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MHC Alloantigens Elicit Secondary, But Not Primary, Indirect In Vitro Proliferative Responses

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The relative contributions of direct and indirect pathways of allorecognition to graft rejection remain controversial. Recent reports suggest that the indirect pathway may play a prominent role in both acute and chronic allograft rejection. Such studies suggest that MHC-derived allopeptides are more immunogenic than those derived from minor histocompatibility or other nominal Ags. The aim of this study was to characterize the immunogenicity of MHC alloantigens in MHC-defined miniature swine via primary and secondary MLR culture assays. APCs were selectively depleted from either responder or stimulator cell populations to specifically analyze direct and indirect proliferative responses, respectively. Radio-resistant cytokine secretion and subsequent backstimulation of responder cells was eliminated by using stimulators that were either lysed or unresponsive to the responder MHC haplotypes. When the effect of backstimulation was eliminated from MLR culture assays, indirect proliferative responses were not observed among naïve responders. Only after in vivo priming of responder animals could indirect proliferation be detected. These data do not refute the potential importance of indirect allorecognition in graft rejection. However, they suggest that MHC-derived alloantigens behave similarly in vitro to minor histocompatibility Ags, with comparable immunogenicity. These data also suggest that the MLR culture assay does not accurately reflect the importance of indirect mechanisms that have previously been reported in experimental models of graft rejection. A greater understanding of the indirect pathway and the associated immunogenicity of MHC allopeptides has the potential benefit of enabling the development of therapeutic interventions to prevent or halt allograft rejection. The Journal of Immunology, 2002, 169: 3613–3621.

Host responses to a transplanted organ can involve both direct and indirect pathways of alloantigen recognition. There is an abundance of experimental evidence to suggest that both mechanisms are involved in the process of allograft rejection, but the relative contributions of each to this process, or the interplay that may exist between them, has not been precisely defined. In the “direct” pathway, host T cells are stimulated by intact allogeneic MHC molecules on the surface of donor APCs (1). Conversely, allogeneic MHC Ags can be processed into peptides and presented by host APCs to responding T cells via the “indirect” pathway of alloantigen recognition (2, 3). In the past, experimental data have emphasized the direct pathway as the predominant mechanism mediating antigrant cytotoxic T cell responses and subsequent graft rejection. This emphasis has been substantiated by a number of in vivo findings which include the following: 1) the importance of MHC Ag matching in prolonging allograft survival; 2) the prolongation of allograft survival via depletion of donor APCs (4); and 3) the restoration of passenger cell-depleted renal graft immunogenicity by addition of donor strain dendritic cells (5).

The contribution of the indirect pathway to allorecognition and to both acute (6) and chronic (7, 8) forms of allograft rejection has also been validated by numerous studies. One pertinent example is that of Auchincloss et al. (9), who reported the rapid rejection of skin allografts derived from MHC class II-knockout mice, thereby demonstrating that indirect recognition alone can be an effective pathway of alloreactivity in this small animal model. The Ag-specific, tolerogenic effect of MHC allopeptide administration in the rat model, reflected by suppression of proliferative and cytotoxic responses in vitro, provided additional experimental evidence for the importance of indirect allorecognition (10).

Recent evidence indicates that processed MHC allopeptides may be more potent stimulators of an indirect immune response than minor Ags based on their capacity to elicit primary indirect proliferative responses in vitro (11). Using a water lysis technique to remove viable stimulating cells and thus eliminate direct allore cognition in vitro, Gould and Auchincloss (11) compared the indirect response to MHC-derived allopeptides with those from minor histocompatibility Ags in MLR culture assays. A primary indirect response (proliferative) against allogeneic MHC peptides, but not against minor histocompatibility Ags, was observed.

Understanding further the mechanism of indirect recognition and the underlying immunogenicity of MHC alloantigens may facilitate the development of interventional strategies to interrupt this pathway and prolong allograft survival. Therefore, in the present study, we have chosen a partially inbred miniature swine model to examine further, via MLR culture assays, the contribution of indirect responses to alloreactivity across selective MHC (swine leukocyte Ag; SLA) and minor histocompatibility barriers. 8

1 Abbreviations used in this paper: SLA, swine leukocyte Ag; PAEC, porcine aortic endothelial cell; MLEC, mixed lymphocyte-endothelial cell culture; SI, stimulation index; F/T, freezing and thawing.
Massachusetts General Hospital miniature swine used in these experiments provide a unique opportunity to reproducibly perform studies across selective MHC disparities in a preclinical large animal model (12). By selectively depleting APCs from the responder and stimulator cell populations, we were able to compare and contrast the proliferative responses observed in the direct vs indirect pathway, respectively, across various combinations of SLA-mismatched responder/stimulator combinations. However, given that IL-2 secretion by stimulating cells is partially radioresistant (13, 14), several experimental measures were taken to avoid backward stimulation of responder cells which could lead to falsely elevated stimulation indices for the indirect pathway.

Materials and Methods

Animal

MGH MHC-inbred miniature swine were used in these experiments. The immunogenetic characteristics of this herd and intra-MHC recombinant haplotypes have been described previously (12, 15).

Isolation of PBMC

Heparinized whole blood was drawn from miniature swine and diluted with HBSS (Life Technologies, Gaithersburg, MD), and mononuclear cells were obtained by gradient centrifugation using lymphocyte separation medium (Organon Teknika, Durham, NC). The mononuclear cells were washed once with HBSS before the contaminating red cells were lysed with ACK Lysis buffer (BioWhittaker, Walkersville, MD). Cells were then washed in HBSS again and resuspended in MLR medium consisting of RPMI 1640 (Life Technologies) supplemented with 6% fetal porcine serum, 100 U/ml penicillin (Life Technologies), 135 µg/ml streptomycin (Life Technologies), 50 µg/ml gentamicin (Life Technologies), 10 mM HEPES (Fisher Scientific, Pittsburgh, PA), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (BioWhittaker), and 5 × 10⁻³ M 2-ME (Sigma-Aldrich, St. Louis, MO). Cell suspensions were kept at 4°C for up to 48 h before use in cellular assays.

APC depletion

APCs were depleted from the PBMC by a two-step procedure. During the first step, 120 × 10⁶ cells were incubated at 37°C in 20 ml standard MLR media (16) on 75-cm plastic flasks (Falcon no. 3023; Beckson Labware, Lincoln Park, NJ) for 3–4 h. Nonadherent cells were then collected and replenated at 37°C for an additional 3–4 h. The remaining nonadherent cells were then adjusted to 3 × 10⁶ in 2 ml MLR media and incubated on 1.2 g of sterile nylon wool (Fenwal Laboratories, Deerfield, IL) at 37°C for 1 h. The cell-containing nylon wool was then rinsed with 8 ml of sterile nylon wool (Fenwal Laboratories, Deerfield, IL) at 37°C for up to 24 h until usage.

Mixed lymphocyte reaction

MLR assays, to test for proliferative response to alloantigen, have been described previously (17). Briefly, 4 × 10⁵ responders and an equal number of irradiated (25 Gy) stimulators were incubated in 200 µl of standard MLR media using flat-bottomed 96-well plates (Costar, Cambridge, MA). After 5 days of incubation, one µCi of [³H]thymidine was added to each well, followed by an additional 5-h incubation. For time series experiments, MLR plates were incubated for 1, 2, 3, 4, and 5 days before harvest. [³H]-incorporation was determined in triplicate samples by liquid scintillation. Stimulation indices were calculated by dividing the average cpm of the experiment represented in Fig. 5A. Background counts for this experiment were 583 ± 80 cpm, still <10% of the relevant back stimulation demonstrated by that figure.

Porcine aortic endothelial cell (PAEC) line transduced with allo-class II

PEDSV.15, an SV40-transformed SLAΔ PAEC clone, was derived and maintained in culture as previously described (18). Retroviral constructs used in this study included the pBPM14 recombinant genome which contained cDNA sequences for the pig DRα and β-polypeptide chains of the SLAα allele together with that of the neomycin resistance. Internal ribo-
some entry sequences derived from the Ig H chain binding protein and the encephalomyocarditis virus were intercalated between class II cDNA to ensure coordinate translation from a single transcript (19). The second construct (N2) contained the neomycin resistance gene cloned into the Moloney retrovirus backbone (20). Cells were transduced according to published protocols (21), and selected in G418 (400 µg/ml active; Medi-atech, Herndon, VA) for 10–14 days for further use in in vitro assays. The presence of retroviral transcripts in transduced cells was analyzed by Northern blot analysis of electrophoresized total RNA probed with radio- active NeoR or DRβ cDNA fragments.

Mixed lymphocyte-endothelial cell culture (MLEC)

MLECs using SLA class II-transduced PAEC were performed to test for recognition of processed allogeneic SLA class II peptides presented by self-SLA class II molecules. MLECs were performed as described above for MLR cultures, using APC-depleted responders and irradiated, responder-matched PAECs (cell concentration: 1 × 10⁶/well) as stimulators and incubating for a total of 4 days.

In vivo sensitization

For secondary proliferation assays, cells were obtained from animals that were sensitized to allogeneic SLA. Animals were grafted with fresh split thickness skin grafts (40 × 40 × 2.2 mm) that were harvested from donors using a Zimmer dermatome and placed on graft beds, also prepared with a dermatome, on the lateral thorax as previously described (4). Skin grafts were allowed to reject. The day of rejection was defined as the time at which <10% of the skin graft showed signs of viability as judged by color, texture, and warmth to touch. Every 3–5 mo, animals were reimmunized by s.c. injection of 2 × 10⁶ PBMC (from skin-graft donor) in 2 ml HBSS.

In vivo tolerance induction

To avoid backstimulation in some assays, stimulator cells were obtained from animals that had beentolerized to the MHC of the responder used in the MLR. The tolerizing regimen consisted of fully SLA-mismatched kidney allograft along with a 12-day course of continuous i.v. FK506 starting on the day of transplant (22). FK506 trough levels were measured and maintained between 40 and 60 ng/dl. FK506-treatment was discontinued 12 days after the transplant, with no additional immunosuppressive treatment provided. Tolerance was defined on the basis of graft survival beyond the 100 days with normal organ function and no pathological evidence of rejection.

Flow cytometric analysis

Flow cytometry was used to detect APCs in PBMC before and after APC depletion and for detection of SLA class II on transduced PAECs. APCs were detected by one-color, indirect flow cytometry analysis. Cells were stained by incubation for 30 min with a B cell-specific mouse-anti-swine CD21 Ab (BB6-11C9, IgG1) or a mouse Ab specific for swine monocytes, SWE3 (74-22-15, IgG1). Polyclonal, FITC-conjugated goat-anti-mouse IgG (Sigma-Aldrich) was used as a secondary reagent after washing the cells twice. All steps were performed at 4°C using flow cytometry buffer (HBSS containing 0.5% BSA and 0.5% sodium azide).

Results

MLR responses across various histocompatibility disparities in miniature swine

Proliferative responses of naive animals to a variety of alloantigens in standard 5-day MLR assays are shown in Fig. 1. No proliferative responses were detected when responders and stimulators were SLA-matched but differed at multiple minor histocompatibility Ag loci. Only weak responses were noted when responders and stimulators were mismatched at SLA class I loci only. In contrast, SLA class II disparate stimulators elicited very high responses, similar to those seen to fully mismatched (class I and II disparate) stimulators (23).

APC depletion did not affect SLA class II-positive T cell populations

APCs were depleted from PBMC based on plastic and nylon wool adherence properties. The effectiveness of the APC depletion was assessed phenotypically by FACS analysis. Representative data for
one experiment are shown in Fig. 2, indicating that a marked reduction of both monocytes and B cells was achieved following APC depletion. This procedure had no effect on the relative percentage of normal swine T cell subpopulations, which included CD4\(^+\)CD8\(^-\) and CD4\(^+\)CD8\(^-\) as well as CD4\(^+\)CD8\(^+\) and CD4\(^-\)CD8\(^+\) T cells (24, 25). In addition, surface SLA class II expression on resting T cells, also normal in swine (26), was unchanged following APC depletion.

**FIGURE 2.** Reduction of monocytes and B cells following APC depletion of PBL. Surface SLA class II expression on resting T cells was not altered or reduced following APC depletion. Depletion of APCs from PBMC: representative FACS analyses of PBMC before (A) and after (B) APC depletion show a marked reduction of monocytes and B cells following this procedure. The forward/side scatter plot (top left panel) indicates a significant decrease in monocytes outside the lymphocyte gate R1 (B) compared with (A). Rows 2 and 3, first columns, histograms show a significant decrease in SWC3 (74-22-15) -positive monocytes, as well as CD21 (BB6-11C9) -positive B cells after APC depletion. SWC3 was measured on nongated cells, whereas CD21 was measured on cells gated for lymphocytes (R1). Second column, Contour plots reflect class II-positive CD3 (74-22-15), CD4 (74-12-4), and CD8 (76-2-11) -positive lymphocytes (R1) before (A) and after (B) APC depletion. Percentages displayed on each contour plot represent the relative numbers of SLA class II positive CD3, CD4, and CD8 cells, respectively.

**FIGURE 3.** Effect of APC depletion on in vitro proliferative responses across class II and I mismatch barriers. Representative MLR data of SLA class II (A) and SLA class I (B) mismatched responder-stimulator pairs, with all APCs, only stimulator APCs, only responder APCs, or no APCs present. Box diagrams above each figure illustrate the haplotypes of responder (left) and stimulator cells (right). For each SLA mismatch, multiple haplotype combinations of MGH swine were used, all yielding similar results.

**APC depletion of responders and stimulators eliminated the MLR response**

As depicted in Fig. 3, the removal of either class I or II disparate responder APCs to specifically measure the direct pathway resulted in an increase in the stimulation index (SI), compared with that observed with bulk PBMC. In contrast, when the stimulators were APC-depleted to measure indirect responses, proliferation was reduced from an SI of 302 with bulk PBMC to 118 in a class II mismatch, and from 18 to 2.7 in a class I mismatch. Removing both responder and stimulator APCs reduced the SI to background levels, confirming that APC depletion of both responder and stimulator populations was complete. This result also demonstrated that following APC depletion, residual SLA class II-expressing T cells could not provide sufficient stimulus for a detectable proliferative response. Therefore, these T cells could serve as an adequate source of SLA class II Ags without directly stimulating responder cells to proliferate, thereby enabling the specific measurement of the indirect proliferative response.
In the absence of backstimulation, indirect MLR responses were not detected among naive responders.

To eliminate any effect of backstimulation on the proliferative response attributed to the indirect pathway in Fig. 4, several experimental measures were taken to prevent responder Ag recognition by stimulating T cells. The first set of experiments included stimulators lysed by repetitive (three times) freezing and thawing (F/T) to remove viable cells potentially capable of causing a backstimulating effect. Lysis of stimulator cells was visually confirmed by light microscopy. No proliferative responses were observed in either class I or II mismatched combinations when non-backstimulating suspensions of stimulator cell remnants were used as stimulators in MLR assays, despite the presence of responder APCs (Fig. 4). Again, the depletion of APCs from the responder cell population resulted in a significantly augmented proliferative response to nonlysed stimulators compared with that of bulk PBMC.
Given the potential for diminished efficiency of Ag source and presentation by lysed allogeneic stimulators, the next series of experiments used structurally intact stimulator cells that were incapable of responding to the responder MHC. The response of heterozygous (F₁) T cells to SLA matched, homozygous parental stimulators (P), such as SLA\textsuperscript{ac} anti-SLA\textsuperscript{cc} PBMC, was examined. There were no measurable responses with bulk PBMC or APC-depleted responders (direct pathway; Fig. 5A). This experiment was also repeated using PBMC from SLA\textsuperscript{cc} swine tolerized in vivo to SLA\textsuperscript{dd} (see Materials and Methods) as responders and stimulators (Fig. 6). As observed with F₁ responders, no MLR responses were observed when bulk or APC-depleted PBMC from these tolerized animals were used as responders. However, the effect of backstimulation on the measurement of the indirect pathway in both of these experiments can be noted in Figs. 5A and 6A. Despite the absence of a direct proliferative response given the use of tolerant responders, APC-depleted parental or nontolerant stimulators induced a significant MLR response, presumably via recognition by stimulator

![Diagram](image-url)

**FIGURE 5.** Backstimulation accounts for the proliferative response of naive responders to APC-depleted SLA-mismatched stimulators. A, MLR using F₁ responders (SLA AC) and parental stimulators (SLA CC). Proliferation in response to APC-depleted stimulators can only be seen when parental stimulators capable of responding to the F₁ responder cells are used. B, MLR responses of naive parental responders to F₁ stimulators. No proliferation of parental responders is seen when F₁ APC-depleted stimulators are used.
cells and subsequent cytokine release which we have termed back-stimulation. No proliferative response of tolerant SLA dd responders was observed following lysis of naive SLA cc stimulator cells by repeated F/T (data not shown).

The results obtained when these responder/stimulator cell populations were reversed, such that parental or nontolerant responders were stimulated by F1 or tolerant cells, is shown in Figs. 5B and 6B, respectively. In both series, the use of bulk PBMC led to a sizable proliferative response that was augmented significantly upon isolation of the direct pathway via removal of the responder APCs. The design of these latter experiments eliminated any potential contribution from backstimulation, and no detectable MLR responses were observed in either series when the indirect pathway was analyzed.

**Priming was required to detect indirect MLR responses**

The objective of the next experiment was to determine whether a secondary indirect response could be achieved by priming responder cells. Proliferation of primed and unprimed parental responders against F1 stimulators are shown in Fig. 7. Similar to the observations presented in the previous figures (Figs. 5B and 6B), no indirect response was observed with unprimed responders against tolerant stimulators. However, primed responders from swine sensitized with class II mismatched skin grafts and s.c. boosting with donor-matched PBMC, mounted substantial indirect antidonor MLR response, with nearly a 6-fold increase in SI compared with unprimed responders. With bulk PBMC and APC-depleted responders, the MLR responses of these in vivo-primed responders were also elevated compared with those observed using naive responders.

**FIGURE 6.** Proliferation in response to mismatched APC-depleted stimulators can only be seen when stimulators (naive cc) capable of responding to the responder cells (tolerant dd) are used. A, Proliferation of in vivo-tolerized dd responders against fully mismatched nontolerant cc stimulators. Backstimulation accounts for the proliferative response of tolerant dd responders to APC-depleted SLA mismatched naive cc stimulators. B, Proliferation of naive responders to fully mismatched, nonbackstimulating (tolerant) stimulators. No proliferation is seen when stimulators (tolerant dd) tolerant to the responder population (naive cc) are used.

**FIGURE 7.** Proliferation of naive and primed parental responders (SLA^cc to F1 stimulators (SLA^cd). Parental SLA^cc responders were sensitized in vivo to SLA^cd by skin grafting as described in Materials and Methods.

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A liferative response was observed in the absence of a direct response among native responders (Fig. 4). However, to test whether the secondary indirect responses were due to a change in the activation properties of the T cells and not of the responder APCs, we removed the APCs from the primed responders and added irradiated, MHC-matched stimulators that presented processed alloantigen. For this purpose, we used responder-matched PAECs that were transduced with an MHC gene matched to the priming cells. Resting PAECs express nearly undetectable levels of endogenous MHC class II and no detectable levels of endogenous MHC class II in PAECs (data not shown). Only primed T cells responded specifically to the transgene-expressing PAECs (Fig. 9A). This response did not require up-regulation of endogenous MHC class II, a result consistent with the absence of the transgene to be regulated by IFN-γ. We hypothesize that the total amount of processed transgene expression on endogenous MHC of resting PAEC was equal to the expression after up-regulation of endogenous MHC class II. A possible response to processed transgene expressed on MHC class I could be ruled out by selective blockage of MHC molecules with mAbs to class I but not class II Ags. Although unlikely, a direct response by primed T cells to low-level surface expression of the transgene, undetectable by flow cytometry or immunoprecipitation, could not definitively be ruled out in this system.

Our results confirm previous data showing that recognition of SLA-class II alloantigens via the direct pathway causes vigorous proliferative responses that do not require Ag priming (16, 28). Similarly, SLA class I-mismatched responder/stimulator pairs also resulted in a primary, although diminished, proliferative response, compared with the nonexistent primary response observed across minor histocompatibility barriers. Similar to class II-induced human T cells (23, 29), a regulatory or tolerizing effect of naive class II-positive swine T cells may underlie the augmented proliferative responses observed following removal of responder APCs (direct response). This indirect response was eliminated when parental responders and F1 stimulators (Figs. 5B and 6B) were used, suggesting that the response previously attributed to the indirect pathway was actually caused by backstimulation.

When the effect of backstimulation was carefully eliminated from the MLR culture, indirect proliferative responses to SLA class II-derived alloantigens occurred only following in vivo or in vitro priming, suggesting that the immunogenicity of MHC allopeptides parallels that of processed nominal Ags (Fig. 7). To test whether the secondary indirect responses were due to a change in the activation properties of the T cells and not of the responder APCs, we removed the APCs from the primed responders and added irradiated, MHC-matched stimulators that presented processed alloantigen. For this purpose, we used responder-matched PAECs that were transduced with an MHC gene matched to the priming cells. Resting PAECs express nearly undetectable levels of endogenous MHC class II and no detectable levels of endogenous MHC class II in PAECs (data not shown). Only primed T cells responded specifically to the transgene-expressing PAECs (Fig. 9A). This response did not require up-regulation of endogenous MHC class II, a result consistent with the absence of the transgene to be regulated by IFN-γ. We hypothesize that the total amount of processed transgene expression on endogenous MHC of resting PAEC was equal to the expression after up-regulation of endogenous MHC class II. A possible response to processed transgene expressed on MHC class I could be ruled out by selective blockage of MHC molecules with mAbs to class I but not class II Ags. Although unlikely, a direct response by primed T cells to low-level surface expression of the transgene, undetectable by flow cytometry or immunoprecipitation, could not definitively be ruled out in this system.
Given the possibility of nylon wool adherence by some class II-expressing T cells, this increase in the SI suggests that these accessory T cells (with concomitant nylon wool adherence properties) in the responder population may have had a suppressive effect on the proliferative responses observed with bulk PBMC.

In summary, these results demonstrate that an in vitro proliferative response to MHC Ags through the indirect pathway requires priming of the responder T cells. Therefore, in the MLR culture assay, MHC alloantigens are indistinguishable from minor Ags. These results contradict those obtained in the mouse model (11) in which MHC-derived allopeptides exhibited an apparently greater immunogenicity in the MLR assay, compared with nominal/minor Ags. Continued study is necessary to determine the mechanism of indirect allorecognition, or more importantly, why and how this process is so important in allograft rejection. A better understanding of indirect mechanisms may eventually enable the development of effective therapeutic strategies to prevent or interrupt these immunological responses to foreign tissues.

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References

**FIGURE 9.** Only primed T cells can proliferate in response to matched PAECs transduced with SLA-disparate class II DRβ-chain. Proliferation of naive and primed T cells (SLA-matched) to SLA-matched PAEC stimulators transduced with SLA DR chains. Only in vivo-primed T cells responded to the SLA transgene matched to the priming cells both in the absence (A) and presence (B) of IFN-γ. Values expressed in terms of cpm to illustrate the relatively high level of background or thymidine uptake among PAECs. TDx, transduction; media, no responders present.


