGGACCTGGC	600–622	X03672	55	30
	R AGCATTTGCGGTGCACGATGGA GGG	1179–1203			
IL-2	F GACACTTGTGCTCCTTGTCA	84–103	K02292	60	40
	R TCAATTCTGTGGCCTGCTTG	291–311			
IL-4	F TCGGCATTTTGAACGAGGTC	188–208	M25892	58	40
	R GAAAAGCCCGAAAGAGTCTC	384–404			
IL-10	F ATGCAGGACTTTAAGGGTTACTTG	285–309	M37897	60	40
	R TAGACACCTTGGTCTTGGAGCTTA	05–539			
IFN-γ	F TGAACGCTACACACTGCATCTTGG	111–135	M28621	58	35
	R TGACTCCTTTTCCGCTTCCTGAG	549–570			
IL-12p40	F CAGTACACCTGCCACAAAGGA	293–313	M86671	60	40
	R GTGTGACCTTCTCTGCAGACA	549–569			
iNOS	F CCACCTTGTTCAGCTACGCC	846–1865	M92649	60	40
	R GGACATCAAAGGTCTCACAG	2213–2232			
TNF-α	F ATGAGCACAGAAAGCATGATC	157–177	M20155	61	35
	R TACAGGCTTGTCACTCGAATT	412–432			
Fas-L	F CAGCTCTTCCACCTGCAGAAGG	425–446	U06948	60	45
	R AGATTCCTCAAAATTGATCAGAGAGAG	908–934			
Perforin	F AGCTGAGAAGACCTATCAGG	665–684	M23182	60	40
	R GATAAAGTGCGTGCCATAGG	844–863			

Data analysis

Kaplan-Meier plots for graft survival were generated and analysed using GraphPad Prism and Instat software (GraphPad Software, San Diego, USA).

Results

Graft survival

Skin isografts all survived to 100 days (Fig. 1). Heart grafts which stopped beating within 48 h of grafting were considered to be technical failures and were excluded from further analysis. The technical success rate for heart grafts was 80%. Heart isografts also showed 100% survival to 100 days (Fig. 2). Minor antigen-mismatched skin grafts had a median survival time of 11 days, and fully allogeneic skin grafts a median survival time of 10 days (MHC vs. mHC mismatch, not significant; Fig. 1). Minor antigen-mismatched heart grafts had a median survival time of 9 days and fully allogeneic heart grafts a median survival time of 6.5 days ($P = 0.0003$; Fig. 2).

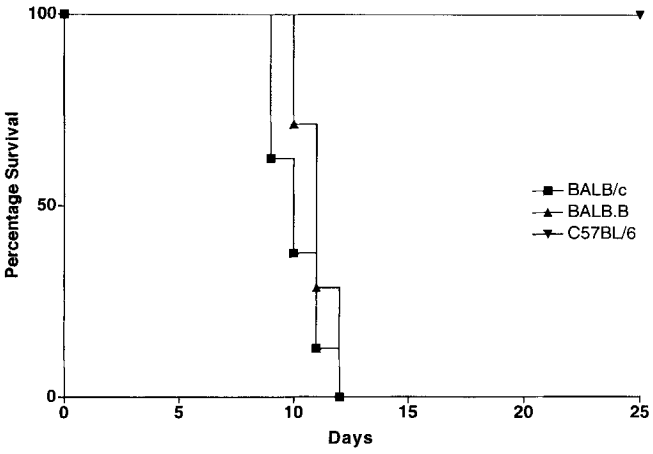


Fig. 1. Skin graft survival. Syngeneic (*C57/BL6*), minor histocompatibility antigen-mismatched (*BALB.B*) or fully allogeneic (*BALB/c*) skin was grafted onto C57/BL6 recipients. Graft survival was monitored daily for 3 weeks and then weekly thereafter. The day of rejection was taken as the day when 90% necrosis was seen

Histology

Skin isografts showed a mononuclear cell infiltrate which had resolved by days 9–11 (Fig. 3A). Skin allografts (fully allogeneic and minor antigen-mismatched) showed a mononuclear cell infiltrate, composed predominantly of macrophages and lymphocytes. In contrast to isografts, skin allografts showed acanthosis, hydropic degeneration of the basal cells, spongiosis and dyskeratotic keratinocytes within the epidermis. By days 9–11, skin allografts showed necrosis (Fig. 3B). Heart isografts demonstrated only a mild mononuclear cell infiltrate which resolved without myocyte necrosis (Fig. 3C). Heart allografts (fully allogeneic and minor antigen-mismatched) showed a severe and diffuse mononuclear cell infiltrate composed of macrophages, lymphocytes and occasional eosinophils with associated interstitial oedema, myocyte degeneration and necrosis (Fig. 3D).

RT-PCR

The central question addressed in this work is whether transcripts characteristic of specific effector mechanisms

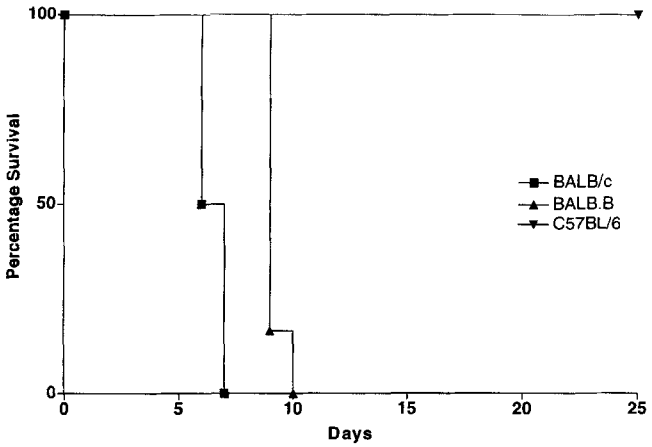


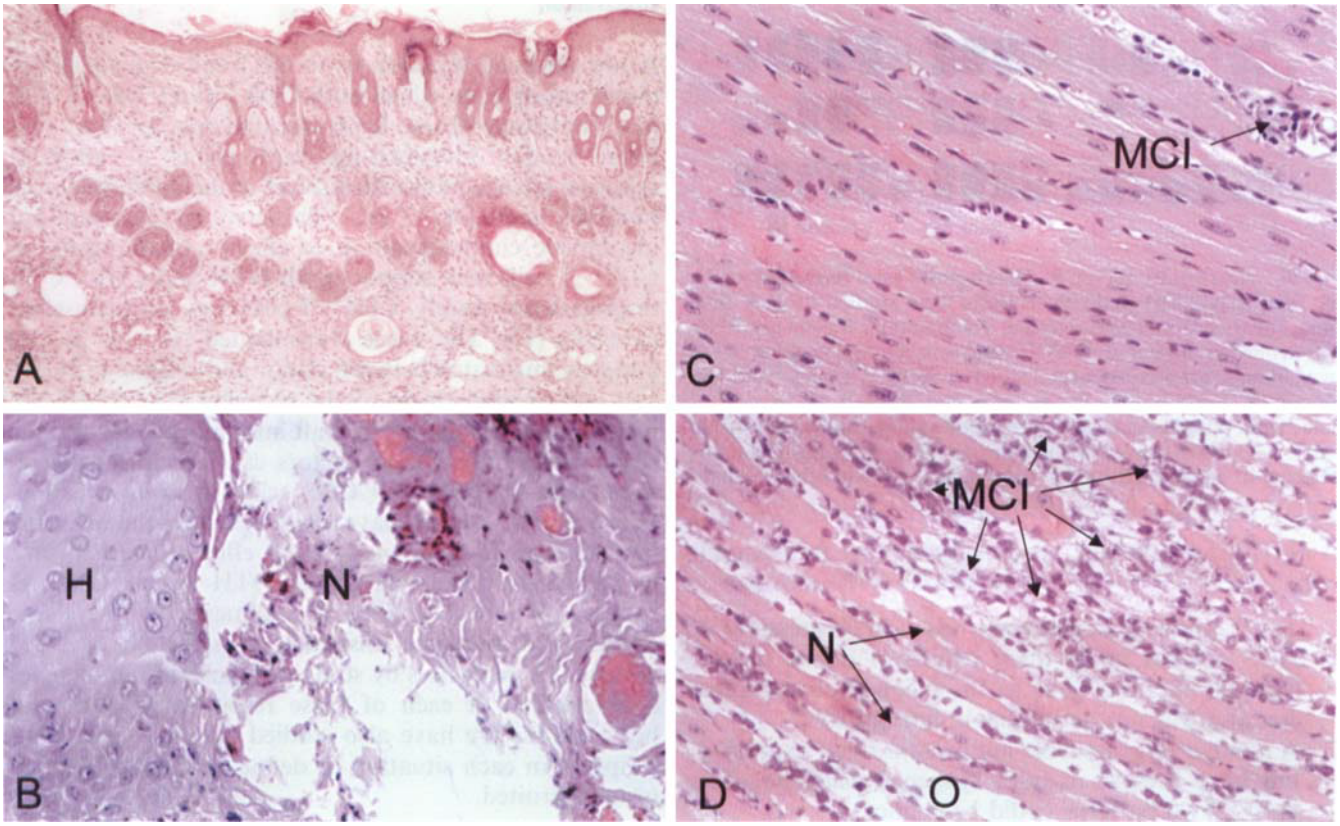
Fig. 2. Heart graft survival. Syngeneic (*C57/BL6*), minor histocompatibility antigen-mismatched (*BALB.B*) or fully allogeneic (*BALB/c*) skin was grafted onto C57/BL6 recipients. Graft survival was monitored daily for 3 weeks and then weekly thereafter. The day of rejection was taken as the first day when no heart beat was palpable. Minor histocompatibility antigen-mismatched grafts were rejected more slowly than fully allogeneic grafts ($P = 0.0003$)

were present in skin or hearts grafted across defined histocompatibility barriers. Absence of a specific transcript would preclude involvement of a given effector mechanism in graft rejection. Semi-quantitative RT-PCR is an appropriate technique to address this question. We have classified each transcript as 'absent', 'weak' or 'present' based on an examination of results in all animals in each group ($n = 3$ for skin and $n = 2$ for heart). Transcripts were classified as 'absent' if no band was visible in any animal. If a faint band was present in at least one animal, the product was classified as 'weak'. Transcripts were classified as 'present' if a strong band was present in at least one animal; in all these cases a band was visible in all animals.

The results for skin grafts are shown in Fig. 4, and for heart grafts in Fig. 5.

We found no significant differences in the pattern of gene expression between fully allogeneic and mHC mismatched grafts; these different degrees of antigenic mismatch will be considered together under the title of 'allografts'. The results from this study fit into three patterns of expression previously described by Dallman

Fig. 3A–D. Histology of heart and skin grafts. Histology is shown for fully allogeneic allografts on the day of rejection (day 10 for skin and day 6 for heart). Isografts are shown from the same day. **A** Skin isografts (× 10 magnification) showed only minor histological abnormalities. **B** Skin allografts (× 40) show hydropic degeneration (*H*), spongiosis and necrosis (*N*). **C** Heart isografts (× 40) show mild mononuclear cellular infiltrate (*MCI*) without myocyte necrosis. **D** Heart allografts (× 40) show diffuse mononuclear cellular infiltrate (*MCI*), interstitial oedema (*O*), myocyte degeneration and necrosis (*N*)



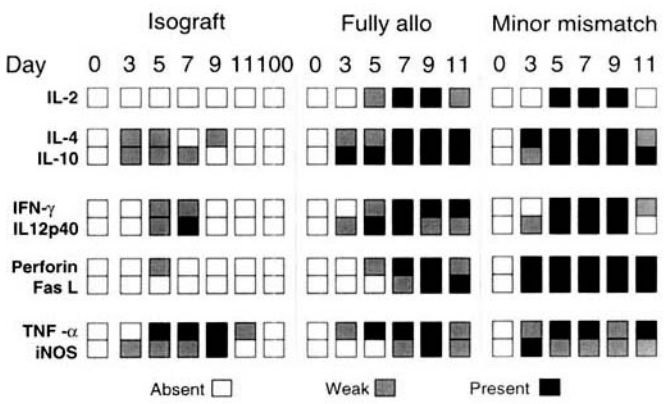


Fig. 4. Skin graft RT-PCR. Skin grafts were harvested at the indicated days. Three individual grafts were studied at each time point for each donor and recipient combination. Messenger RNA was defined as 'absent' if no PCR band was detected in any of the three animals. Transcripts were defined as 'weak' if a weak band was present in 1–3 animals. Bands were only defined as 'present' if detected in all three animals, with at least one animal showing a strong band. There was no group of animals where a strong band

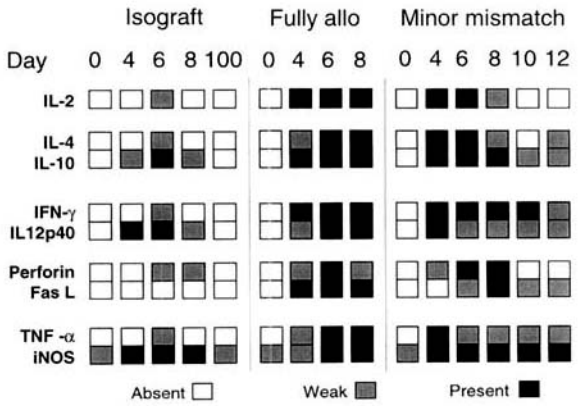


Fig. 5. Heart graft RT-PCR. Heart grafts were harvested at the indicated days. Two individual grafts were studied at each time point for each donor and recipient combination. Messenger RNA was defined as 'absent' if no PCR band was detected in either of the two animals. Transcripts were defined as 'weak' if a weak band was present in 1–2 animals. Bands were only defined as 'present' if detected in both animals, with at least one animal showing a strong band. There was no group of animals where a strong band was detected in one animal but no band detected in the other animal

et al. [11] for cytokine gene expression in transplantation.

1. Hearts showed constitutive (day 0) expression of iNOS mRNA in all three strains of donor mouse. This was the only gene to show constitutive expression and was not detectable in normal skin.
2. A number of genes were not constitutively expressed, but were present in both syngeneic *and* allogeneic grafts. Two patterns could be defined:

Weak expression in isografts with upregulation in allografts

(a) Heart isografts showed weak and transient expression of IL-2, IL-4, IFN-γ, perforin and TNF-α mRNA with significant upregulation and prolonged expression in allografts.
(b) Skin isografts showed weak expression of IL-10, IL-4, IFN-γ and perforin transcripts with pronounced upregulation in allografts.

Significant expression in both isografts and allografts

(a) TNF-α and iNOS mRNA were detectable at similar levels and over a similar time course in skin isografts and allografts.
(b) IL12p40 mRNA was present at similar levels in heart isografts and allografts.
(c) IL-12p40 mRNA showed significant expression in skin, and this expression was prolonged in allografts.
(d) IL-10 mRNA showed significant expression in heart isografts, but strong expression was prolonged in allografts.

3. The only transcripts unique to allogeneic grafts were IL-2 and Fas L in skin grafts and Fas L in heart grafts.

Discussion

Acute allograft rejection is an αβ T-cell dependent immune response, to which both $CD4^+$ and $CD8^+$ T cells may contribute. There is no essential role for antibody. Most studies investigating mechanisms of allograft rejection have confined themselves to the differential role of $CD4^+$ and $CD8^+$ T cells. This gives an important but incomplete characterisation of effector mechanisms, as $CD4^+$ T cells may support both DTH and $CD8^+$ CTL responses and, rarely, $CD4^+$ T cells may act themselves as CTLs [17]. The tissue transplanted [18, 19, 24, 31] antigenic mismatch between donor and recipient [24, 50] and background strain of the recipient [23, 24, 38] are important determinants of graft immunogenicity and the speed of rejection. These factors can also affect the requirement for CD4 and CD8 cells for graft rejection. However, few studies have examined how these factors influence recruitment of different effector mechanisms, in particular the activation of DTH versus CTL responses. We have studied the influence of the tissue grafted and antigenic mismatch on selection of DTH versus CTL responses by studying transcription of genes characteristic of each of these responses in skin and heart grafts. We have also studied cytokine gene transcription in each situation to define further the mechanisms recruited.

Macrophages are the effector cells in DTH responses. Historically, DTH responses were believed to mediate allograft rejection in a manner akin to the 'tuberculin reaction' [5]. However, macrophages are not able to mediate cell-specific killing. Thus, the exquisite specificity seen in rejection of allophenic skin grafts [1, 33], mixed tumour grafts [16] and pancreatic islet grafts [45], argued against rejection of these grafts by DTH responses and supported a role for CTLs. $CD8^+$ CTL-mediated rejection has subsequently been confirmed in many animal models. However, this debate has been reopened by the demonstration that DTH responses most probably mediate rejection of MHC class-I mismatched skin grafts by $CD4^+$ T cells [12, 51] and by experiments demonstrating rejection of grafts by CD8 knockout mice Youssef et al., unpublished observation and [27]. Macrophage depletion has been shown to prevent primary non-function of pancreatic islet allografts [25] and rejection of tumour allografts [49]. However, macrophage depletion is difficult to achieve and the effect of macrophage depletion on graft rejection has thus not been extensively studied.

We studied expression of four mRNAs attributable to macrophage activation (IL-10, IL-12p40, TNF-α and iNOS). None of these genes can be considered specific for macrophage activation or for DTH responses. In addition to production by macrophages, IL-10 is produced by T cells [34], IL-12 by B cells [48], iNOS is readily induced in cardiac myocytes [20, 29], and TNF-α may have a role in CTL effector function [39]. TNF-α is also produced by neutrophils [14], fibroblasts [13], and keratinocytes [4, 26], as a non-specific response to injury. Furthermore, upregulation of TNF-α and iNOS transcripts is seen in isografts without graft destruction. However, the concomitant upregulation of expression of IL-10, IL-12p40, TNF-α and iNOS mRNA seen in this study, and their increased expression in allografts, compared with isografts, strongly suggest macrophage activation, supporting a role for DTH responses in rejection of skin and heart mHC antigen-mismatched and fully allogeneic grafts. Cytotoxic T cells can mediate killing by both granule exocytosis and Fas-dependent pathways [21]. Perforin, granzyme and Fas L transcription correlate well with acute rejection of renal allografts [30]. Perforin transcription correlates also with rejection of murine mHC mismatched skin grafts [4]. In our work, evidence of CTL activation was evident in both fully allogeneic and mHC antigen-mismatched strain combinations and for both skin and heart grafts. Thus, our results do not allow elucidation of criteria predicting the involvement of CTL versus DTH responses on the basis of the tissue grafted or antigeneic mismatch because markers of CTL and macrophage activation were detected in all allografts studied.

Allograft rejection is classically described as a type-1 cytokine response, with these cytokines supporting both CTL and DTH responses. In keeping with this, upregulation of type-2 cytokines correlates with resolution or inhibition [35, 43, 46] of the rejection process. However, in CD8-deficient animals, type-2 responses have been demonstrated to mediate graft rejection not attributable to humoral responses [6] and in IL-4 and anti-IFN-γ treated animals, $CD8^+$ CTLs, which can still mediate graft rejection, survive [32]. Furthermore, eosinophil activation is dependent on type 2-cytokines and eosinophils are detectable in rejecting kidney [47] and heart allografts. In our work, type-1 and type-2 cytokines were upregulated with a similar time course. Our results for IL-2, IL-4 and IFN-γ transcription in vascularised heart isografts and allografts are similar to those reported by Dallman et al. (20), although these workers found no expression, rather than weak expression, of IL-2 and IL-4 in heart isografts. These workers also demonstrated early upregulation of another type-2 cytokine, IL-5, that is essential for eosinophil activation.

In summary, we found no significant difference between the speed of rejection of mHC antigen-mismatched and fully allogeneic skin grafts. Minor histocompatibility antigen-mismatched heart grafts were rejected more slowly than fully allogeneic grafts. Compared with isografts, skin and heart grafts transplanted across fully allogeneic and mHC antigen mismatches all showed increased expression of type-1 and type-2 cytokines, increased expression of markers of cytotoxic T-cell effector function and markers of macrophage activation. Thus, the slower rate of rejection in mHC antigen-mismatched heart grafts did not correlate with a different pattern of intragraft gene expression, and no effector mechanism is precluded in these strain combinations. This is important, as the mHC mismatched grafts used in this study give a situation similar to the best match that could be hoped for in a clinical transplant. Thus, when developing novel immunosuppressive agents for clinical transplantation it may be necessary to ensure inhibition of the full range of cellular immune responses in order to ensure prevention of graft rejection in outbred populations.

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