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Targeting MHC Class I Monomers to Dendritic Cells Inhibits the Indirect Pathway of Allorecognition and the Production of IgG Alloantibodies Leading to Long-Term Allograft Survival

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T cell depletion strategies are an efficient therapy for the treatment of acute rejections and are an essential part of tolerance induction protocols in various animal models; however, they are usually nonselective and cause wholesale T cell depletion leaving the individual in a severely immunocompromised state. So far it has been difficult to selectively delete alloreactive T cells because the majority of protocols either delete all T cells, subsets of T cells, or subpopulations of T cells expressing certain activation markers, ignoring the Ag specificity of the TCR. We have developed a model in which we were able to selectively deplete alloreactive T cells with an indirect specificity by targeting intact MHC molecules to quiescent dendritic cells using 33D1 as the targeting Ab. This strategy enabled us to inhibit the indirect alloresponse against MHC-mismatched skin grafts and hence the generation of IgG alloantibodies, which depends on indirectly activated T cells. In combination with the temporary abrogation of the direct alloresponse, we were able to induce indefinite skin graft survival. Importantly, the targeting strategy had no detrimental effect on CD4+CD25+ FoxP3+ T cells, which could potentially be used as an adjunctive cellular therapy. Transplantation tolerance depends on the right balance between deletion and regulation. For the former this approach may be a useful tool in the development of future tolerance induction protocols in non-sensitized patients. The Journal of Immunology, 2010, 184: 1757–1764.

The ability of the immune system to distinguish between self and non-self is the driving impetus for allograft rejection. This process is directed by T lymphocytes, which recognize graft MHC alloantigens directly and indirectly and subsequently orchestrate a response that is rapid, specific, and ultimately leads to the destruction of the allograft. The inhibition or abrogation of this early T cell activation is pivotal to induce a state of tolerance against the allograft (1). Anatomically tolerance can be divided into central and peripheral compartments. Central tolerance involves deletion of self-reactive T cells during thymic development and the generation of CD4+CD25+ regulatory T cells (2–4). Peripheral tolerance ensures that self-reactive T cells that escape central tolerance checkpoints remain innocuous in peripheral organs mainly by mechanisms of regulation, deletion, and anergy (5, 6). Currently, there is considerable interest in harnessing strategies of peripheral tolerance for the induction of transplantation tolerance. Finkelman et al. (7) introduced the concept that targeting dendritic cells (DCs) in vivo in a quiescent state can lead to tolerance rather than active immunity. DCs are the sentinels of the immune system, and by sampling their environment, they acquire and integrate information that initiates and guides the adaptive immune response. DCs can be distinguished by various cell surface markers and differ in their origin and specialized function. Generally they are divided into plasmacytoid DCs and conventional/classical DCs (cDCs) (8, 9). On the basis of surface markers and intrinsic function mouse cDCs of splenic origin consist of two major subsets: one subset is CD4+CD8−CD205+ and the other is CD4+CD8+DC inhibitory receptor-2 (DCIR2)−. Although both subsets can activate CD4+ and CD8+ T cells, CD205+ DCs are more suited to activating CD8+ T cells, whereas DCIR2+ DCs preferentially activate CD4+ T cells (10–15).

Delivering Ags specifically to cDCs via DEC-205 (targeted at CD205) or 33D1 (targeted at DCIR2) leads to effective presentation by MHC class I and class II molecules, respectively (11, 14–16). This is followed by a transient activation and proliferation of Ag-specific T cells, which are subsequently deleted. Besides this mere deletion, there is evidence to suggest that targeting cDCs can also induce/expand regulatory T cells (17–20), which might contribute to unresponsiveness upon further challenge. The usefulness of this concept was shown in experimental mouse models of type 1 diabetes where targeting of autoantigens to CD205 could prevent the onset and progression of CD4+ and CD8+ T cell-mediated disease (17, 21).

Although the above results are promising, it is difficult to translate these findings into the field of transplantation tolerance. First, despite the fact that these studies showed an increase in percentage and absolute numbers of regulatory T cells, they failed to show the induction of a state of dominant tolerance/linked suppression in vivo. Hence, it is possible that the observed effects are the result of the deletion of effector T cells and the relative increase in CD4+CD25+ regulatory T cells played only a minor role in preventing disease. In organ transplantation, there is the added complexity that the

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recipient's T cells respond to donor MHC molecules that are presented by two distinct routes, direct and indirect (22).

On the basis of these reports, we decided to develop a transplant model where we targeted cDCs with either a single MHC class I-derived allopeptide or the complete MHC class I molecule. We reasoned that in the case of efficient-linked suppression, the delivery of a single allopeptide could be sufficient to induce tolerance to skin grafts. However, if deletion is the main mechanism of tolerance, delivery of an entire MHC class I molecule would be necessary to induce tolerance in a single MHC class I-mismatched model.

**Materials and Methods**

**Mice, culture media, reagents, and Abs**

Female C57BL/6 (B6; H2b) and (H2bd), 6–8 wk old, were purchased from Charles River Laboratories (Margate, U.K.). C57BL/6-Tg(TcrαTcrβ) TCR75Rpb (TCR75), which are Thy1.1+ and RAG−/−, C57BL/6-Tg(Kd) Rpb (B6.Kd), and B6.KdF1 mice were generated by Dr. P. Bucy and coworkers and have been described previously (23). The animals were bred and maintained in the Biological Services Unit of King’s College London. Mice were kept under sterile conditions. Mouse handling and experimental procedures were conducted in accordance with national and institutional guidelines for animal care and use. The Kd peptide (QEGPEYWEEQTQRAK; EZBiolab) is an immunodominant epitope of the α1-chain of the class I molecule Kd. The TCR75 T cells have an indirect specificity for the Kd peptide restricted by Ab (24).

**Preparation of mouse T cells and adoptive transfer experiments**

Spleen and lymph nodes were harvested from female TCR75 mice (H2b+) and were passed through a cell strainer and CD4+ T cells were prepared by using Dynal CD4 Negative Isolation Kit (Dynal Biotech). The survival of transferred TCR75 T cells and the development of CD4+CD25− regulatory T cells were determined by staining 5 × 10⁶ cells for CD4, Thy1.1, CD25, and FoxP3. For blocking of CTLA-4 and programmed death ligand 1 (PD-L1), experiments were performed as above. Additionally, mice received 500 μg relevant Ab or its isotype on days −1, 0, +1, and +5. Mice were culled on day +14 and pooled lymph nodes and the spleen were enriched for CD4+ T cells as described above.

**T cell proliferation assays**

For in vitro proliferation assay of nonsensitized animals 2 × 10⁶ CD4+ T cells from adoptively transferred animals were stimulated with 2 × 10⁵ gamma-irradiated CD4+CD8−depleted splenocytes from B6 mice. Kd+ peptide was added at 0, 1, and 10 μg/ml, and proliferation was assessed by [³H]thymidine incorporation for the last 18 h of 4-d culture in 96-well plates.

**Generation of conjugates**

Kd+ peptide conjugate. For conjugate formation the Kd+ peptide was modified by adding an extra cystein (Cystein[C]-QEGPEYWEEQTQRAK; EZBiolab, Carmel, IN) residue at the N-terminal end. For conjugate formation, 1 mg/ml 33D1 was incubated overnight at 4°C with the cross-linker Sulfo-SMCC (molar ratio 1:5, no-Weigh format; Thermo Scientific, Watford, U.K.). The mixture was then spun through a desalting column (Thermo Scientific, Cramlington, U.K.) to remove any free Sulfo-SMCC and incubated with the modified Kd+ peptide (molar ratio 1:5) for 1 h at room temperature. To remove any unbound Kd+ peptide, the product was finally spun through a centrifugal filter unit with a membrane cutoff of 100 kDa (Millipore, Watford, U.K.). The concentration of the protein was measured by using a Pierce bicinchoninic acid protein assay (Thermo Scientific) and concentrated at 0.5 mg/ml. Every batch of generated conjugate was checked for relevant endotoxin contamination by injecting naive mice with 50 μg of the conjugate. The absence of any upregulation of CD80 and CD86 24 h after injection was necessary for any further experiments.

Kd+ monomer conjugate. A modified protocol as previously described (25, 26) was used to generate Kd+ monomer conjugates. In brief, 33D1 at 2 mg/ml was avidinated at a molar ration of 1:5 according to the manufacturer's guidelines (Innova Biosciences, Cambridge, U.K.). It was then mixed with the biotinylated Kd+ monomer (m ≈ 60 kDa), which was provided by Dr. J. Fry (ProImmune, Oxford, U.K.), at a ratio of 1:1.

**FIGURE 1.** Injection of the 33D1 Ab into mice does not lead to deletion of CD11c+CD4+ cDCs, and its chemical modification does not change its specificity. A, Diagrammatic representation of the targeting Ab, note that the number of conjugated Ag is arbitrarily chosen. B, Three groups of B6 mice (n = 4/group) were injected with 200 μl of saline, 500 μg of rat IgG2b isotype/200 μl of saline, or 500 μg of 33D1 Ab/200 μl of saline. Forty-eight hours later, the mice were culled, and the distribution of the CD4+ and CD8+ DC subsets within the gated CD11c+ population of the spleen was determined. The experiment was repeated twice with similar results; one representative result is shown. C, B6 splenocytes (3 × 10⁶) were resuspended in 600 μl of supplemented RPMI 1640 medium and kept on ice throughout the experiment. First, they were incubated for 30 min with no Ab, a rat IgG2b isotype, the unconjugated 33D1 Ab, or the 33D1-Kd+ peptide conjugate. Next, the cells were washed three times in cold RPMI 1640 medium and then stained for 20 min with 33D1-PE or isotype-PE to visualize the specific binding during the first incubation. The experiments were repeated twice with similar results; one representative result is shown.
In vivo sensitization and in vitro readout

Naive B6 animals were treated with saline, 10 μg of Kd peptide or 10 μg 33D1-Kd peptide and 10 d later were sensitized by the injection 100 μg Kd peptide/IFA into the hind leg of these animals. Eight days later, the draining inguinal and popliteal lymph nodes were recovered. The Kd peptide-specific response was tested by stimulating 2 × 10^6 resuspended lymph node cells with 10 μg/ml Kd peptide, and the proliferation was assessed by [3H]thymidine incorporation for the last 18 h of 3-d culture in 96-well plates.

Flow cytometric analysis

All flow cytometry acquisition was conducted on a BD Biosciences FACSCalibur running CellQuest software (BD Biosciences, Oxford, U.K.). For analysis we used FlowJo software (Tree Star, Ashland, OR). For surface staining, 5 × 10^6 cells were incubated with saturating concentrations of appropriate Abs for 30 min at 4˚C in the dark and then washed twice in cold FACS buffer (PBS with 1% [v/v] FCS and 0.01% [w/v] sodium azide) before analysis. FoxP3 staining was performed according to the manufacturer’s guidelines.

Skin transplants

Skin transplants were performed as described previously (27). In brief, full-thickness tail skin grafts were transplanted onto the dorsal thorax of recipient mice and secured by gauze and a plaster for 7 d. Rejection was defined as >90% necrosis of graft tissue.

Detection of IgG alloantibodies

Sera from skin transplant recipients and untransplanted controls were analyzed for the presence of Kd-specific IgG Abs. B6.Kd spleenocytes were incubated with PBS/2% FCS/5% goat serum containing rat Abs against CD16/32 (0.5 μg/10^6 cells; BD Biosciences) for 20 min on ice. Hamster anti mouse CD3-PE (BD Biosciences) was then added directly to the cells to enable subsequent gating on CD3+ T cells. After further incubation for 20 min on ice cells were washed twice in staining buffer. Recipient’s serum or control was added (5 μL/well; final dilution 1/10) to appropriate wells and incubated as before. Cells were washed twice before the addition of goat anti-mouse IgG FITC (1/200; Sigma-Aldrich, Dorset, U.K.) for 20 min on ice. Cells were washed again twice, resuspended in 1% paraformaldehyde, and analyzed on a BD Biosciences FACSCalibur running CellQuest software (BD Biosciences).

FIGURE 2. Targeting of DCs with alloantigen conjugated to the 33D1 Ab leads to the specific deletion of Ag-specific T cells and to a suppressed response in a subsequent Ag challenge. A, Three groups of B6 mice (n = 3/group) were adoptively transferred with 8 × 10^6 TCR75 CD4+ T cells. Twenty-four hours later, the mice received 200 μl of saline, 10 μg of isotype-Kd peptide/200 μl of saline, or 10 μg of 33D1-Kd peptide/200 μl of saline. Two weeks later, the mice were culled. B, The removed spleen and lymph nodes were enriched for CD4+ T cells. The cells were stained for CD4, Thy1.1, CD25, and FoxP3 to determine the percentage of surviving TCR75 CD4+ T cells and the development of regulatory T cells. C, Experiments as performed in A and B; upper panel shows percentage of Thy1.1 FoxP3+CD4+ T cells out of all Thy1.1 CD4+ T cells as summary of two independent experiments with n = 3/group/experiment; groups were compared using a Student t test. *p < 0.05. Lower panel shows absolute numbers of Thy1.1 FoxP3+CD4+ T cells 14 d after adoptive transfer with or without conjugate; presented result is representative for two independent experiments with n = 3/group/experiment. D, Two groups of B6 mice (n = 3/group) were adoptively transferred with 4 × 10^6 TCR75 CD4+ T cells. Twenty-four hours later, the mice received 200 μl of saline or 10 μg of 33D1-Kd peptide/200 μl of saline. Two weeks later, the mice were culled, and the removed spleen and lymph nodes were negatively selected for CD4+ T cells. CD4+ and CD8+ depleted B6 splenocytes (2 × 10^5) were cultured in 96-well plates with 2 × 10^5 CD4+ cells from each experimental group. Synthetic Kd peptide at a final concentration of 10 μg/ml was added to half of the cultures. Cultures were maintained for 96 h with [3H]thymidine added for the last 18 h of the culture. E, Three groups of B6 mice (n = 3/group) were i.v. injected with 200 μl of saline, 10 μg of Kd peptide/200 μl of saline, or 10 μg of 33D1-Kd peptide. Ten days later, all mice were sensitized with 100 μg of Kd peptide/IFA into the hind leg. Eight days after sensitization the mice were culled and the draining lymph node cells resuspended and tested for Kd peptide specificity by [3H]thymidine incorporation. All experiments were done twice with similar results, one representative result is shown.
for CD4+ T cells and stained for CD4 and Thy1.1 to determine the survival. Cullage were culled on day 14. Pooled lymph nodes and splenocytes were enriched and then Kd peptide-specific deletion was induced by injecting 10 µg of 33D1-Kd peptide/200 µl of saline (day 0). Each group was divided into two subgroups (n = 30 subgroup), and the subgroups then received either 500 µg of aCTLA-4 or isotype Ab on days −1, 0, +1, and +5. Animals were culled on day 14. Pooled lymph nodes and splenocytes were enriched for CD4 T cells and stained for CD4 and Thy1.1 to determine the survival of TCR75 T cells. B. Same as in A, instead of aCTLA-4, aPD-L1 and its appropriate isotype was used. The experiments were repeated twice with similar results, one representative result is shown.

**FIGURE 3.** CTLA-4 and PD-L1 do not play a role in the deletional process of Ag-specific T cells. A. Two groups of B6 mice (n = 6/group) were adaptively transferred with 4 × 10^6 TCR75 CD4+ T cells (day −1) and then Kd peptide-specific deletion was induced by injecting 10 µg of 33D1-Kd peptide/200 µl of saline (day 0). Each group was divided into two subgroups (n = 3 subgroup), and the subgroups then received either 500 µg of aCTLA-4 or isotype Ab on days −1, 0, +1, and +5. Animals were culled on day 14. Pooled lymph node and splenocytes were enriched for CD4 T cells and stained for CD4 and Thy1.1 to determine the survival of TCR75 T cells. B. Same as in A, instead of aCTLA-4, aPD-L1 and its appropriate isotype was used. The experiments were repeated twice with similar results, one representative result is shown.

**Statistical analysis**

All statistical tests were performed using GraphPad Prism version 4.0b for Macintosh (GraphPad Software, San Diego, CA; www.graphpad.com). The applied statistical tests are noted in the figure legends or Results, and a value of p < 0.05 was deemed significant.

**Results**

**Injection of the 33D1 Ab into mice does not lead to deletion of CD11c^{hi}CD4+ DC and its chemical modification does not change its specificity**

Various studies have been performed investigating the potential role of DC-targeted delivery of Ags with or without the combination of TLR ligands. We chose 33D1 (rlg22b) as our model Ab, which binds DCIR2 on conventional DCs. It has been shown that CD11c^{hi}CD4+DC4*DCIR2* DCs primarily interact with CD4+ T cells, which are central to transplant rejection. Next, we conjugated the chemically modified immunodominant epitope Kd^{A,a,b} (Kd) peptide of the hypervariable region of the α1 domain of the class I molecule Kd to the 33D1 Ab (Fig. 1A) as described in Materials and Methods. To reduce any possible loss of Ag specificity as a result of promiscuous conjugation, we used a low ratio between Ab and conjugate (1:5). Because rlg22b Abs have deleting properties when used in vivo (28), we injected mice with high doses (500 µg) of intact 33D1 Ab. To assess possible deletion, we determined the ratio of CD4+ and CD8+ splenic CD11c^{hi}DCs and used the percentage of CD11c^{hi}CD4+ DCs as a surrogate marker for CD11c^{hi}CD4*DCIR2* DCs (11). As demonstrated in Fig. 1B, 48 h after injection of 33D1 Ab, the ratio between CD4+ and CD8+ DCs did not change, and the injection also had no influence on the percentage of CD11c^{hi} DCs recovered from splenocytes (data not shown). Therefore, we can conclude that 33D1 has no deleting properties when used in vivo. A novel rlg22b Ab against mouse CD40 (29), which is expressed on cDCs, was also not able to induce deletion in a recent report supporting the notion that cDCs can be resistant to Ab-mediated deletion. To verify that conjugating peptide to the Ab did not alter its binding specificity we performed a competitive binding assay in which we first incubated splenocytes with the conjugated 33D1 Ab and then second with 33D1-PE. As compared with the unmanipulated Ab, the conjugated Ab was still able to completely abolish the binding of 33D1-PE (Fig. 1C), proving that the conjugation technique used in this study did not alter the specificity of the Ab.

**Targeting of DC with allopeptide conjugated to the 33D1 Ab leads to the deletion of alloantigen-specific T cells and to a suppressed response in a subsequent antigenic challenge**

Using the Kd peptide, we took advantage of TCR75 RAG−/− mice, which carry a transgenic TCR specific for the Kd peptide restricted by Aβ. TCR75 T cells can be traced because they express the congenic marker Thy1.1. Of note is the fact that although TCR75 mice are on a RAG−/− background, they can still harbor low but detectable numbers of CD4+CD25*FoxP3* T cells (Fig. 2A). This gave us the additional opportunity to investigate how previously unstimulated naturally occurring regulatory T cells behave when stimulated by quiescent DCs. In line with published reports (15, 16), the injection of 10 µg of conjugated Ab (33D1-Kd peptide) led to the profound deletion of TCR75 T cells (92.56 ± 4.02% SD; p < 0.005; n = 10) when adoptively transferred into B6 animals (Fig. 2A, 2B and data not shown). Next, we determined the expression of the regulatory markers CD25 and FoxP3 14 d after the injection of the conjugated Ab. We could show that among the remaining Thy1.1+ T cells there was an increased percentage of CD4+CD25*FoxP3* T cells (Fig. 2B); however, at the dose used, there was no difference in the absolute numbers of Thy1.1+CD4*CD25*FoxP3* T cells between the individual groups as previously shown (19). This result suggests that naturally occurring regulatory T cells have a survival advantage when they encounter Ag presented by quiescent CD11c^{dh}CD4+ DCs, whereas effector T cells are deleted.

To further assess the responsiveness of the surviving Thy1.1+ T cells after treatment with the 33D1-Kd peptide conjugate, we enriched spleen and pooled lymph node cells for CD4+ T cells and stimulated them with Kd peptide-pulsed APCs in vitro. The proliferation data in Fig. 2D confirms the expected hyporesponsiveness of the remaining T cells. To substantiate our data in a nontransgenic system, we tolerized naive B6 animals with either 10 µg of Kd peptide or 10 µg of 33D1-Kd peptide conjugate. Ten days later, the animals were sensitized with Kd peptide in IFA. After 8 additional days, the draining lymph nodes were explanted, resuspended in RPMI 1640 medium, and tested for Kd peptide-specific proliferation. Again, the pretreatment with 33D1-Kd peptide inhibited the sensitization against the Kd peptide in this nontransgenic model and was as efficient as peptide induced hyporesponsiveness (Fig. 2E) (30).

**Neutralizing Abs against CTLA-4 and PD-L1 do not prevent the deletion of alloantigen-specific T cells**

Reports by Probst et al. (31) suggested that signaling via CTLA-4 and PD-L1 might be involved in the deletional process of Ag-specific CD8+ T cells when they encounter Ag presented by quiescent DCs. To address this issue we adoptively transferred TCR75 T cells into B6 animals and treated them with the 33D1-Kd peptide conjugate. To block signaling via CTLA-4 (Fig. 3A) or PD-L1 (Fig. 3B), the animals additionally received 500 µg of neutralizing Abs against CTLA-4 [clone UC10-4F10-11 (32)], PD-L1 [clone 10F.9G2 (33)], or the corresponding isotype control on days −1, 0, +1, and +5. The injection of those blocking Abs had no significant impact on the capacity of 33D1-Kd peptide conjugate to induce deletion of TCR75 T cells. Therefore, we concluded that signaling via CTLA-4 and PD-L1 are not essential.
transplants. Additionally, one allogeneic transplant group received 20 μg of 33D1-Kd peptide at a final concentration of 10 μg/ml was added to half of the cultures. Cultures were maintained for 96 h with [3H]thymidine added fort the last 18 h of the culture. D. Three groups of B6 mice (H2b, n = 5/group) received either no treatment, 20 μg of 33D1-Kd peptide/200 μl of saline, or 20 μg of 33D1-Kd monomer/200 μl of saline. Fourteen days later, all mice received a B6.Kd skin transplant (H2d-Kd), and transplant survival was monitored daily. E. Four groups of B6 mice (n = 5/group) received either syngeneic (B6) or allogeneic (B6.Kd) skin transplants. Additionally, one allogeneic transplant group received 20 μg of 33D1-Kd monomer/200 μl of saline 14 d before the transplantation, and unmanipulated B6 animals served as a control. The development of Kd specific IgG Abs was measured 4 wk after transplantation by flow cytometry and is expressed as the mean fluorescence intensity using sera from individual mice against B6.Kd T cells and detecting allospecific Abs by using a FITC-conjugated goat anti-mouse-IgG Ab.

For the deletion of Ag-specific CD4+ cells, we cannot totally exclude the possibility that, despite the large amount of neutralizing Ab used, signaling via these two ligands still occurs. Kd monomer conjugated to the 33D1 Ab can induce deletion of Ag-specific T cells and inhibit the generation of IgG alloantibodies against Kd.

Because the Kd peptide represents only a single immunodominant epitope out of the complete Kd molecule, our strategy so far solely deleted effector T cells against this epitope without expanding the corresponding regulatory T cell pool. This approach would only have a limited application in transplantation given the multiple epitopes derived from a single MHC molecule. To delete all possible effector T cells against Kd, we generated a conjugate, which consisted of the 33D1 Ab linked to a complete Kd monomer connected via an avidin-biotin interaction (33D1-Kd monomer; Fig. 4A) (25). This approach should ensure that the targeted DCs present all possible epitopes from Kd restricted by Ab. To test this assumption, we adoptively transferred B6 mice with TCR75 CD4+ T cells by stimulating them with autologous APCs pulsed with the Kd peptide in vitro (Fig. 4C). Although we could only see a 50% deletion at the low dose of the 33D1-Kd monomer conjugate (2 μg), we were not able to detect a proliferative response upon rechallenge in vitro (Fig. 4C). This might indicate that the observed deletion in the high dose group (20 μg) is preceded by a state of anergy.

Next, we performed skin grafts by transplanting full-thickness tail skin from B6.Kd mice to the flank of B6 mice after we had treated them with the 33D1-Kd peptide conjugate or the 33D1-Kd monomer conjugate or left them untreated. Neither the pretreatment with the 33D1-Kd peptide conjugate nor with the 33D1-Kd monomer conjugate led to a significant prolongation of survival of the transplanted B6.Kd skin (Fig. 4D). We concluded that regardless of the high percentage of CD4+CD25+FoxP3+ T cells (Fig. 2) we could not promote a state of dominant tolerance by targeting DCs with alloantigens. Furthermore, we reasoned that the injection of the conjugate would only deplete T cells with an indirect specificity but would not be able to delete CD8+ T cells with a direct specificity for Kd, which could account for the observed rejection. To demonstrate the deletion of CD4+ T cells with an indirect specificity with the 33D1-Kd monomer, we performed a further set of transplants and measured the appearance of IgG alloantibodies.
against Kd. It has been shown that the production of IgG alloantibodies by B cells depends on indirectly activated CD4+ T cells (34, 35). We were able to demonstrate the development of an anti-Kd IgG alloantibody response 4 wk after transplantation (B6.Kd → B6), which could be suppressed by the pretreatment of mice with the 33D1-Kd monomer conjugate. Collectively, we had sufficient evidence that the pretreatment with the 33D1-Kd monomer conjugate prevented the development of an efficient indirect allosresponse.

**Abrogation of the direct CD8+ allosresponse together with 33D1-Kd monomer induces indefinite skin graft survival**

Having identified CD8+ T cells with a direct specificity as a possible mediator of rejection in our model, we combined the 33D1-Kd monomer and 33D1-Kd peptide conjugate pretreatment with the weekly depletion of CD8+ T cells for 4 wk. By using this approach, we were able to induce indefinite graft survival in the majority of animals who were treated with the 33D1-Kd monomer conjugate in combination with anti-CD8 (Fig. 5A), whereas the depletion of CD8+ T cells in combination with or without 33D1-Kd peptide did not lead to a significant prolongation. The finding that the combination of the 33D1-Kd peptide and anti-CD8 did not prolong transplant survival implies that the Kd-specific regulatory T cells, which should be still present after the treatment with the 33D1-Kd peptide (Fig. 2B, 2C), are not sufficient to suppress CD4+ effector T cells specific for Kd-derived epitopes other than Kd.

The fact that some animals who received deleting Abs against CD8 in combination with the 33D1-Kd monomer still rejected could be either explained by a failure of anti-CD8 to totally deplete all circulating CD8+ T cells, by the few surviving CD4+ T cells, which were still able to mount an indirect allosresponse in vivo, or by a combination of both. To discriminate between these possibilities, we used B6.Kd2-M+ mice as donor mice. Because these animals lack β2-microglobulin, their cells are not able to express MHC class I molecules on their surface and cannot stimulate CD8+ T cells, whereas their DCs are still able to directly present the Kd peptide and stimulate TCR75 T cells (23). As shown in Fig. 5B, skin transplants from these animals placed on B6 recipients led to indefinite graft survival in all animals after the pretreatment with the 33D1-Kd monomer confirming that the direct allosresponse by CD8+ T cells must be suppressed to permit indefinite survival of skin grafts in our model.

**Discussion**

The presented data provide a proof-of-concept study showing that it is possible to target quiescent DCs with intact MHC molecules to inhibit the development of an indirect allosresponse and the production of IgG alloantibodies. These two effects can have a major impact on tolerance induction protocols. Recent clinical and experimental data has shown that alloreactive B cells and alloantibodies induce local complement activation and result in poor graft survival (36, 37). In addition alloantibodies might form immune complexes with shed alloantigen. These are preferentially taken up by APCs via their Fc or complement receptors, eventually leading to their maturation and potent stimulation of alloreactive T cells (38, 39). A study by Noorchashm et al. (40) confirmed that B cells themselves can effectively act as APCs and are an important contributor to T cell activation and allograft rejection in a murine cardiac allograft model. This work was extended by Burns et al. (41), who showed that preformed alloantibodies caused resistance to tolerance induction protocols. The avoidance of alloantibody production and memory B cell formation would also reduce the risk of Ab-mediated rejection (42, 43). Indirectly activated CD4+ T cells play a key role in the activation of allo-specific B cells and IgG alloantibody production in transplantation (34, 35). We were able to inhibit the indirect interaction between B cells and CD4+ T cells by selective deletion of T cells with an indirect allospecificity, leaving B cells without the necessary help.

The finding that the neutralizing anti–CTLA-4 or –PD-L1 Abs do not play a role in interfering with the deletion of Ag-specific T cells appears to contradict the recent report by Probst et al. (31). However, although their study investigated the mechanisms by which CD8+ T cells are deleted (31), we describe the deletion of Ag-specific CD4+ T cells. The assumption that the peripheral deletion of CD4+ and CD8+ T cells has different requirements is supported by data from the recent report by Haspot et al. (44). In their model of mixed bone marrow chimeras, the authors could show that only the peripheral deletion of alloreactive CD8+ T cells but not of alloreactive CD4+ T cells depended on the interaction between programmed death-1 and PD-L1. Currently, the mechanisms involved in the peripheral deletion of mature CD4+ T cells remain elusive. Apoptosis during negative selection in the thymus is mediated by the proapoptotic Bcl-2 family member Bcl-2 interacting mediator of death (Bim) (45). Bim has also been shown to be essential for the superantigen staphylococcal enterotoxin B-induced deletion of mature Vβ8+ T cells (46) or in the termination of an acute immune response (47). Further studies will be needed to test the role of Bim in the deletional properties of quiescent DCs and to determine whether extrinsic death ligands, such as members of the TNF superfamily (e.g., CD95) are pivotal (48).

Although we were not able to suppress the direct response the abrogation of the indirect allosresponse has a wider implication. CD4+ and CD8+ T cells recognize the allograft primarily by either directly interacting with intact MHC molecules on donor cells (direct pathway) or by interacting with recipient DCs which present donor-derived allopeptides (indirect pathway). The high frequency of T cells with direct allospecificity and the relatively low frequency of T cells with indirect specificity in the normal T cell repertoire has historically led to the concept that the direct allosresponse dominates the early posttransplant period and then...
gradually wanes off with the eventual death and removal of migrating donor APCs. In contrary, the indirect pathway starts slowly, requiring Ag capture and processing, gains momentum, and continues as long as the graft is in situ and donor Ags are processed and presented by recipient APCs (49–51). On the basis of this, there is a strong correlation between long-term graft function and outcome and the frequency of T cells with an indirect allospecificity (52, 53). In support of this assumption, ELISPOT analysis of recipient T cells prospectively performed after murine skin transplantation showed a decline of allospecific T cells with direct specificity over time. Most importantly, these CD4+ T cells became incapable of allograft rejection once MHC class II-expressing donor leukocytes were no longer present (24, 54). However, T cells with an indirect specificity showed an increase in frequency, especially in the graft and developed superior effector function over time compared with T cells with direct specificity (24, 54). Human studies from our own group and others have confirmed this inverse relationship between the direct and indirect allopeptides (52, 55, 56). Collectively, these observations suggest that the importance of the direct pathway diminishes with time after transplantation and makes T cells with an indirect specificity the main targets of any tolerance induction protocol (57, 58). Against this backdrop, it is important to note that the conjugate used in this study had no deleterious effect on regulatory T cells, which could therefore be combined with this strategy to regulate the direct allopeptide. Finally, it might be advantageous to combine the targeting of DCIR2 and CD205 because the former regulates the direct alloresponse. Furthermore, it might be advantageous to T cells, which could therefore be combined with this strategy to promote experimental transplantation tolerance. J. Exp. Med. 209: 3395–3401.


