Modified Dendritic Cells Coexpressing Self and Allogeneic Major Histocompatibility Complex Molecules: An Efficient Way to Induce Indirect Pathway Regulation

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Abstract. A feature of the tolerance that has been described in experimental models is that it can be transferred by CD4+ T cells to a naive recipient. Described is a novel approach to induce indirect pathway regulatory T cells in a rat model that exploits the natural processing and presentation of major histocompatibility complex (MHC) molecules as peptide by the MHC class II molecules of the same cell. Dendritic cells (DC) coexpressing donor (AUG) and recipient (LEW) MHC molecules were rendered tolerogenic by treatment with dexamethasone. After injection into LEW animals followed by a single low dose of CTLA4-Ig, T cells were rendered unresponsive to indirectly presented AUG alloantigens, but retained direct pathway responsiveness to fully allogeneic AUG cells. The T cells from the DC-injected rats were unresponsive to (LEW × AUG) F1 stimulator cells, suggesting the presence of indirect pathway regulatory cells whose activity depended on the presence of LEW MHC molecules. Depletion of CD25+ cells from the responder population led to a marked increase in proliferation, and the T cells from the DC-injected rats inhibited the response of naive LEW T cells to (LEW × AUG) F1, but not to AUG, stimulator cells, further indicating indirect pathway-mediated regulation. Most importantly, pretreatment of LEW rats with the dexamethasone-treated DC led to the indefinite survival of AUG kidney grafts after a short course of cyclosporin to inhibit the early direct pathway response. Similarly treated AUG DC had no effect, confirming the privileged status of F1 cells in the induction of indirect pathway regulation.

Major histocompatibility complex (MHC) alloantigens are recognized via two distinct pathways, referred to as direct and indirect (1–3); the relative contribution of these two pathways to allorecognition has been extensively studied (4–6). The direct pathway is responsible for the high-frequency responses that provoke the mixed leukocyte reaction in vitro (7) and that cause early acute transplant rejection (8). The indirect pathway (9) corresponds to the manner in which all conventional protein antigens are recognized by T lymphocytes—namely, the foreign MHC molecules are internalized, processed, and presented as peptides by recipient MHC molecules (10–12). In contrast to the direct pathway, the indirect pathway is characterized by low-frequency responses and is thought to play a dominant role in later, more chronic, forms of transplant rejection (13–15).

We (16) and others (17,18) have examined the evolution of these two pathways of the alloimmune response with time after clinical transplantation. Although the strength of the direct response declines with time in most patients (19,20), the indirect response has been noted to increase, selectively in patients with evidence of chronic rejection (21). These data suggest that it is the indirect pathway of the antidonor alloimmune response that poses the major threat to long-term transplant survival.

The central goal in transplantation biology, ever since Madawar’s demonstration that transplantation tolerance could be achieved by neonatal injection of donor cells (22), has been to induce robust, donor-specific tolerance using a clinically applicable protocol. Where this has been achieved in experimental rodent models, one salient feature of the tolerant state is that it can often be transferred, via T cells, to a naive host. Interestingly, given the threat posed by the indirect alloresponse, the specificity of the regulatory cells that are instrumental in maintaining and transferring tolerance appears to be for the indirect, rather than for the direct, pathway. Indeed, in a model of heart transplantation, tolerance could not be achieved in recipient animals that were able to mount a direct response, but were incapable of indirect antidonor responses, suggesting that indirect allorecognition is necessary to induce stable tolerance (23).

In this study, we describe a novel strategy for the induction of tolerance and regulation specifically in the indirect pathway. This approach was predicated on the contrasting fates of fully allogeneic versus semiallogeneic rat kidneys after retransplantation (9). When an (AS × AUG) F1 kidney was retransplanted...
from an intermediate, immunosuppressed, parental strain AS recipient into a second AS rat after 1 mo, all of the grafts were spontaneously accepted, without the need for any immunosuppression therapy. However, if the same protocol was followed using a fully allogeneic (AUG) kidney, all the retransplanted grafts were rejected, albeit at a slower pace than for a primary graft. The interpretation of those early findings, which triggered the experiments described here, is that the parenchymal cells of the F1 kidney, which coexpressed AUG (allogeneic) and AS (self) MHC molecules, had the ability to silence AS T cells with indirect anti-AUG specificity. It is well known that a substantial fraction of the peptides presented by MHC molecules are derived from other MHC molecules as a result of natural antigen processing; thus, on an F1 cell, the self MHC molecules will constitutively be displaying allogeneic MHC molecules in peptide form. Given that the tissue parenchymal cells of an allograft are poorly immunogenic as a result of inefficient costimulation, this constitutive indirect presentation may provide a mechanism for the silencing of T cells with indirect allospecificity as they traffic through the graft. On the basis of this interpretation, we have tested the ability of F1 immature DC to induce tolerance and regulation via the indirect pathway, in vitro and in vivo.

### Materials and Methods

#### Animals

Inbred Lewis (LEW, RT1-\(A\)^3), August (AUG, RT1-A^3), and Brown Norway (BN, RT1-A^3) rats were purchased from Harlan and Charles River (Bicester, UK), Hybrid (LEW × AUG)F1 rats were bred and maintained in the specific pathogen-free Biologic Services Unit at the Hammersmith Campus of Imperial College (London, UK). Male rats weighing 200 to 250 g were used for all of the experiments.

#### Recipient Pretreatment and Kidney Transplantation

Operational tolerance was induced by injecting adult LEW rats intravenously with 10^7 (LEW × AUG)F1 DC modified in vitro with dexamethasone (Dex) (Sigma, Dorset, UK). Recipient rats also received a single dose of murine CTLA4-Ig (R&D Systems, Abingdon, UK) (200 \(\mu\)g) intraperitoneally 24 h later. Orthotopic kidney transplants from AUG and BN donors were performed 10 d after dendritic cells (DC) vaccination by conventional microsurgical technique as described previously (24). All recipient rats underwent a 10-d course of immunosuppression with cyclosporin A (Novartis Pharma AG, Basle, Switzerland) at a dosage of 10 mg/kg/d administered by oral gavage. Recipients’ contralateral native kidneys were removed 7 d later. Animals were regularly inspected for survival and bled for serum urea and creatinine estimations at weekly intervals. Spleens were harvested from the tolerant rats 100 d after transplantation for in vitro alloresponses in T cell proliferation assays. Kidney allograft rejection was defined when blood urea levels were \(\approx 80\) mmol/L or when recipient animals were unwell.

Kidney allografts were harvested from tolerant rats 100 d after transplantation and on the day of rejection from control animals. Hematoxylin-eosin staining was performed on graft specimens.

#### Preparation of DC

Bone marrow–derived DC were prepared as described by Talmor et al. (25) using rat recombinant granulocyte macrophage–colony stimulating factor and IL-4 (Peprotech EC, London, UK) at a concentration of 1 ng/ml. Dex was added to the medium from day 5 of culture at a concentration of 10^\(-7\) M, and DC were harvested at day 9.

### Antibodies and Flow Cytometric Analysis

The following mouse anti-rat monoclonal antibodies were used for flow cytometry and cell isolation: anti-MHC class II (OX-6), anti-CD11c (8A2) anti-CD45 RC (OX-22), anti-CD25 (OX-39), and isotype control mouse IgG2a were obtained from Serotec (Kidlington, UK). Anti-CD4, anti-CD80 and anti-CD86 were purchased from Pharmingen (BD Biosciences, Oxford, UK). Anti-MHC class I (OX-18) was purified from supernatants of a B cell hybridoma purchased from the European Collection of Animal Cell Culture (ECACC, London, UK), F17-23-2 anti-MHC class II antibody was purified from hybridoma culture supernatant (kindly provided by Prof. Fabre, Kings College, London, UK), isotype control mouse IgG1, and FITC-conjugated sheep anti-mouse IgG were obtained from Sigma. Dex-treated and mature DC were stained with the appropriate antibodies and analyzed in a Becton Dickinson FACS-Calibur running CellQuest software.

#### Two-Stage Culture for Anergy Induction

T cells from naive LEW rats were purified by using rat T cell enrichment columns (R&D Systems) following the protocol provided by the manufacturer. Responder T cells (2 × 10^5/well) were cocultured in 24-well plates with either Dex-treated or mature (LEW × AUG)F1 DC (2 × 10^5/well), treated with 30 Gy X-irradiation. After 4 d, the T cells were purified by isolation on a Lympho-Sep gradient (Oxford Biotechnology, Oxford, UK) and rested for 24 h in complete medium. In the second stage, recovered T cells were rechallenged (2 × 10^5/well) with the same (F1) or third-party (BN) irradiated splenocytes (4 × 10^5/well). After 3, 5, and 7 d, cells were pulsed with 1 \(\mu\)Ci 3H-Thymidine (TdR) (Amersham, Buckinghamshire, UK) for the last 18 h of culture. Proliferation was measured as 3H-TdR incorporation by liquid scintillation spectroscopy. Results are expressed as mean cpm ± SEM of triplicate cultures.

### Induction of Indirect Pathway T Cell Tolerance In Vivo and Indirect Alloresponse Assay In Vitro

#### Assays to Reveal the Induction of Regulatory T Cells In Vivo

T cells from animals immunized with (LEW × AUG)F1 mature DC were rechallenged in the indirect pathway assay before and after depletion of CD25^+ cells with a Dynabead technique (Dynal, Merseyside, UK), according to the manufacturer’s instructions. T cell proliferation was measured by 3H-TdR incorporation on days 3, 5, and 7 after culture.
derived from DC-treated animals. After 5 d, T cell proliferation was measured and expressed as described above. In this experimental system the possibility that regulatory T cells were self MHC class II restricted was analyzed by blocking the T cell proliferation with either F17-23-2 or OX-6 monoclonal antibodies.

Statistical Analyses

Statistical analysis was performed by Mann-Whitney U test. The graft survival was assessed in the experimental groups by a nonparametric ANOVA by Kruskall-Wallis test. A value of P < 0.05 was considered statistically significant.

Results

Dex-Treated DC Induce Allospecific Nonresponsiveness

Bone marrow–derived DC from (LEW × AUG)F1 donors were used in all of the experiments after 8 to 9 d of culture. Dex was added to the culture at day 5 when DC were fully differentiated but in an immature state. As shown in Figure 1, Dex-treated DC expressed low levels of MHC classes I and II, and of the costimulatory molecules CD80 and CD86, compared with mature, nontreated DC.

Untreated and treated DC were tested for their capacity to stimulate a primary T cell alloresponse in vitro. In contrast with the mature DC, the Dex-treated DC were unable to induce a primary T cell alloresponse by LEW T cells (Figure 2a). Moreover, modified DC induced allospecific hyporesponsiveness to (LEW × AUG)F1 splenocytes, as demonstrated in the two-stage culture system (Figure 2b). Figure 2b (i) shows that T cells cultured in the presence of allogeneic Dex-treated (LEW × AUG)F1 splenocytes were significantly inhibited in a titratable manner by the addition to the culture of an equal number of T cells from Dex-treated, DC-treated animals (Figure 4d). In contrast, when AUG stimulators were used, no inhibition was seen (Figure 4e). These results further indicate that the regulatory cells induced by this approach could suppress direct pathway anti-AUG T cells, and suggest that indirect presentation of AUG (RT1-A) MHC molecules was required to activate the regulatory T cells from tolerized animals. T cells from naive animals were unable to inhibit the primary alloresponse induced by F1 or AUG stimulators (data not shown).

To determine whether the regulatory cells in rats injected with Dex-treated DC were restricted by self MHC class II molecules, we exploited the specificity of an anti–RT1-A monoclonal antibody, F17-23-2, that recognizes LEW (RT1-A) but not AUG (RT1-A) MHC molecules. Addition of F17-23-2 to the cocultures of naive and Dex-treated cells significantly amplified the anti-AUG response (Figure 4f). In contrast, the OX-6 monoclonal antibody, which recognizes all rat MHC class II alleles, caused further inhibition (Figure 4g).
Figure 1. Freshly isolated bone marrow derived dendritic cells (DC) exhibit an immature phenotype when cultured in the presence of dexamethasone (Dex). Bone marrow cells were cultured for 9 d in RPMI 10% FCS with 1 ng/ml GM-CSF and IL-4. Dex was added at day 5 of culture. Cells were labeled with the designed antibody and then with FITC-labeled sheep anti-mouse IgG. Mean fluorescence intensity expressed as arbitrary units is represented in each panel. Shown is a representative of six experiments.
Injection of Dex-Treated (LEW × AUG)F1 DC Caused Indefinite Survival of AUG Kidney Allografts

To investigate whether Dex-treated DC were able to induce operational tolerance to donor alloantigens in vivo, we analyzed kidney allograft survival in LEW recipients under different conditions. To prevent rejection due to the direct alloresponse, all recipients underwent a short course (10 d) of immunosuppression with cyclosporin A. Results in Figure 5 show that indefinite survival of AUG kidney grafts was achieved in LEW recipients injected with Dex-treated (LEW × AUG)F1 DC. Furthermore, serum urea and creatinine measurements revealed excellent graft function (data not shown), and histologic analysis of the kidneys from tolerant animals showed normal tubular and glomerular morphology with no evidence of chronic vascular rejection (Figure 5ii, a). In contrast, we observed a diffuse infiltrate of mononuclear cells and signs of tubulitis in the rejected grafts (Figure 5ii, b and c).

Notably, no prolongation of graft survival was seen in rats injected with Dex-treated AUG DC. Rejection occurred at between 20 and 30 d ($P < 0.05$), confirming the privileged role of F1 DC in the induction of indirect pathway regulation. Finally, graft prolongation was donor specific, as third-party BN kidneys were acutely rejected in animals injected with Dex-treated (AUG × LEW)F1 DC.

To further characterize the tolerance achieved in the animals treated with Dex-treated F1 DC, T cells, purified from spleens, were harvested from the recipients 100 d after transplantation and rechallenged in vitro in an indirect pathway assay, or were
cocultured in a proliferation assay with (AUG × LEW)F1 or AUG splenocytes as stimulators. Results from one representative animal are shown in Figure 6. T cells from tolerant animals did not proliferate to AUG antigens presented indirectly by LEW APC. Tolerance induced in the indirect alloresponse was antigen-specific, as demonstrated by a preserved T cell response to BN antigens and third-party stimulators (Figure 6, a and c). A primary response to AUG and BN indirectly presented antigens was detected for T cells from a naive animal (Figure 6b).

The response of T cells from tolerant animals was also severely diminished to F1 splenocytes and partially reduced to fully allogeneic AUG splenocytes (P < 0.05) (Figure 6c) as compared with the response observed when T cells were obtained from a naive animal (Figure 6d). This finding suggests that the prolonged residence of the AUG kidney graft had induced a degree of direct pathway hyporesponsiveness.

**Discussion**

The key observations made in the study presented here were that Dex-treated, immature DC obtained from F1 hybrids induced allospecific regulatory cells with indirect specificity for the expressed MHC alloantigens, that these regulatory cells were capable of inhibiting direct and indirect pathway T cells, and that they resulted in indefinite survival of fully mismatched kidney grafts after short course immunosuppression. The value of these results is that they demonstrate that indirect pathway tolerance can be achieved using a clinically applicable protocol.

A substantial literature has accumulated in recent years concerning the tolerogenic properties of immature DC (26, 27). Clearly it is desirable to treat the immature DC in some way that “freezes” the cells in the immature state and that prevents their maturation after injection in vivo for pretransplant immunotherapy. It has been shown that chemically modified DC are resistant to further differentiation in response to maturation stimuli. Of the many treatments that have been described (28–33), we found that Dex was the least toxic agent that had the desired phenotypic and functional effects. The low toxicity may be an important consideration, bearing in mind the fate of the injected cells, as discussed below.

Part of the protocol used in our model was to inject a low dose of the costimulation blocking fusion protein, CTLA4-Ig.
Figure 4. Dexamethasone (Dex)-treated dendritic cells (DC) induce in vivo a subset of allospecific and self-restricted regulatory T cells. The whole population of T cells (diamonds) or CD25⁺ depleted (squares) (2 × 10⁵/well) from rats immunized with Dex-treated (LEW × AUG)F₁ DC were rechallenged in a proliferation assay with LEW DC (10⁴/well) pulsed with AUG (a), BN (b), and LEW (c) antigens derived from multiple cycles of freezing and thawing of their respective splenocytes, to detect in vitro the indirect allospecific T cell response. T cells (2 × 10⁵/well) from rats immunized with (LEW × AUG)F₁ Dex-treated DC were cocultured with naive T cells (2 × 10⁵/well) in the presence of DC (10⁴/well) from either (LEW × AUG)F₁ (d) or AUG (e) rats. The ratio of T cells from immunized rats is shown at the bottom of each column. Self MHC restriction of regulatory T cells induced in vivo by (LEW × AUG)F₁ Dex-treated DC was tested by blocking their activity with an allospecific antibody in the same experimental conditions described in Panels a and b, using F1 DCs as stimulators. F17-23-2 monoclonal antibody (f) interacts with RT1-A¹ strains (LEW) but not with RT1-A² (AUG), OX 6 (g) recognizes MHC class II determinants of both strains. The plates were incubated for 3, 5, and 7 d (a, b, c) or 5 d (d through g). ³H-TdR was added for the last 18 h. Data are expressed as mean counts per minute (cpm) ± SEM of triplicate cultures ×10⁻³. Similar results were obtained in three independent experiments.
1 d after the DC injection. This was based on both theoretical and empirical grounds. One of the limitations of DC as tolerogens is that it is difficult to control their fate in vivo. It is clear from the work of Inaba and colleagues (34) that injected DC are efficiently taken up and re-presented by recipient DC. This runs the risk of reversing the potential tolerance-promoting effects of the injected cells themselves. The CTLA4-Ig was administered to avoid the confounding effects of presentation of the alloantigens expressed by the injected DC by recipient DC. This theoretical concern was justified by the finding that pretreatment with Dex-treated (LEW × AUG)F1 DC without the CTLA4-Ig failed to prolong the survival of AUG kidney grafts (data not shown). Given the recent finding that CTLA4-Ig can induce indoleamine-2,3-dioxygenase (IDO) release by DC (35), it is conceivable that this mechanism also contributed to the effects of injecting this fusion protein.

One striking feature of the results obtained using this protocol was that the regulation was selectively induced in the indirect pathway. This was evidenced by the in vitro assays; although marked hyporesponsiveness was observed when

Figure 5. Onset of kidney rejection. (i) Kidney graft survival in Lewis (LEW) rats injected iv with (LEW × AUG)F1 dexamethasone (Dex)-treated dendritic cells (DC) 10 d before receiving a kidney transplant from August (AUG) rats (n = 5) (diamonds). Control animals were injected with mature (LEW × AUG)F1 DC (n = 5, solid squares) or AUG Dex-treated DC (n = 5, open squares) followed by a single injection of CTLA4-Ig or CTLA4-Ig only (n = 5, x) 10 d before receiving AUG kidney graft. To test the specificity of DC vaccination, kidney grafts were isolated from Brown Norway (BN) third-party controls and transplanted in LEW recipients previously injected with (LEW × AUG)F1 Dex-treated DC followed by a single administration of CTLA4-Ig (n = 5, circles). All recipient rats underwent a 10-d short-course immunosuppression with 10 mg/kg/d cyclosporin A. (ii) (a) LEW rat injected with (LEW × AUG)F1 Dex-treated DC before receiving AUG kidney. Kidney histology at 100 d after transplantation. The kidney shows normal tubular and glomerular morphology. An interlobular artery (inset, *) appears normal with no evidence of chronic vascular rejection. (b) LEW rat injected with AUG Dex-treated DC before receiving AUG kidney. Kidney histology at 27 d. There is a diffuse interstitial infiltrate of mononuclear inflammatory cells with tubulitis indicative of severe acute rejection. (c) LEW rat injected with (LEW × AUG)F1 Dex-treated DC before receiving BN kidney. Kidney histology at 28 d. There is tubular damage associated with a widespread mononuclear cell infiltrate and focal tubulitis indicative of acute rejection.
LEW DC were pulsed with AUG cell lysates (Figure 3) or (LEW × AUG)F1 DC were used as stimulator cells, T cell responses to fully allogeneic AUG stimulator cells were unaffected (data not shown). These T cells were rechallenged in a proliferation assay either with autologous dendritic cells (DC) (10^4/well) expressing allogeneic (AUG), syngeneic (LEW), or third-party (BN) antigens (indicated within each graphs in Panels a and b), as described in Materials and Methods, or with fully (AUG), semiallogeneic (LEW × AUG)F1, and third-party (BN) splenocytes (indicated within each graphs in Panels c and d). Culture plates were harvested on days 3, 5, and 7 to detect response with primary versus secondary kinetics. [^-3]H-TdR was added for the last 18 h. Results are expressed as the mean counts per minute (cpm) ± SEM for triplicate cultures × 10^-3. The stimulator cells used in the assays are indicated within each graph. The data shown are from one tolerant animal and are representative of five independent experiments, which gave similar results.

**Figure 6.** Indirect allospecific T cells from tolerant rats do not proliferate, and direct allospecific T cells from the same recipients are hyporesponsive on rechallenge *in vitro* with the alloantigen. T cells (2 × 10^5/well) were purified from the spleen of rats with a long-term kidney allograft survival (a, c) or from naive Lewis (LEW) rats (b, d). These T cells were rechallenged in a proliferation assay either with autologous dendritic cells (DC) (10^4/well) expressing allogeneic (AUG), syngeneic (LEW), or third-party (BN) antigens (indicated within each graphs in Panels a and b), as described in Materials and Methods, or with fully (AUG), semiallogeneic (LEW × AUG)F1, and third-party (BN) splenocytes (indicated within each graphs in Panels c and d). Culture plates were harvested on days 3, 5, and 7 to detect response with primary versus secondary kinetics. [^-3]H-TdR was added for the last 18 h. Results are expressed as the mean counts per minute (cpm) ± SEM for triplicate cultures × 10^-3. The stimulator cells used in the assays are indicated within each graph. The data shown are from one tolerant animal and are representative of five independent experiments, which gave similar results.

the initial direct anti-AUG response. This interpretation is consistent with the thesis promoted by Wells et al. (36): that a wave of deletion is a necessary prerequisite for tolerance. Indeed, we have recently argued that deletion and regulation are highly complementary and interdependent mechanisms in the induction and maintenance of tolerance to MHC-mismatched allografts (37).

One other key feature of the regulatory cells induced by the Dex-treated DC was that they exhibited “linked suppression” (38). This was most clearly shown when F1 cells were used to stimulate the T cells from the F1 DC-treated recipients. Although these animals contained abundant numbers of T cells capable of responding to AUG MHC alloantigens via the direct pathway, these T cells were inhibited when stimulator cells...
coexpressing AUG and LEW (self) MHC types were used. That this was the result of stimulation of indirect pathway regulatory cells, presumably regulating direct pathway responder T cells, was confirmed by the loss of inhibition seen when the F17-23-2 antibody (specific for LEW but not AUG RT1-A alleles) was added to the cultures (Figure 5c).

In conclusion, these data demonstrate that pharmacologically modified immature DC that coexpress self and donor MHC class II molecules are able to induce tolerance in the indirect pathway of the alloimmune response *in vitro* and *in vivo*. Furthermore, when this approach is combined with short-term immunosuppression, it can culminate in indefinite transplant survival. Interestingly, the effects of the modified DC include the expansion of regulatory T cells with specificity for the indirect antidonor alloresponse. Although these findings need further investigation in other experimental models, the approach has obvious clinical applicability in living donor organ transplantation and could represent an important step forward in the pursuit of clinical transplantation tolerance.

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### References


