MIGRATION OF DENDRITIC LEUKOCYTES FROM CARDIAC ALLOGRAFTS INTO HOST SPLEENS

A Novel Pathway for Initiation of Rejection

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Dendritic leukocytes (DL)[1] from lymphoid tissues are specialized antigen-presenting cells that initiate immune responses (1). There is evidence that related cells in transplanted tissues (passenger leukocytes) are the principal stimulators of rejection but it is not known where they initially activate host T cells (2). The prevailing view is that for fully vascularized organ allografts sensitization occurs within the graft itself (i.e., peripherally) (3–5), while for skin grafts it occurs in the draining lymph nodes (i.e., centrally) (6–8). In this report we show that donor-derived MHC class II-positive (Ia^+) DL migrate rapidly out of mouse cardiac allografts into the recipients' spleens where they home to the peripheral white pulp and associate predominantly with $CD4^+$ T lymphocytes. This provides a novel route for central sensitization against fully vascularized allografts, and most likely represents a pathway by which immune responses are generated against antigens on blood-borne DL emigrating from peripheral tissues.

While dendritic cells (DC) isolated from lymphoid tissues, lymph, and blood can initiate immune responses, their precursors in skin, Langerhans cells (LC), lack this ability. LC, however, can mature into potent stimulatory cells (9) and migrate as veiled cells via the lymph into lymph nodes (10, 11); by a second route purified lymphoid DC that are administered intravenously can migrate from blood into spleen and home to T areas (12, 13). Bone marrow-derived Ia^+ cells with dendritic morphology have been identified in a wide variety of nonlymphoid organs and are presumed to be DL (14). Recent work suggests these cells in kidney, heart, and perhaps other nonlymphoid organs are immature precursors more closely resembling LC than lymphoid DC (Austyn, J., and C. Larsen, unpublished data).

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[1] *Abbreviations used in this paper:* DC, lymphoid dendritic cells; DL, dendritic leukocytes; LC, Langerhans cells.

Materials and Methods

Mice

Male C57BL/10 (H-2^b), BALB/c (H-2^d), CBA/Ca (H-2^k), and C3H/He (H-2^k), mice were obtained from Olac Ltd. (Bicester, Oxon., UK) or from the Biomedical Services Unit, John Radcliffe Hospital, University of Oxford.

Cardiac Transplantation

BALB/c hearts were transplanted as fully vascularized, heterotopic grafts into C3H/He recipients essentially as described (15). Technical success was >95% and the total ischemic time was generally 20–25 min. The amount of donor blood transferred to recipients in the grafts was minimized by removing ~0.7 ml blood from the vena cava, injecting 1 ml heparinized saline (300 U/ml) at 4°C, and allowing this to circulate before severing the abdominal aorta and vena cava. Donor hearts were then dissected out and gently massaged between cotton buds in saline at 4°C.

Measurement of the Volume of Blood Contained in a Transplanted Heart

The average volume of blood contained within a graft was determined using ^{51}Cr-labeled mouse erythrocytes essentially as described (16): cells containing 150,000 cpm in 0.25 ml PBS were injected intravenously into three mice and after 20–30 min a sample of peripheral blood was taken and the heart was prepared as for transplantation (Table I).

Immunohistology

Monoclonal Antibodies. The following mAbs were used as tissue culture supernatants for first stage antibodies: TIB122 anti-leukocyte common antigen, 2.4G2 anti-Fc receptor type II, M1/70 (TIB 128) anti-C3bi receptor, GK1.5 anti-CD4, TIB 105 anti-CD8, TIB 104 anti-CD5, TIB 145 anti-B220, TIB 146 anti-B p50, 2D2C anti-Pgp-1.1 (Ly24), B21-2 (TIB 229) anti-I$a^{b,d}$, all of which are rat monoclonals, and MKD6 anti-Ia^d which is a mouse mAb (antibodies in reference 17). For two-color staining biotinylated YTS 191, rat IgG2b anti-CD4 and YTS 169, and rat IgG2b anti-CD8 were used (18; both kind gifts from K. Wood, Nuffield Dept. of Surgery).

Tissue Preparation. Multiple cryosections (7 μm) of heart and spleen from transplanted and normal mice embedded in OCT compound (Miles Scientific) were prepared and stored at −30°C until use. For staining, the sections were fixed in acetone for 10 min, air dried and rehydrated in PBS with 1% fetal calf serum.

Two-Color Immunofluorescent Staining

Resident cardiac leukocytes (see text) were examined by two-color immunofluorescence using MKD6 mouse anti-Ia^d and TRITC-conjugated goat anti-mouse Ig (Sigma Chemical Co., St. Louis, MO) as a first step. The sections were then blocked with normal mouse serum before staining with various rat anti-mouse mAbs followed by FITC-mouse anti-rat Ig (Boehringer-Mannheim Biochemicals, Indianapolis, IN).

Localization of Migratory DL within Spleen. Sections of recipient spleens 2–3 d after trans-

TABLE I

Passenger Blood Volume Transferred in Cardiac Allografts

Animal	Blood activity	Heart activity	Heart weight	Calculated blood volume	Blood volume/ 0.1 g heart
	cpm/μl	*cpm*	*g*	*μl*	*μl*
1	101	223	0.1086	2.2	2.0
2	134	184	0.0874	1.4	1.6
3	100	191	0.0995	1.9	1.9
Mean			0.0985	1.8	1.8

plantation were first stained with TRITC-conjugated goat anti-mouse Ig and blocked with normal mouse serum before staining donor DL with B21-2 and FITC mouse anti-rat Ig.

Immunoperoxidase Staining. Frozen sections were stained using B21-2 anti-$Ia^{b,d}$ or 2D2C anti-Pgp-1.1 (not shown) followed by peroxidase-conjugated goat anti-rat Ig (Sigma Chemical Co.) adsorbed with normal mouse serum, after first eliminating endogenous peroxidase by incubating the sections in 10 U/ml glucose oxidase type VS (Sigma Chemical Co.) in 10 mM glucose for 30 min at 37°C. Peroxidase activity was detected using H_2O_2-DAB (3,3' diaminobenzidine tetrahydrochloride; Polysciences Inc., Warrington, PA) as a chromogen. After counterstaining with Harris' hematoxylin (BDH, Ltd. Poole, UK) the slides were dehydrated and mounted in DPX (BDH, Ltd.).

Two-color Immunogold-Peroxidase Staining. Sections were prepared as for one-color peroxidase staining and Ia^d-positive cells were stained with B21-2 and gold-conjugated goat anti-rat Ig (Janssen Life Sciences Products, Olen, Belgium) adsorbed with normal mouse serum. After blocking with 5% normal rat serum, sections were incubated with biotinylated YTS 191 (anti-CD4) or YTS 169 (anti-CD8) followed by peroxidase-conjugated goat anti-biotin (Sigma Chemical Co.). Silver enhancement (IntenSE-M; Janssen Life Sciences Products) was performed before development with DAB.

An ocular grid was used to enumerate discrete Ia^d-positive cells in tissue sections of heart grafts (2 hearts/time point, 10 fields/sample). From day 3 onwards Ia^d was induced on other graft elements, as noted by others (19), so DL could only be quantified in areas lacking generalized Ia expression; a similar approach has been used elsewhere (20).

Ia^d-positive cells in recipient spleens were counted similarly (6–12 sections/sample) and cross-sectional areas were measured using an Optimax V image analyzer in order to standardize the number of cells per unit area. Statistical analysis was carried out using Student's *t*-test or χ^2 with Yates correction.

Results and Discussion

Using two-color immunofluorescent staining we identified two resident leukocyte common antigen-positive populations in mouse hearts: a strongly Ia^+, Fc receptor type II^+ ($FcRII^+$), phagocyte glycoprotein-1^+ ($Pgp\text{-}1^+$), and complement receptor type III (CR3)-weak population of similar phenotype to LC, comprising the presumptive DL; and a smaller group which is Ia^- $FcRII^+$, $Pgp\text{-}1^+$, and CR3-strong, perhaps comprising tissue macrophages or precursors of the Ia^+ population. Normal hearts contained no cells that stained for B cell markers (anti-Ig, B220, and TIB146) or T cell markers (CD4, CD5, CD8).

We wished to determine whether donor DL could migrate via the blood from vascularized organ allografts into recipients' spleens after transplantation (an efflux of these cells in the draining lymph having been reported previously [5]). BALB/c hearts (H-2d, Pgp-1.1) were transplanted into C3H/He recipients (H-2k, Pgp-1.2) and we examined the grafts for changes in density of resident DL, as well as the recipient spleens for entry of cells bearing donor alloantigens between 1–6 d later (rejection normally occurs at 8–12 d).

Over the first 4 d after transplantation there was a marked reduction in the number of Ia^+ DL in the graft, from a mean of 95.7 mm^{-2} (at day 0) to 9.2 mm^{-2} (Table II). At the same time that donor DL were leaving the grafts, strongly Ia^d-positive cells with dendritic morphology entered the recipients' spleens (Fig. 1 *a* and *b*, and Table II). These cells also labeled for the donor-strain Pgp-1 allele that is expressed by DL, further suggesting the cells were of donor origin, although we can not exclude the less likely possibility they were host-derived cells that had acquired donor alloantigens in a form recognizable by the antibodies. Donor cells were readily apparent by day 1, and maximum numbers were present between days 2 and 4 after transplantation, representing a total of $\sim 10^4$ cells per spleen at any given time (Table

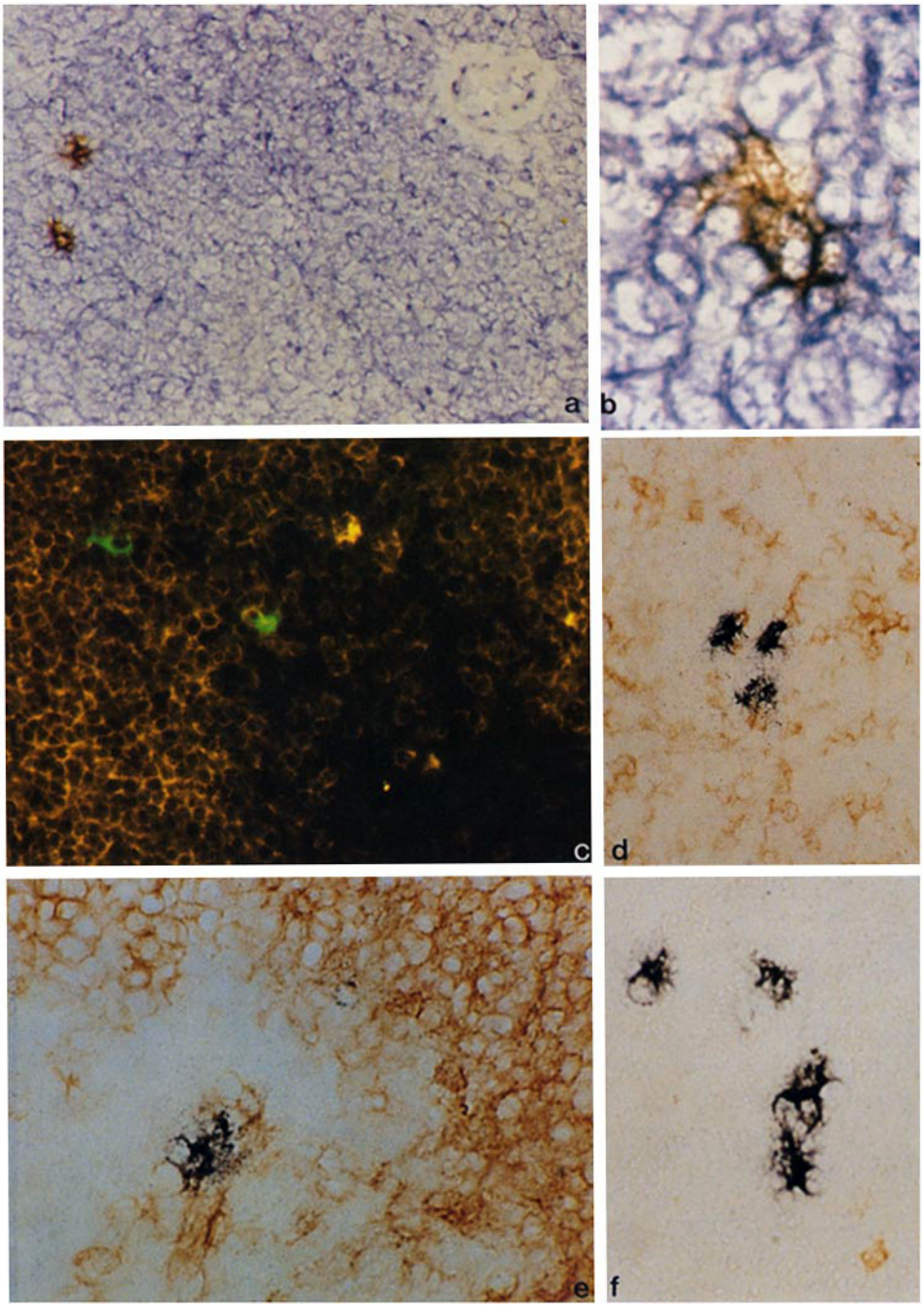

FIGURE 1. Donor dendritic leukocytes enter recipient spleens after transplantation. Sections of C3H/He spleens from recipients of BALB/c hearts 2 d after transplantation stained to show the presence of Ia^d-positive cells, note the central arteriole (*a*, ×250), and their dendritic morphology (*b*, ×1,000). Legend continued on facing page.

TABLE II

Frequencies of Donor Ia$^+$ DL in Cardiac Allografts and Recipient Spleens

Time after transplantation	Mean DL ± SE in cardiac allografts per mm^2 section	Mean donor DL ± SE in recipient spleens per 10 mm^2 cross-section
d		
0	95.7 ± 8.2	0
1	47.5 ± 7.3*	5.0 ± 0.8
2	17.0 ± 3.0*	11.8 ± 1.3
3	9.2 ± 2.0*	10.4 ± 0.8
4	20.5 ± 5.1*	9.6 ± 1.7
6	‡	0

Discrete Ia^d positive cells in tissue sections of heart grafts (2 hearts/time point, 10 fields/sample) were enumerated using an ocular grid. Ia^d-positive cells in recipient spleens were counted similarly (6–12 sections/sample) and cross-sectional areas were measured. By multiplying the mean number of stained cells per 10-mm^2 section (7 μm thick) by the approximate number of sections in a spleen 1.5 cm in length (2,000), it is possible to estimate $\geq 10^4$ cells/spleen are present at any time on days 2–4.

* A significant difference compared with normal nontransplanted BALB/c hearts (day 0) ($p \leq 0.001$).

‡ At day 6 generalized Ia induction precluded assessment of the density of DL.

II, legend), but they were not detectable at day 6. Similar findings were made in other strain combinations (BALB/c → CBA/Ca, C57BL/10 → CBA/Ca and BALB/c → C57BL/10; data not shown).

The donor cells in the spleen were not cells such as B cells or monocytes derived from the very small amount of blood remaining in the heart graft. First, precautions were taken to avoid significant transfer of blood in the donor heart (see above). Second, experiments revealed the volume of blood transferred in a graft to be 1.8 ± 0.2 μl. (Table I). When 2.5 μl of BALB/c blood (diluted to 0.25 ml) was administered to each of three C3H/He mice, the frequency of Ia^d-positive cells in spleen 2 d later was 0.2 ± 0.1/10 mm^2, indicating the donor cells in spleens of transplant recipients were not derived from blood. Hence, the donor Ia^d positive cells found in the recipient spleen must have come from the resident Ia$^+$ leukocytes of grafted heart which we would term dendritic leukocytes, there being no resident B cells in normal cardiac tissues.

To determine where donor Ia$^+$ cells were localized within the spleen, double-labeling techniques were used (Fig. 1). Donor cells were found exclusively within the peripheral white pulp that contains primarily B cells and some T cells, or at the border of this with central white pulp (dense T areas) (Fig. 1 *c*). On close inspection, donor cells did not appear to express surface or cytoplasmic Ig, providing fur-

Donor dendritic leukocytes localize in splenic peripheral white pulp and associate with CD4$^+$ T cells. Immunofluorescence to show donor Ia^d-positive cells (green) within the B cell rich areas (red) and at the border of B cell and T cell areas (*c*, ×250). Immunogold-peroxidase staining to show donor DL (black) in association with CD4$^+$ T cells (brown) (*d*, ×250) and an Ia^d-positive DL in a cluster of CD4$^+$ T cells (*e*, ×400), and in contrast, donor DL not in contact with CD8$^+$ T cells (*f*, ×400).

ther evidence against their being B cells. Double staining with Ia and CD4 or CD8 mAbs revealed that 78% of donor cells were associated with $CD4^+$ T cells (Fig. 1, *d* and *e*), either singly or in a cluster, but only 14% appeared to be in contact with $CD8^+$ T cells (Fig. 1 *f*), a highly significant difference ($\chi^2 = 39$, $p = 0.0001$).

These findings demonstrate a novel migratory route for DL that express high levels of alloantigens and/or foreign peptide/MHC complexes from a vascularized organ allograft into the spleen (via blood, since the spleen lacks a lymphatic supply), concomitant with their disappearance from the graft. Previous investigators have noted such a reduction in both allografts and isografts, but failed to detect migration into lymphoid tissues, perhaps because endogenous peroxidase activity was not sufficiently blocked to allow detection of the relatively small numbers of cells (20). While the number of DL in the spleen at any time point is only $\sim 10^4$ there may be a continuous flux into and out of this tissue, and a single injection of even this number of donor-strain lymphoid DC alone can precipitate allograft rejection (16, 21).

Studies have shown that depletion of DL from transplanted tissues not only has a profound influence on cellular rejection (22, 23), but also prevents the development of graft-specific antibodies (24). This is particularly interesting in light of the juxtaposition of donor DL with both T and B cells, consistent with the idea that DC play a role in the initiation of cell-mediated and T cell–dependent antibody responses in vivo, as has been shown in vitro (25). This distribution is somewhat different from that seen after intravenous administration of syngeneic splenic DC, which appear to home to the central white pulp (12). Possibly after transplantation the allogeneic DL encounter alloreactive T cells in the peripheral white pulp, soon after their migration out of the marginal zone, and their subsequent movement into the central white pulp is inhibited. Alternatively, the migratory properties of DL from nonlymphoid tissues may be distinct from those of lymphoid DC. Additional studies will be required to distinguish between these possibilities.

DL may migrate into blood in response to cytokines that are released during the inflammatory reaction that occurs after transplantation. Results of older studies favoring peripheral sensitization against fully vascularized organ allografts can now be reinterpreted as central sensitization due to migration of blood-borne DL into the spleen, and in physiological situations this most likely provides a pathway for inducing responses against antigens (e.g., viruses) derived from peripheral tissues. It is quite conceivable that DL reside in nonlymphoid organs as precursors and process antigens from the environment, before they develop into mature DC and subsequently migrate into lymphoid tissues with the ability to present these peptide/MHC complexes and activate resting T cells (9, 26). Studies to define the signals (e.g., cytokines) that regulate maturation and migration of DL from peripheral tissues may provide new strategies to overcome allograft rejection and manipulate antigen-dependent immune responses, e.g., vaccination.

Summary

It has been a long-standing dogma that host sensitization against fully-vascularized organ allografts occurs peripherally within the graft itself. In this report we show that donor-derived MHC class II–positive (Ia^+) DL migrate rapidly out of mouse cardiac allografts into the recipients' spleens where they home to the peripheral white

pulp and associate predominantly with $CD4^+$ T lymphocytes. This provides a novel route for central sensitization against fully vascularized allografts, and most likely represents a pathway by which immune responses are generated against antigens on blood-borne DL emigrating from peripheral tissues.

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