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Transplant surgery injury recruits recipient MHC class II-positive leukocytes into the kidney

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Transplant surgery injury recruits recipient MHC class II-positive leukocytes into the kidney.

Background. CD4 T cells, which are stimulated by the "indirect pathway" of antigen-presentation, participate in rejection. These T cells are sensitized by recipient major histocompatibility complex (MHC) class II-positive leukocytes that migrate into the transplant. Therefore, an important early step in rejection is the immigration of these recipient MHC class II-positive leukocytes into the renal transplant. The regulation of this early step is not understood. We now test the hypothesis that such leukocytes immigrate into the renal transplant in response to ischemic injury occurring during the transplant procedure.

Methods. We transplanted Brown Norway (BN) kidneys into F1 Lewis/Brown Norway (L/BN) recipients. The F1 recipients are tolerant to the parental BN antigens, and any infiltration of recipient MHC class II-positive leukocytes results from injury occurring during transplantation surgery. In addition, ischemia/reperfusion injury was also induced by temporarily occluding the native renal arteries for 30 minutes. Transplanted kidneys and native kidneys, which suffered ischemia/reperfusion injury, were studied by immunohistochemistry on days 3, 7, 14, and 28 after surgery. Staining by the new monoclonal antibody (mAb) OX62 and antibodies to MHC class II identified dendritic cells. In addition, the following monoclonal antibodies identified: gamma/delta T cells, V65; B cells, OX33; cells that may be macrophages, dendritic cells, or dendritic cell precursors, ED1 (+) and OX62 (−); and recipient class II MHC, OX3.

Results. After transplantation, the serum creatinine increased to 4 mg/dl and then decreased, which was consistent with reversible injury during transplantation and the absence of rejection. We found that the injury of transplantation itself resulted in the infiltration of recipient MHC class II-positive leukocytes into the transplanted kidney. This infiltrate peaked at days 7 to 14 after surgery. The inflammation was peritubular and patchy and involved cortex and outer medulla. Double staining for OX62 and OX3 identified some of the infiltrating leukocytes as dendritic cells. Other recipient leukocytes were MHC class II positive, ED1 positive, and OX62 negative. We also found that MHC class II leukocytes, including dendritic cells, infiltrated native kidneys injured by ischemia/reperfusion injury.

Conclusion. To our knowledge, this is the first demonstration that injury to the kidney during transplantation recruits recipient MHC class II-positive leukocytes into the kidney. Some of these leukocytes are dendritic cells.

Key words: dendritic cell, transplantation, ischemia, acute renal failure, major histocompatibility complex.

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Recipient major histocompatibility complex (MHC) class II-positive leukocytes, which immigrate into the renal transplant, stimulate recipient alloreactive CD4 T cells via the "indirect antigen presentation pathway" [reviewed in 1–3]. Unlike T cells stimulated "directly" by donor MHC class II molecules on donor "passenger" leukocytes, these T cells are stimulated "indirectly" by allopeptides bound to recipient MHC class II molecules on the cell surfaces of recipient leukocytes. These T cells contribute to acute and chronic rejection of kidney, liver, skin, and heart transplants in humans [4–8] and rodents [9–14].

Despite the importance of recipient MHC class II-positive leukocytes in initiating CD4 T cell activation via the indirect pathway, the signals that recruit these antigen-presenting cells into the transplanted kidney are not completely understood. Two possibilities, which are not mutually exclusive, may be considered. One possibility is that these cells are recruited by cytokines, chemokines, and adhesion molecules produced during the initial rejection of alloantigens via the "direct pathway." Another possibility is that recipient antigen-presenting cells are recruited into the transplant by the response of the kidney to injury occurring during the transplant procedures. The latter possibility is the focus of this article.

We now report two new major findings. First, we show that the injury of transplantation is itself sufficient to recruit MHC class II recipient leukocytes into the transplant. We transplanted parental (BN) kidneys into Lewis/Brown Norway F1 (L/BN) recipients. In this model, there should be no rejection because the F1 recipients are tolerant to parental antigens, and recipient MHC

Table 1. Monoclonal antibodies used to identify inflammatory cells in BN kidneys transplanted into L/BN recipients

Monoclonal antibody	Cells identified by monoclonal antibody	Reference
OX3	Lewis class II MHC—present only on recipient (L/BN) cells	[15]
OX6	Class II MHC—present on both donor and recipient cells	[15]
ED1	Dendritic cells and macrophages	[26]
OX62	An integrin or integrin like molecule present on dendritic cells[a]	[18]
OX33	CD45RA or A/B present only on B cells	[27]
V65	Gamma/delta T cells	[28]

[a] OX62 is also present on gamma/delta T cells. In our experiments, OX62 identified dendritic cells, since the positive cells were negative for a gamma/delta T cell marker (V65). Other references also indicate that OX62 cells are dendritic cells [19–25]

class II leukocytes are identified by virtue of a monoclonal antibodies specific for recipient class II MHC (OX3) [15]. Our experiments demonstrate that the increased number of MHC class II leukocytes result from injury, not alloreactivity, and from the immigration of recipient leukocytes, not from the proliferation and differentiation of donor leukocytes resident in the transplant. To our knowledge, this is the first demonstration that injury, occurring during transplantation, recruits recipient MHC class II leukocytes into the transplanted kidney. Although others have shown increased MHC class II-positive cells after temporarily clamping the renal artery of native kidneys [16, 17], these experiments do not exactly mimic the injury of transplantation, nor do they exclude the possibility that the MHC class II-positive leukocytes resulted from the differentiation and proliferation of MHC class II-negative precursors already residing in the kidney. Second, we examined the transplanted kidneys by double staining with monoclonal antibodies specific for recipient MHC class II and also specific for dendritic cells (OX62) [18–25]. We found that at days 7 and 14, some of the infiltrating cells were recipient dendritic cells. In addition, there were a number of OX62-negative, recipient MHC class II-positive, and ED1-positive cells. These may have been recipient OX62-negative dendritic cells, their precursors, or macrophages.

METHODS

Animals

Male BN and L/BN rats (Harlan Sprague Dawley, Houston, TX, USA) were maintained according to guidelines of the National Institutes of Health and the University of Texas Southwestern Medical Center Institutional Animal Care and Research Advisory Committee.

Antibodies

We used murine antirat monoclonal antibodies shown in Table 1. OX3, OX6, ED1, and OX62 were purchased from Serotec (Kidlington, UK). OX33 and V65 were from Pharmingen (San Diego, CA, USA).

Table 2. Serum creatinine measurements after transplantation of BN kidneys into L/BN recipients

	Serum creatinine *mg/dl*			
Rat	Day 1	Day 3	Day 7	Day 14
JGP28	3.6	1.6	0.8	Not done
JGP29	4.0	3.1	2.3	1.0
JGP30	4.4	1.6	1.6	2.0

Bilateral native nephrectomies were performed on the recipients at the time of transplant surgery.

Renal transplantation

Male BN kidneys were transplanted into male L/BN recipients. Donors and recipients were anesthetized with intraperitoneal pentobarbital. The donor kidneys were harvested with a cuff of aorta and vena cava and perfused with 3 ml of cold (4°C) University of Wisconsin (UW) solution. End to side anastomoses of donor aorta and vena cava to recipient aorta and vena cava were performed. The donor ureter was anastomosed to the dome of the recipient bladder. Unilateral native nephrectomies were performed in the recipients The transplants were initially divided into two equal groups to determine the effects of overnight cold storage. One group of kidneys was transplanted immediately after harvest. The average vascular anastomosis time was 20 to 30 minutes, and the renal allograft was covered with saline ice during most of this time. The other group of kidneys was stored in UW solution at 4°C for 20 hours prior to transplantation. We found no difference in the immunohistochemical studies of these two groups of transplants, and the data analysis considers all of the kidneys as a single group. An additional three delayed recipients had bilateral native nephrectomies at the time of transplantation in order to evaluate renal allograft function with serial serum creatinines (Table 2).

Renal ischemia/reperfusion injury

To study the effects of renal ischemia, as opposed to renal transplantation, male L/BN rats were anesthetized with intraperitoneal pentobarbital, and the right native renal artery was temporarily occluded with a vascular clamp for 30 minutes. The left kidney was used as a control.

Immunohistology

Transplanted kidneys and native control kidneys were harvested at day 3, 7, 14, and 28 post-transplant. Ischemic and nonischemic L/BN kidneys were harvested at day 14 only. Transplanted kidneys and the native control kidneys were perfused via the recipient aorta with 25 ml of heparinized saline (10 units heparin/ml) using a perfusion pump. This removed any contaminating donor

blood from the vasculature of the kidney. Ischemic kidneys and their nonischemic controls were not perfused. Each kidney was removed and sectioned transversely. One section was snap frozen in OCT compound, and the other section was fixed in paraformaldehyde-lysine-periodate (PLP) for four hours, followed by 7% sucrose in phosphate-buffered saline (PBS) overnight and then snap frozen in OCT compound.

Eight micron sections of each kidney were cut on a cryostat and placed on glass slides and air dried. The sections were air dried and fixed in cold ethanol for 10 minutes. Sections were treated with avidin and biotin to block endogenous biotin. Primary antibodies are listed in Table 1. Tissue sections were incubated with primary antibody for 60 minutes and biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 60 minutes. Endogenous peroxidase was blocked with 0.5% H_2O_2 in methanol for five minutes. The slides were incubated in an avidin-biotin horseradish peroxidase complex (ABC peroxidase; Vector Laboratories) for 60 minutes. The color was developed with DAB (Vector Laboratories). Nuclei were counterstained with Gill's hematoxylin (Fisher, Pittsburgh, PA, USA). The slides were dehydrated in ethanol, cleared in xylene, and mounted with Permount. Tissues fixed in PLP were treated identically except that the eight micron sections were fixed in PLP instead of ethanol. All cell counts were done on fresh frozen tissue. Grading of tubular MHC class II (OX6) was done on PLP-fixed tissue because the better morphology using this fixation allowed a differentiation between interstitial and renal tubular cells.

Double staining of sections was performed on transplant tissue from days 3, 7, and 14. Fresh frozen tissue was again cut in eight micron sections and fixed in cold ethanol. The first primary antibody was OX62. The secondary antibody was the same as described earlier in this article. The avidin-biotin-complex was ABC phosphatase (Vector Laboratories) and the color was developed with Vector Blue (Vector Laboratories). Levamisole was used to block endogenous phosphatase. The same sections were stained with the second antibody using the technique described earlier in this article for single staining. The second primary antibodies were either OX6 or OX3. The nuclei were counterstained with methyl green instead of hematoxylin.

Cell counting and grading

For each antibody, a blinded observer counted the number of cells per high-power field (×40 objective), which were evenly distributed throughout the cortex. The average number of cells per high power field in a single kidney was used as a single data point in the graphs. Tubular staining was graded on a scale of 0 to 3 instead of counted. Nine low power fields (×100 objective) were graded for each kidney. As discussed earlier in this article, the average grade for each kidney was used as a single data point. The data were analyzed with analysis of variance on ranks using the software program Sigma Stat. A P value of < 0.05 was considered statistically significant.

Determination of the percentage of recipient dendritic cells in double stained sections

The total number of OX62 staining cells (blue) and the number of cells double staining for both OX3 and OX62 (blue and brown) were determined in nine high-power fields of each kidney section at days 3, 7, and 14. The ratio of double-stained cells (recipient dendritic cells) to single blue-staining cells (total dendritic cells) was calculated for each kidney and used as a single data point for each time point. The day 7 and 14 data were compared with day 3 data for statistical significance.

RESULTS

BN kidney transplants not rejected by F1 (L/BN) recipients

The F1 (L/BN) recipient was tolerant to the parental BN renal transplant. The absence of rejection was manifested as a continued decrease in the serum creatinine of recipients that had bilateral nephrectomies at the time of transplantation (Table 2). Also consistent with the absence of rejection was the decreasing inflammation in the transplanted kidneys by 28 days, as discussed later in this article.

Recipient dendritic cells immigrate into the transplanted kidneys

Figure 1 shows the time course of the appearance and then the disappearance of OX62-positive cells in the transplanted kidney. We used OX62 to show immigration of dendritic cells into the transplanted kidney [18, 29]. Although OX62 also stains gamma-delta T cells, we found no gamma-delta T cells in the kidney. At day 14, when the number of OX62-positive cells peaked (Fig. 1), we found that none of the OX62-positive cells stained with the monoclonal antibody V65, which detects such gamma-delta T cells [28]. As a positive control, we did find V65 + cells in the spleen, which does contain gamma delta T cells (Fig. 2) [28]. A number of laboratories [19–25] have confirmed the original report [18] that OX62 is a marker of dendritic cells. As further confirmation that OX62 was detecting dendritic cells, double immunostaining indicated that the OX62-positive cells were also positive for MHC class II, which is another marker of dendritic cells (Fig. 3) [29].

As shown in Figure 1, we found small numbers of OX62-positive dendritic cells on day 3 after transplant. These increased at day 7, peaked at day 14, and then

Fig. 2. Renal and splenic staining with the monocolonal antibody V65. V65 is a monoclonal antibody that identifies gamma delta T cells. (*A*) Negative staining of a donor kidney harvested at day 14 (BN donor, L/BN recipient). The white arrow shows negatively staining inflammatory cells and "g" labels a glomerulus. (*B*) Positive staining in a L/BN spleen. The arrowhead shows a splenic arteriole and the arrow shows some of the gamma delta T cell that stains positive (red) for V65. Ethanol fixation, vector red staining, hematoxylin counterstain.

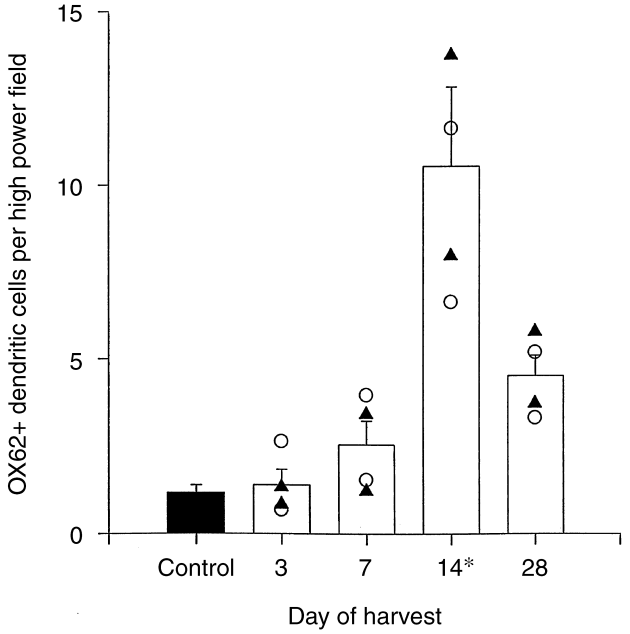

Fig. 1. Quantitative summary of OX62-positive dendritic cells in transplanted kidneys. OX62 is a monoclonal antibody that is specific for dendritic cells and gamma delta T cells. Because gamma delta T-cell staining was not detected with the monoclonal antibody V65 (data not shown), OX62 in our experiments detects dendritic cells. The x-axis is the post-transplant day and the y-axis is the number of cells that stained positively per high-power field (×400). Each circle or triangle represents a data point for an individual kidney and is the average count for nine high-powered fields. The open circles represent kidneys transplanted immediately; the triangles represent kidneys transplanted after overnight storage in UW solution. The bars and lines represent the average and standard error, respectively, for pooled immediate and stored groups of kidneys at each time point. The controls were the native L/BN kidneys harvested with the transplanted kidneys ($N = 16$ for the controls, $N = 4$ for each transplant group). $^*P < 0.05$ vs. corresponding controls.

decreased toward baseline by day 28. Note that each circle or triangle on the graph represents a single rat and is the average cell count from nine high-powered fields taken from the cortex and outer medulla. In all our graphs, the solid triangles represent kidneys transplanted after overnight storage in cold UW solution, and the open circles represent kidneys transplanted immediately. Because there was no significant difference between these groups, the data from all kidneys at a given time after transplantation were pooled; the bars represent the means and standard errors of these pooled kidneys. As controls, we used the right native kidney of the recipient, which was not removed, and was examined for dendritic cells on days 3, 7, 14, and 28 after transplant. We found no increase in dendritic cells in these kidneys compared with kidneys taken from unmanipulated rats. This indicates that the laporatomy itself did not cause dendritic cell immigration into the kidney. We did find small baseline numbers of dendritic cells in these control kidneys as previously reported [30–33].

The OX62-positive dendritic cells found in the transplanted kidneys could be derived from OX62-negative precursor cells from the donor ("passenger leukocytes"), or they could have immigrated from the recipient. To determine the origin of these dendritic cells, we double stained sections from 11 transplanted kidneys for both OX3 (recipient class II MHC) and OX62 (dendritic cell) at days 3, 7, and 14. Cells that double stained were recipient dendritic cells. By counting the number of double-staining cells per high-power field and dividing by the total number of dendritic cells per high-power field, we determined the percentage of recipient dendritic cells. As shown in Table 3, at day 3, 43% of the dendritic cells double stained with recipient-specific OX3 and dendritic-cell specific OX62. At days 7 and 14, the percentage of these recipient dendritic cells had increased to 93 and 94%, respectively. Sections were also double stained with both OX62 and OX6 (a monoclonal antibody that detects both recipient and donor class II MHC) and showed that all OX62-positive dendritic cells were also class II MHC positive. Representative photomicrographs are shown in Figure 3. In all panels of Figure 3, the blue stained cells represent OX62 (dendritic cells), and the green is the counterstained nuclei (methyl green). The brown staining is either recipient class II MHC (OX3; Fig. 3 A, B) or both recipient and donor class II MHC (OX6; Fig. 3 C, D). At day 3, there were few dendritic cells, and many of these were from the donor. Serial sections show that they were negative for OX3 (Fig. 3A, single arrow) and positive for OX6 (Fig. 3C, double arrowhead). At day 7, all the dendritic cells double stain with the recipient specific class II MHC marker (OX3; Fig. 3B, double arrows). Note that the dendritic cell infiltrate was interstitial and did not involve the glomeruli.

Infiltration of recipient MHC class II-positive, OX62-negative cells into the transplanted kidneys

We now discuss these cells in greater detail. The time course of infiltration of recipient class II MHC (OX3)-positive cells is summarized in semiquantitative fashion in Figure 4. There was a mild infiltrate at day 3 followed by a heavy infiltrate at day 7. The infiltrate had declined

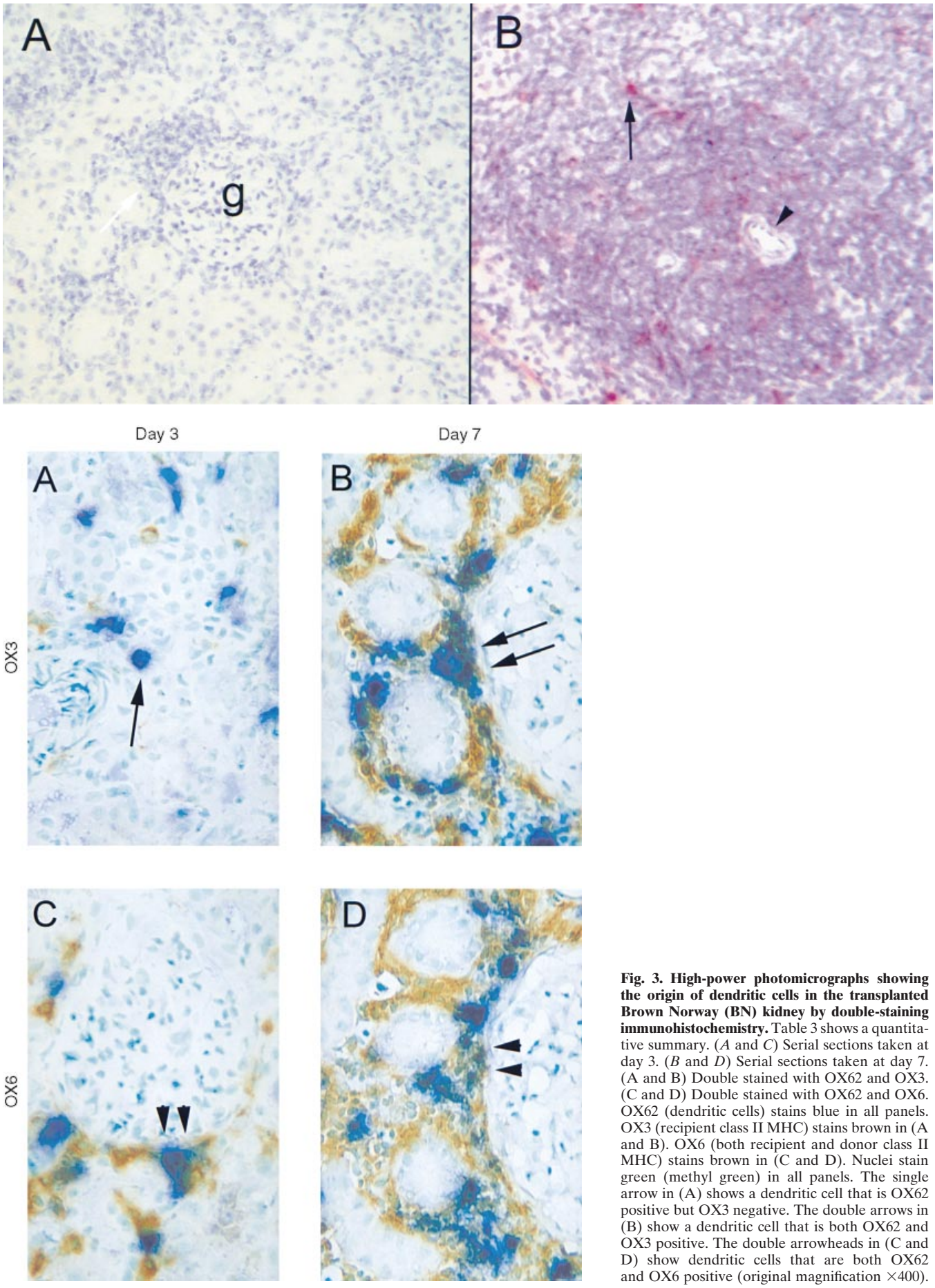

Fig. 3. High-power photomicrographs showing the origin of dendritic cells in the transplanted Brown Norway (BN) kidney by double-staining immunohistochemistry. Table 3 shows a quantitative summary. (*A* and *C*) Serial sections taken at day 3. (*B* and *D*) Serial sections taken at day 7. (A and B) Double stained with OX62 and OX3. (C and D) Double stained with OX62 and OX6. OX62 (dendritic cells) stains blue in all panels. OX3 (recipient class II MHC) stains brown in (A and B). OX6 (both recipient and donor class II MHC) stains brown in (C and D). Nuclei stain green (methyl green) in all panels. The single arrow in (A) shows a dendritic cell that is OX62 positive but OX3 negative. The double arrows in (B) show a dendritic cell that is both OX62 and OX3 positive. The double arrowheads in (C and D) show dendritic cells that are both OX62 and OX6 positive (original magnification ×400).

Table 3. Recipient dendritic cells immigrate into the renal transplant

	Day after transplantation		
	Day 3	Day 7	Day 14
Ratio[a] of recipient dendritic cells to total dendritic cells (recipient plus donor)	0.43	0.93	0.94
Number of transplanted BN kidneys	4	4	3
P value compared to day 3	—	0.00002	0.0007

Transplanted kidney sections at days 3, 7, and 14 were double stained with OX3 and OX62. Double stained cells were counted as recipient dendritic cells and compared to the total number of OX62 positive dendritic cells. See Figure 3 for photomicrographs of double-stained kidney sections.

$$^{a}\text{Ratio} = \frac{\text{Recipient dendritic cells}}{\text{All dendritic cells}} = \frac{\text{Number of cells double staining for both OX3 as well as OX62}}{\text{Total number of OX62 positive cells}}$$

by day 28. We also stained the kidneys for ED1. Although ED1 is often considered a marker for macrophages [26], it is also found on some dendritic cells [34]. The time course of infiltration of ED1 cells is shown in Figure 5 and is similar to that of recipient MHC class II-positive cells. Although B cells may be class II MHC positive, B cell (OX33) staining was absent at day 3 and barely detectable at days 7, 14, and 28 (data not shown).

We compared the staining of OX62 (dendritic cells), ED1 (macrophages and dendritic cells), OX3 (recipient class II MHC), and OX6 (recipient and donor class II MHC) in serial sections from a kidney at day 14, the time of maximal inflammation (Fig. 6). OX62 appeared to stain a subset of the ED1-positive cells. Many of the ED1-positive cells were also OX3 positive.

Altogether our data indicate that there are many OX62-negative, recipient MHC class II-positive, ED1-positive cells in the transplanted kidney. We cannot determine if these are macrophages, OX62-negative dendritic cells, or dendritic cell precursors. These cells are closely related and have common precursors [34].

Class II MHC on renal tubular cells in the transplanted kidneys

We found increased class II MHC on renal tubular cells after transplantation. Figure 6D shows that OX6, which detects class II MHC of both the donor and the recipient, stains both the donor renal tubule cells as well as the recipient interstitial inflammatory cells. In contrast, OX3 detects recipient class II MHC and stains only the recipient inflammatory cells (Fig. 6C). The interstitial staining was ignored when we graded the tubular staining for the graph in Figure 7. The staining was quantitated by grading because of the difficulty in counting individual renal tubular cells. The increased tubular class II MHC may be a response to ischemic injury because it also occurs after clamping the renal artery of native kidneys [16, 17]. Consistent with previous reports, we did find some low-level staining of class II MHC on control tubules [35].

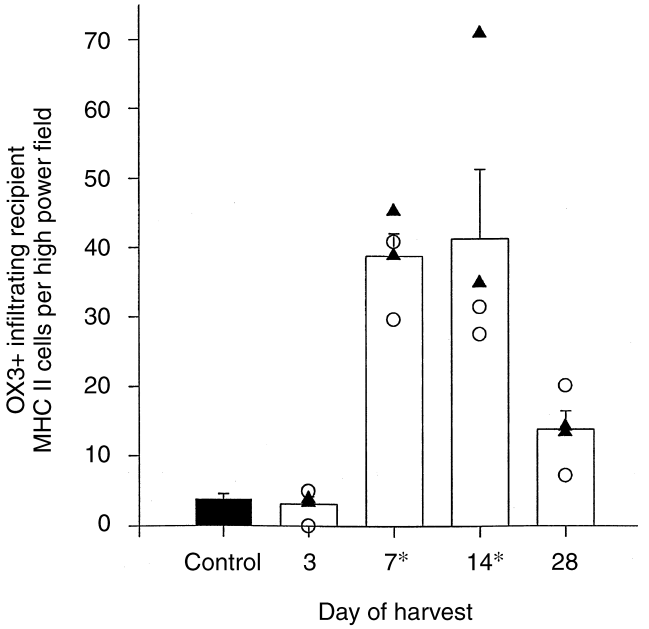

Fig. 4. Quantitative summary of recipient (L/BN) class II major histocompatibility complex (MHC)-positive infiltrating cells in the transplanted BN kidneys. OX3 is a monoclonal antibody that binds to recipient class II MHC, but not donor class II MHC. Each circle or triangle represents a data point for an individual kidney and is the average count for nine high-powered fields: (○) kidneys transplanted immediately; (▲) kidneys transplanted after overnight storage in UW solution. The bars and lines represent the average and standard error, respectively, for pooled immediate and stored groups of kidneys at each time point. The controls were the native L/BN kidneys harvested with the transplanted kidneys ($N = 16$ for the controls, $N = 4$ for each transplant group). $^{*}P < 0.05$ vs. control group. Figure 6C shows a representative photomicrograph at day 14.

Dendritic cells and macrophages are increased in native kidneys injured by ischemia/reperfusion

One explanation for the increased macrophages and dendritic cells in these transplanted kidneys is that ischemia/reperfusion injury recruited these inflammatory cells. To test this hypothesis, we temporarily occluded the right native renal artery of L/BN rats for 30 minutes; the left renal artery was not manipulated. Table 4 shows that there were increased numbers of inflammatory cells in the right native kidney 14 days after the ischemia/reperfusion injury. The infiltrate included cells that were class II MHC-positive, ED1-positive (macrophages and dendritic cells), and OX62 positive (dendritic cells).

DISCUSSION

The data in this article make two new points. First, we show that the injury of transplantation itself is sufficient to recruit MHC class II-positive recipient leukocytes

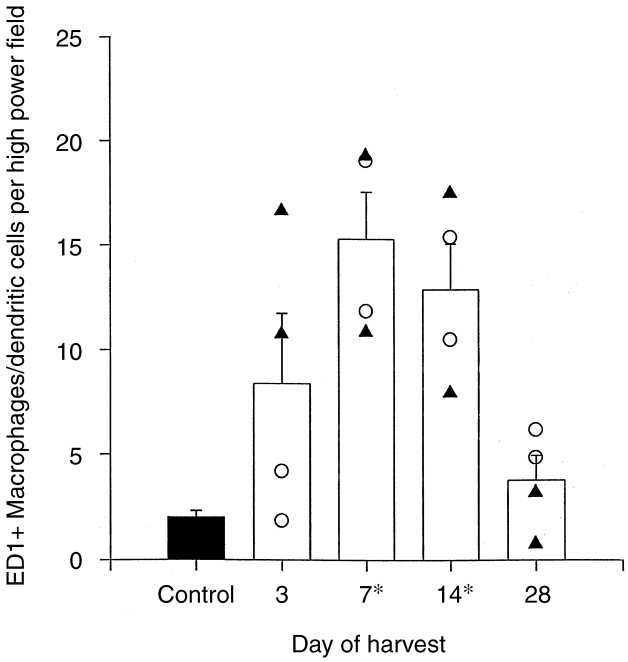

Fig. 5. Quantitative summary of ED1-positive cells in the transplanted kidneys. ED1 is a monoclonal antibody that binds to dendritic cells and mature macrophages. Each circle or triangle represents a data point for an individual kidney and is the average count for nine high-powered fields: (○) kidneys transplanted immediately; (▲) kidneys transplanted after overnight storage in UW solution. The bars and lines represent the average and standard error, respectively, for pooled immediate and stored groups of kidneys at each time point. The controls were the native L/BN kidneys harvested with the transplanted kidneys ($N = 16$ for the controls, $N = 4$ for each transplant group). Figure 6B shows a representative photomicrograph.

into the kidney in a model in which there is no rejection. To our knowledge, this is the first demonstration that injury itself recruits these leukocytes into the transplanted kidney. Second, we show that some of these recipient leukocytes may be dendritic cells because they are positive for MHC class II and for OX62. These two findings are discussed in greater detail in this section.

The injury of transplantation itself is sufficient to recruit MHC class II-positive recipient leukocytes into the transplant. Such leukocytes may activate recipient T cells via the indirect pathway of antigen presentation. They might be recruited into the transplant by cytokines, chemokines, and adhesion molecules produced during the initial rejection of alloantigens via the "direct pathway." In addition, these leukocytes may also be recruited into the transplant by the signal generated by the kidney in response to injury occurring during the transplant procedure. Our experiments were designed to test the latter possibility. In our model, the L/BN recipients should be tolerant of the transplanted parental BN kidneys. The increased numbers of recipient MHC class II leukocytes found in the kidney transplant are not due to rejection. Indeed, the improving renal function after transplant (Table 2) and the decreasing numbers of leukocytes in the transplanted kidneys after two weeks (Figs. 1, 4, and 5) are consistent with the absence of rejection. The MHC class II leukocytes are from the recipient because they react with the monoclonal antibody OX3, which detects only recipient MHC class II [15]. We also analyzed native kidneys after temporary clamping of the native renal artery (Table 4) and found an increased number of MHC class II-positive leukocytes. These data are consistent with that reported by others who also examined MHC class II-positive leukocytes after clamping the native renal artery [16, 17, 36].

The peak infiltration of recipient MHC class II-positive cells is 7 to 14 days after transplant in a strain combination in which there is no rejection, and the immigration of these leukocytes into the kidney is due only to injury (Fig. 4). However, if the recipient is not tolerant to the donor, acute rejection may start prior to this time. There are two explanations of the apparent paradox of acute rejection occurring before the peak infiltration of recipient MHC class II-positive leukocytes in response to the injury of transplant. First, the vigor and tempo of the infiltration of these leukocytes may be increased when recruitment is due both to the cytokines and chemokines released during the initial rejection of alloantigens of the "direct pathway" in addition to the injury of transplantation. Alternatively, the immigration of recipient MHC class II-positive cells may be late, and the acute rejection mediated by T cells, which are activated by these cells via indirect pathway, occurs later—after the first month. Thus, early acute rejection may involve CD4 T cells stimulated by the direct antigen-presentation pathway, whereas later acute rejection may result from CD4 T cells stimulated by the indirect pathway. This occurs in rodent models of transplantation [9, 37]. This hypothesis is also consistent with clinical observations that late acute rejection (7 to 12 months after transplant) has a different prognosis than early acute rejection [38] and that human lymphocyte antigen-DR (HLA-DR) disparities between donor and recipient, which would result in antigen presentation via the direct pathway, have less prognostic significance after the first six months post-transplant [39, 40].

Some of the previously mentioned recipient MHC class II-positive leukocytes are dendritic cells. Some of the recipient MHC class II-positive cells had surface markers consistent with their being dendritic cells. Double staining indicated that some cells were positive for two dendritic cell antigens: recipient MHC class II and OX62 (Fig. 3 and Table 3). The OX62 positivity suggests that these cells are dendritic cells. Although OX62 is found on dendritic cells and gamma-delta T cells [18], we found that our OX62-positive cells were negative for

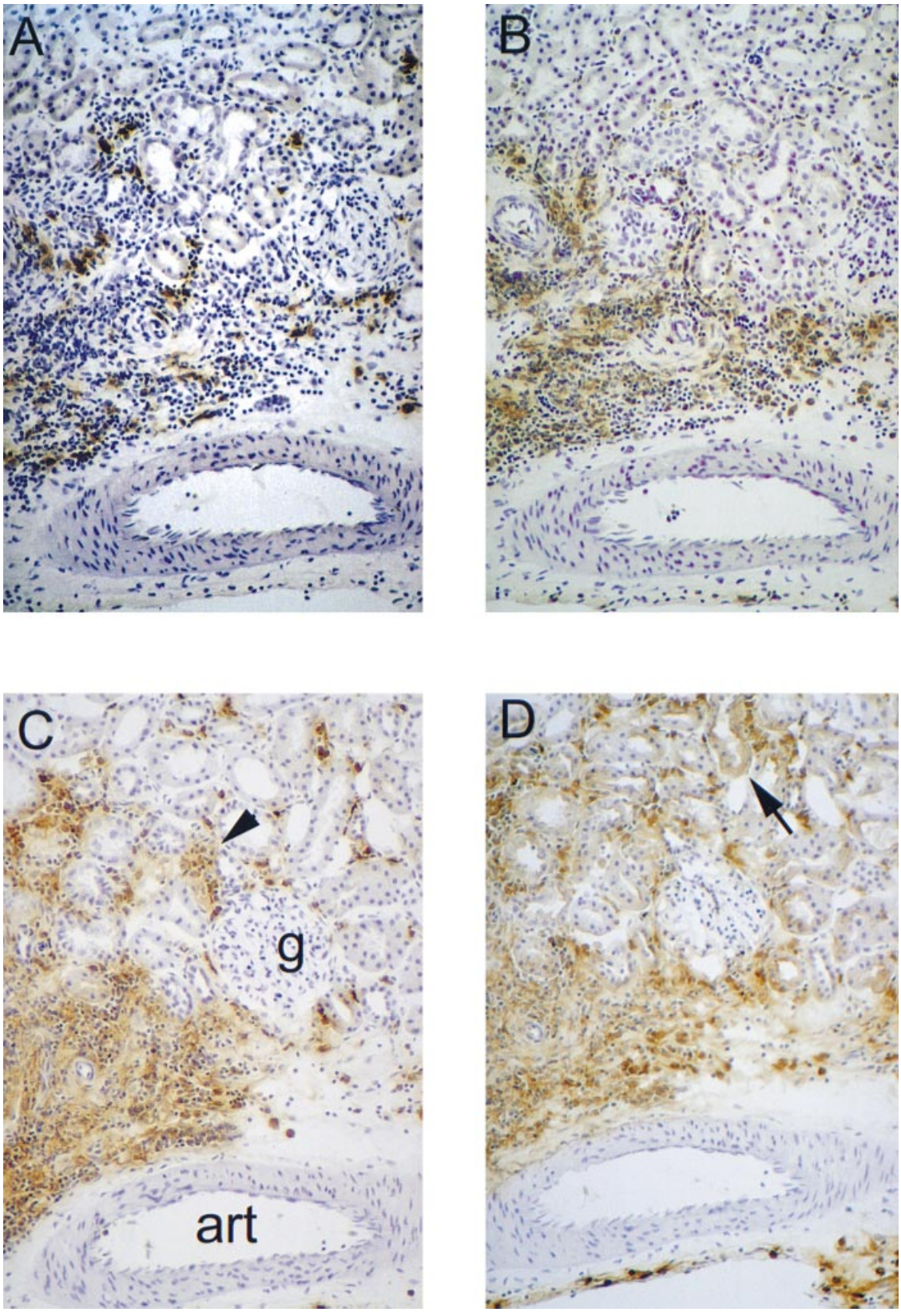

Fig. 6. Serial sections of a transplant kidney harvested at day 14. (*A*) OX62-positive dendritic cells. (*B*) ED1-positive macrophages and dendritic cells. (*C*) OX3-positive recipient-specific class II MHC staining. OX3 stains only interstitial cells (arrowhead indicates examples). A glomerulus is labeled with "g," and an arteriole is labeled with "art." D, OX6-positive class II MHC of donor and recipient. OX6 stains interstitial and tubular cells. The arrow indicates one such tubule. Sections were fixed in PLP and immunostained, and the color was developed with DAB (brown). The nuclei are counterstained blue with hematoxylin. The original magnification was ×200. (Publication of this figure in color was made possible by a grant from Fujisawa USA, Inc., Deerfield, IL.)

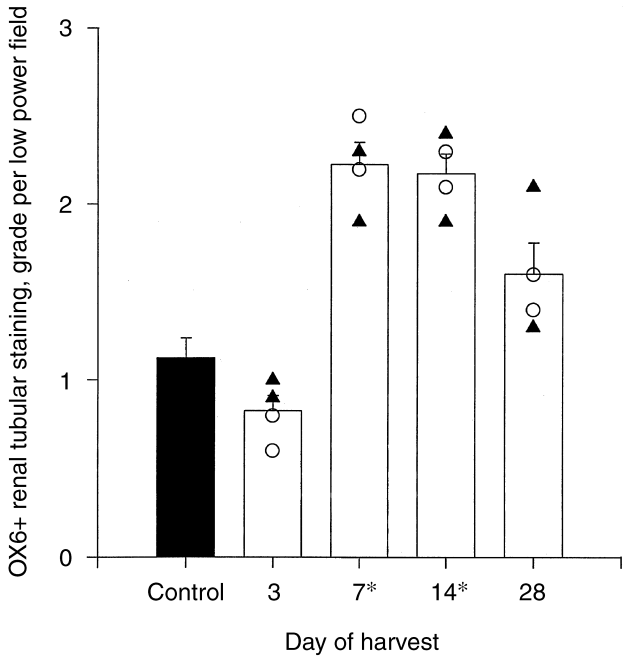

Fig. 7. Semiquantitative summary of tubular staining for class II MHC by OX6 in transplanted kidneys. OX6 binds to class II MHC of both the donor and the recipient. The graph is similar to Figure 1 except that the *Y*-axis is the average grade of tubular staining instead of cells per high-power field. Interstitial staining was ignored during grading. Each circle or triangle represents a data point for an individual kidney and is the average count for nine high-powered fields: (○) kidneys transplanted immediately; (▲) kidneys transplanted after overnight storage in UW solution. The bars and lines represent the average and standard error, respectively, for pooled immediate and stored groups of kidneys at each time point. The controls were the native L/BN kidneys harvested with the transplanted kidneys ($N = 16$ for the controls, $N = 4$ for each transplant group). Figure 6D shows a representative photomicrograph.

V65 [28], a monoclonal antibody that detects gamma-delta T cells (Fig. 2). Furthermore, an increasing literature indicates that cells that are OX62 positive behave in a manner consistent with their being dendritic cells [19–25, 41].

Many of the recipient MHC class II-positive cells in the transplanted kidneys were OX62 negative. Although B cells are MHC class II positive, few of these were found in the transplant. Instead, Figure 6 indicates that many of the MHC class II-positive, OX62-negative cells were ED1 positive. ED1 is a marker of both macrophages and dendritic cells [26]. Early during their differentiation, dendritic cells may be OX62 negative [22]. Thus, the recipient MHC class II-positive, OX62-negative, ED1-positive cells may be macrophages, dendritic cells, or precursors destined to differentiate into OX62-positive dendritic cells [22]. In any event, macrophages and dendritic cells are closely related, as they differentiate from a common precursor [29, 42].

Table 4. Quantitative measurement of cellular infiltrates in ischemic native kidneys

	OX6 + MHC II	OX62 + dendritic cell	EDI + dendritic cells or macrophages
Control	21 ± 0.4	0.5 ± 0.2	5.5 ± 1.3
L/BN ischemic kidneys	163 ± 8.3	5.2 ± 1.5	35 ± 8.8
P value	0.003	0.04	0.03

L/BN kidneys were temporarily clamped for 30 minutes and harvested at day 14. The indicated antibodies were used to stain kidney sections. The control kidneys were contralateral nonischemic kidneys. The numbers represent the average number of cells per high power field for three kidneys. The *P* values were calculated with a *t*-test comparing the ischemic kidneys to the control kidneys. There were 4 kidneys in each group.

Either recipient MHC class II-positive macrophages [43] or dendritic cells would be capable of stimulating CD4 T cells and thus contribute to the indirect pathway of antigen presentation. Increased numbers of dendritic cells may be found after tissue injury [44]. Some authors have suggested that dendritic cells are particularly potent antigen-presenting cells to naive T cells [29, 45]. Although the sensitization of naive T cells might occur in the transplant, some studies suggest that major sites of sensitization are the lymph node and spleen; sensitized T cells then migrate into the transplant [46–49]. Dendritic cells might participate in such sensitization by virtue of their ability to ingest alloantigen in the transplant and then migrate to the lymph node and spleen [29, 45, 50].

All renal allografts suffer some unavoidable injury during transplantation. This includes injury during organ harvesting, injury during cold storage and transport of the kidney, and injury during surgical creation of the vascular anastomoses between allograft and recipient. Cadaveric allografts also are injured by the hemodynamic instability associated with the trauma or acute illness that caused brain death of the donor. A growing body of data suggests the response to this injury [51–56] or the cytokine and autonomic "storm" associated with brain death (abstract; van der Heovon et al, *Transplantation* 65:95, 1998) [57, 58] may play a critical role in initiating allograft rejection. In other words, activation of an immune response, such as allograft rejection, requires not only recognition of non-self–antigen, but also tissue injury [59]. According to this hypothesis, the immune system evolved not to respond to non-self–antigens alone, but to non-self–antigens that cause injury. Ordinarily, such non-self–antigens would be pathogens that injure tissues during the process of infection. In the case of transplantation, the non-self–antigen would be the alloantigens of the allograft, and tissue injury would result from the transplant process.

To our knowledge, this is the first report that injury occurring to the kidney during transplantation itself recruits recipient MHC class II-positive leukocytes into the transplant in a model in which there is no rejection.

These leukocytes may activate CD4 T cells via the indirect pathway. Some of these leukocytes have cell surface markers—class II MHC and OX62—suggesting that they are recipient dendritic cells. This leukocyte population also includes cells that are recipient class II MHC positive, ED1 positive, and OX62 negative. These may be recipient macrophages, dendritic cells, or cells destined to differentiate into OX62-positive dendritic cells.

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REFERENCES

1. Vella JP, Knoflach A, Waaga AM, Sayegh MH: T cell mediated immune responses in chronic allograft rejection: Role of indirect allorecognition and costimulation. *Graft* 1:11–17, 1999
2. Fabre JW: The role of polymorphic donor peptides in allograft recognition and rejection. *Immunol Rev* 154:21–43, 1996
3. Batchelor JR: Transplant immunology. *Transplant Proc* 26:1875–1876, 1994
4. Muluk SC, Clerici M, Via CS, Weir MR, Kimmel PL, Shearer GM: Correlation of in vitro CD4+ T helper cell function with clinical graft status in immunosuppressed kidney transplant recipients. *Transplantation* 52:284–291, 1991
5. Molajoni ER, Cinti P, Orlandini A, Molajoni J, Tugulea S, Ho E, Liu Z, Suciu-Foca N, Cortesini R: Mechanism of liver allograft rejection: The indirect recognition pathway. *Hum Immunol* 53:57–63, 1997
6. Liu Z, Colovai AI, Tugulea S, Reed EF, Fisher PE, Mancini D, Rose EA, Cortesini R, Michler RE, Suciu-Foca N: Indirect recognition of donor HLA-DR peptides in organ allograft rejection. *J Clin Invest* 98:1150–1157, 1996
7. Ciubotariu R, Liu Z, Colvai AI, Ho E, Itescu S, Ravalli S, Hardy MA, Cortesini R, Rose EA, Suciu-Foca N: Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. *J Clin Invest* 101:398–405, 1998
8. Vella JP, Spadafora-Ferreira M, Murphy B, Alexander SI, Harmon W, Carpenter CB, Sayegh MH: Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. *Transplantation* 64:795–800, 1997
9. Vella JP, Vos L, Carpenter CB, Sayegh MH: Role of indirect allorecognition in experimental late acute rejection. *Transplantation* 64:1823–1828, 1997
10. Benham AM, Sawyer GJ, Fabre JW: Indirect T cell allorecognition of donor antigens contributes to the rejection of vascularized kidney allografts. *Transplantation* 59:1028–1032, 1995
11. Shirwan H, Leamer M, Wang HK, Makowaka L, Cramer DV: Peptides derived from alpha-helices of allogenic class I major histocompatibility complex antigens are potent inducers of CD4+ and CD8+ T cell and B cell responses after cardiac allograft rejection. *Transplantation* 59:401–410, 1995
12. Terness P, Dufter C, Otto G, Opelz G: Allograft survival following immunization with membrane-bound or soluble peptide MHC class I donor antigens: Factors relevant for the induction of rejection by indirect recognition. *Transpl Int* 9:2–8, 1996
13. Lee RS, Grusby MJ, Glimcher LH, Winn HJ, Auchincloss H Jr: Indirect recognition by helper cells can induce donor-specific cytotoxic T lymphocytes in vivo. *J Exp Med* 179:865–872, 1994
14. Auchincloss H Jr, Lee R, Shea S, Markowitz JS, Grusby MJ, Glimcher L: The role of "indirect" recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice. *Proc Nat Acad Sci USA* 90:3373–3377, 1993
15. McMaster WR, Williams AF: Identification of Ia glycoproteins in rat thymus and purification from rat spleen. *Eur J Immunol* 9:426–433, 1979
16. Shoskes DA, Parfrey NA, Halloran PF: Increased major histocompatibility complex antigen expression in unilateral ischemic acute tubular necrosis in the mouse. *Transplantation* 49:201–207, 1990
17. Shackleton CR, Ettinger SL, McLoughlin MG, Scudamore CH, Miller RR, Keown PA: Effect of recovery from ischemic injury on class I and class II MHC antigens expression. *Transplantation* 49:641–643, 1990
18. Brenan M, Puklavec M: The MRC OX-62 antigen: A useful marker in the purification of rat veiled cells with the biochemical properties of an integrin. *J Exp Med* 175:1457–1465, 1992
19. Varas A, Vicente A, Sacedon R, Zapata AG: Interleukin-7 influences the development of thymic dendritic cells. *Blood* 92:93–100, 1998
20. Oka M, Furihata C, Kitoh K, Yamamoto M, Tatematsu M, Ichinose M, Miki K, Ito T, Sakaki Y, Reske K: Involvement of dendritic cell response to resistance of stomach carcinogenesis caused by N-methyl-N′-nitro-N-nitrosoguanidine in rats. *Cancer Res* 58: 4107–4112, 1998
21. Brenan M, Rees DJ: Sequence analysis of rat integrin alpha E1 and alpha E2 subunits: Tissue expression reveals phenotypic similarities between intraepithelial lymphocytes and dendritic cells in lymph. *Eur J Immunol* 27:3070–3079, 1997
22. Chen-woan M, Delaney CP, Fournier V, Wakizaka Y, Murase N, Fung J, Starzl TE, Demetris AJ: A new protocol for the propagation of dendritic cells from rat bone marrow using recombinant GM-CSF, and their quantification using the mAb OX-62. *J Immunol Methods* 178:157–171, 1995
23. Gieseler R, Hoffmann PR, Kuhn R, Fayyazi A, Stojanovic T, Schlemminger R, Peters JH: Enrichment and characterization of dendritic cells from rat renal mesangium. *Scand J Immunol* 46:587–596, 1997
24. Chen-woan M, Delaney CP, Founier V, Wakizaka Y, Murase N, Fung J, Starzl TE, Demetris AJ: In vitro characterization of rat bone marrow-derived dendritic cells and their precursors. *J Leukoc Biol* 59:196–206, 1996
25. Matsuno K, Ezaki T, Kudo S, Uehara Y: A live stage of particle-laden rat dendritic cells in vivo: Their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. *J Exp Med* 183:1865–1878, 1996
26. Dijkstra CD, Dopp EA, Joling P, Kraal G: The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, and ED3. *Immunology* 54:589–599, 1985
27. Woollett GR, Barclay AN, Puklavec M, Williams AF: Molecular and antigenic heterogeneity of the rat leukocyte-common antigen from thermocytes and T and B lymphocytes, *Eur J Immunol* 15:168–173, 1985
28. Kuhnlein P, Park JH, Herrmann T, Elbe A, Hunig T: Identification and characterization of rat gamma/delta T lymphocytes in peripheral lymphoid organs, small intestine, and skin with a monoclonal antibody to a constant determinant of the gamma/delta T cell receptor. *J Immunol* 153:979–986, 1994
29. Austyn JM: Commentary: New insights into the mobilization and phagocytic activity of dendritic cells. *J Exp Med* 183:1287–1292, 1996

30. Kaissling B, Hir ML: Characterization and distribution of interstitial cell types in the renal cortex of rats. *Kidney Int* 45:709–720, 1994
31. Welsh KI, Batchelor JR, Maynard A, Burgos H: Failure of long surviving, passively enhanced kidney allografts to provoke T-dependent alloimmunity. II. Retransplantation of (AS × AUG) F1 kidneys from AS primary recipients into (AS × WF) F1 secondary hosts. *J Exp Med* 150:465–470, 1979
32. Gurner AC, Smith J, Cattel V: The origin of Ia antigen-expressing cells in the rat kidney. *Am J Pathol* 127:342–348, 1987
33. Hart DN, Fabre JW: Major histocompatibility complex antigens in rat kidney, ureter, and bladder: Localization with monoclonal antibodies an demonstration of Ia-positive dendritic cells. *Transplantation* 31:318–325, 1981
34. Azuma H, Nadeau K, Takada M, Mackenzie HS, Tilney NL: Cellular and molecular predictors of chronic renal dysfunction after initial ischemia/reperfusion injury of a single kidney. *Transplantation* 64:190–197, 1997
35. Mayrhofer G, Schon-Hegrad MA: Ia antigens in rat kidney, with special reference to their expression in tubular epithelium. *J Exp Med* 157:2097–2109, 1983
36. Ibrahim S, Jacobs F, Zukin Y, Enriquez D, Holt D, Baldwin W, Sanfilippo F, Ratner LE: Immunohistochemical manifestations of unilateral kidney ischemia. *Clin Transplant* 10:646–652, 1996
37. Braun MY, McCormack A, Webb G, Batchelor JR: Mediation of acute but not chronic rejection of MHC-incompatible rat kidney grafts by alloreactive CD4 T cells activated by the direct pathway of sensitization. *Transplantation* 55:177–182, 1993
38. Leggat JE Jr, Ojo AO, Leichtman AB, Port FK, Wolfe RA, Turenne MN, Held PJ: Long-term renal allograft survival: Prognostic implication of the timing of acute rejection episodes. *Transplantation* 63:1268–1272, 1997
39. Zantvoort FA, D'Amaro J, Persijn GG, Cohen B, Schreuder GM, Van Rood JJ, Thorogood J: The impact of HLA-A matching on long-term survival of renal allografts. *Transplantation* 61:841–844, 1996
40. McKenna RM, Lee KR, Gough JC, Jeffery JR, Grimm PC, Rush DN, Nickerson P: Matching for private or public HLA epitopes reduces acute rejection episodes and improves two-year renal allograft function. *Transplantation* 66:38–43, 1998
41. Nelson DJ, McMenamin C, McWilliam AS, Brennan M, Holt PG: Development of the airway intraepithelial dendritic cell network in the rat from class II MHC (Ia) negative precursors: Differential regulation of Ia expression at different levels of the respiratory tract. *J Exp Med* 179:203–212, 1994
42. Inaba K, Inaba M, Deguchi M, Hagi K, Yasumizu R, Ikehara S, Muramatsu S, Steinman R: Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. *Proc Natl Acad Sci USA* 90:3038–3042, 1993
43. Unanue ER: The concept of antigen processing and presentation. *JAMA* 274:1071–1073, 1995
44. Banchereau J, Steinman RM: Dendritic cells and the control of immunity. *Nature* 392:245–252, 1998
45. Steinman RM: The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 9:271–296, 1991
46. Imhof BA, Dunon D: Leukocyte migration and adhesion. *Adv Immunol* 58:345–416, 1995
47. Campbell JJ, Hedrick J, Zlotnik A, Siani MA, Thompson DA, Butcher EC: Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science* 279:381–384, 1998
48. Zinkernagel RM, Ehl S, Aichele P, Oehen S, Kundig T, Hengartner H: Antigen localisation regulates immune responses in a dose- and time-dependent fashion: A geographical view of immune reactivity. *Immunol Rev* 156:199–209, 1997
49. Butcher EC, Picker LJ: Lymphocyte homing and homeostasis. *Science* 272:60–66, 1996
50. Austyn JM, Larsen CP: Migration patterns of dendritic leukocytes: Implications for transplantation. *Transplantation* 49:1–7, 1990
51. Halloran PF, Homik J, Goes N, Lui SL, Urmson J, Ramassar V, Cockfiled SM: The "injury response": A concept linking nonspecific injury, acute rejection, and long-term transplant outcomes. *Transplant Proc* 29:79–81, 1997
52. Land W, Messmer K: The impact of ischemia/reperfusion injury on specific and non-specific, early and late chronic events after organ transplantation. *Transplant Rev* 10:108–127, 1996
53. Nadeau KC, Azuma H, Tilney NL: Cytokines in the pathophysiology of acute and chronic allograft rejection. *Transplant Rev* 10:99–107, 1996
54. Orosz CG, Bergese SD, Wakely E, Xia D, Gordillo GM, Vanbuskirk AM: Acute versus chronic graft rejection: Related manifestations of allosensitization in graft recipients. *Transplant Rev* 11:38–50, 1997
55. Samaniego M, Baldwin WM, Sanfilippo F: Delayed graft function: Immediate and late impact. *Curr Opin Nephrol Hypertens* 6:533–537, 1997
56. Ibrahim MAA, Chain BM, Katz DR: The injured cell: The role of the dendritic cell system as a sentinel receptor pathway. *Immunol Today* 16:181–186, 1995
57. Takada M, Nadeau KC, Hancock WW, Mackenzie HS, Shaw GD, Waaga AM, Chandraker A, Sayegh MH, Tilney NL: Effects of explosive brain death on cytokine activation of peripheral organs in the rat. *Transplantation* 65:1533–1542, 1998
58. Koo DD, Welsh KI, Roake JA, Morris PJ, Fuggle SV: Ischemia/reperfusion injury in human kidney transplantation: An immunohistochemical analysis of changes after reperfusion. *Am J Pathol* 153:557–566, 1998
59. Matzinger P: Tolerance, danger, and the extended family. *Annu Rev Immunol* 12:991–1045, 1994