



Explore Spatial Biology Without Limits
Introducing the Next Generation of Precise Spatial Multiplexing

Meet CellScope™



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

This information is current as of May 26, 2022.

Gary W. Haller, Brian Lima, Shaun M. Kunisaki, Sharon Germana, Christian Leguern, Christene A. Huang and David H. Sachs

J Immunol 2002; 169:3613-3621; ;
doi: 10.4049/jimmunol.169.7.3613
<http://www.jimmunol.org/content/169/7/3613>

References This article **cites 28 articles**, 13 of which you can access for free at:
<http://www.jimmunol.org/content/169/7/3613.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2002 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



MHC Alloantigens Elicit Secondary, But Not Primary, Indirect In Vitro Proliferative Responses¹

Gary W. Haller,² Brian Lima, Shaun M. Kunisaki,³ Sharon Germana, Christian Leguern, Christene A. Huang, and David H. Sachs⁴

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 naive 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.

Most 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 antigraft 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 pas-

senger 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 allorecognition 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)⁵ and minor histocompatibility barriers.

Transplantation Biology Research Center, Massachusetts General Hospital/Harvard Medical School, Boston, MA 02129

Received for publication October 18, 2001. Accepted for publication July 25, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported in part by National Institutes of Health Grants 1R01 CA79988-01A1 and 5R01 HL63430-02. G.W.H. received partial support from the Deutsche Forschungs Gemeinschaft.

² Current address: Klinikum Mannheim, Universitätsklinikum, Chirurgische Klinik, Mannheim, Germany.

³ Current address: Department of Surgery, Massachusetts General Hospital/Harvard Medical School, Boston, MA 02114.

⁴ Address correspondence and reprint requests to Dr. David H. Sachs, Transplantation Biology Research Center, Massachusetts General Hospital/Harvard Medical School, MGH-East, Building 149-9019, 13th Street, Boston, MA 02129. E-mail address: sachs@helix.mgh.harvard.edu

⁵ 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 radio-resistant (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 Teknica, Durham, NC). The mononuclear cells were washed once with HBSS before the contaminating red cells were lysed with ACK Lysing 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 μ g/ml streptomycin (Life Technologies), 50 μ g/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^{-5} 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, 120×10^6 cells were incubated at 37°C in 20 ml standard MLR media (16) on 75-cm plastic flasks (Falcon no. 3023; Beckinon Labware, Lincoln Park, NJ) for 3–4 h. Nonadherent cells were then collected and replated at 37°C for an additional 3–4 h. The remaining nonadherent cells were then adjusted to 3×10^8 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 37°C MLR medium and nonadherent cells were collected and stored at 4°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, 4×10^5 responders and an equal number of irradiated (25 Gy) stimulators were incubated in 200 μ l of standard MLR media using flat-bottom 96-well plates (Costar, Cambridge, MA). After 5 days of incubation, one μ Ci 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 detected for a particular responder-stimulator pair by the average cpm for the same responder stimulated by self stimulators. Each experiment was performed at least three times with reproducible results. Background counts were uniformly low, ranging from 100–300 cpm with the exception 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 SLA^d PAEC clone, was derived and maintained in culture as previously described (18). Retroviral constructs used in this study included the pPBM14 recombinant genome which contained cDNA sequences for the pig DR α and - β polypeptide chains of the SLA^d 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 μ g/ml active; Mediatech, Herndon, VA) for 10–14 days for further use in *in vitro* assays. The presence of retroviral transcripts in transduced cells was monitored by Northern blot analysis of electrophoresized total RNA probed with radioactive *NeoR* or DR^b 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: 1×10^4 /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 ($40 \times 40 \times 2.2$ 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 2×10^7 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 been tolerized 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 100 days with normal organ function and no histological 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. APC 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, SWC3 (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

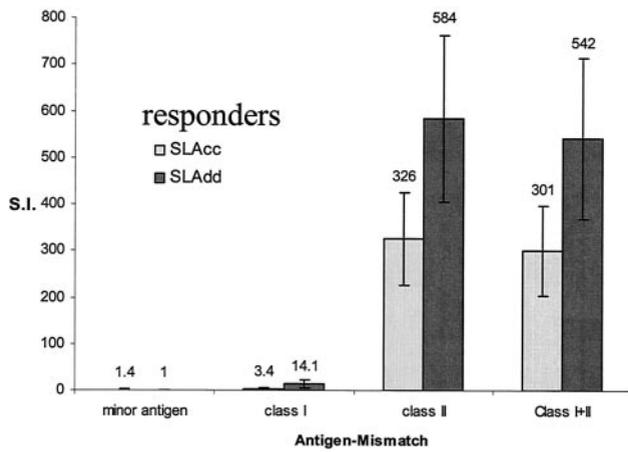


FIGURE 1. Proliferative responses of naive miniature swine across selected MHC barriers. Representative *in vitro* proliferative responses of miniature swine to minor Ag-mismatched but MHC-matched stimulators, MHC class I-mismatched, MHC class II-mismatched, and fully mismatched stimulators. Values are expressed as SI.

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.

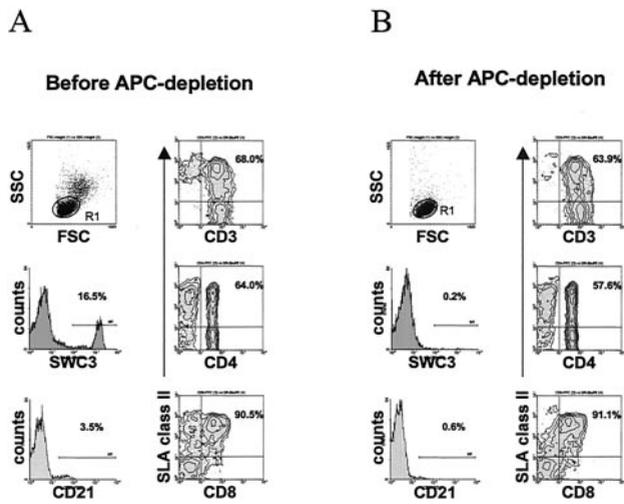


FIGURE 2. Reduction of monocytes and B cells following APC depletion of PBL. Surface SLA class II expression on resting T cells was not altered or reduced following APC depletion. Depletion of APCs from PBMC: representative FACS analyses of PBMC before (A) and after (B) APC depletion show a marked reduction 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 (89H2-6-15), 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.

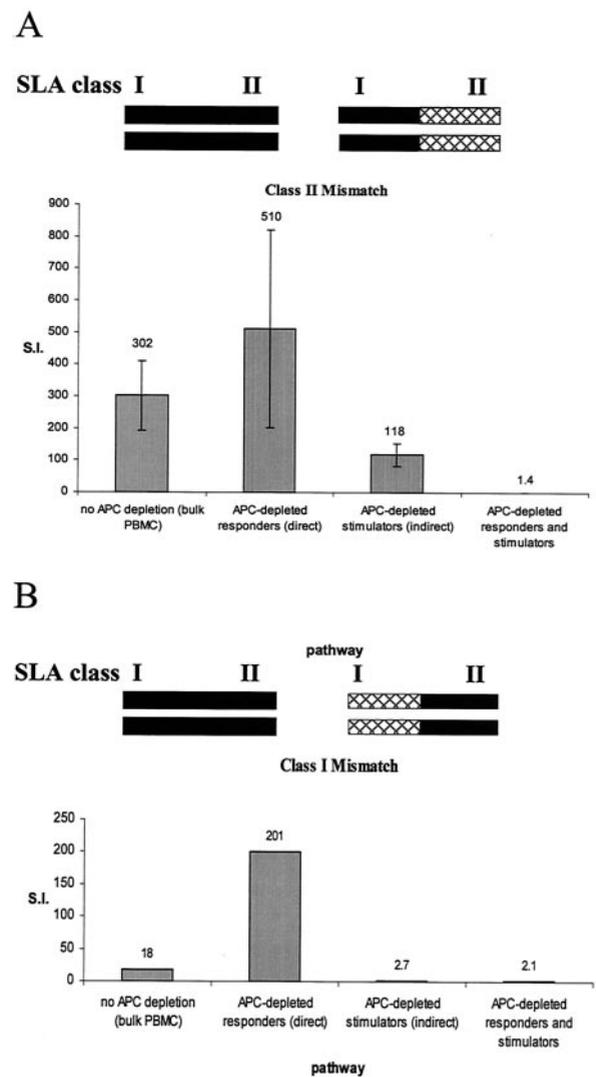


FIGURE 3. Effect of APC depletion on *in vitro* proliferative responses across class II and I mismatch barriers. Representative MLR data of SLA class II (A) and SLA class I (B) mismatched responder-stimulator pairs, with all APCs, only stimulator APCs, only responder APCs, or no APCs present. Box diagrams 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.

A

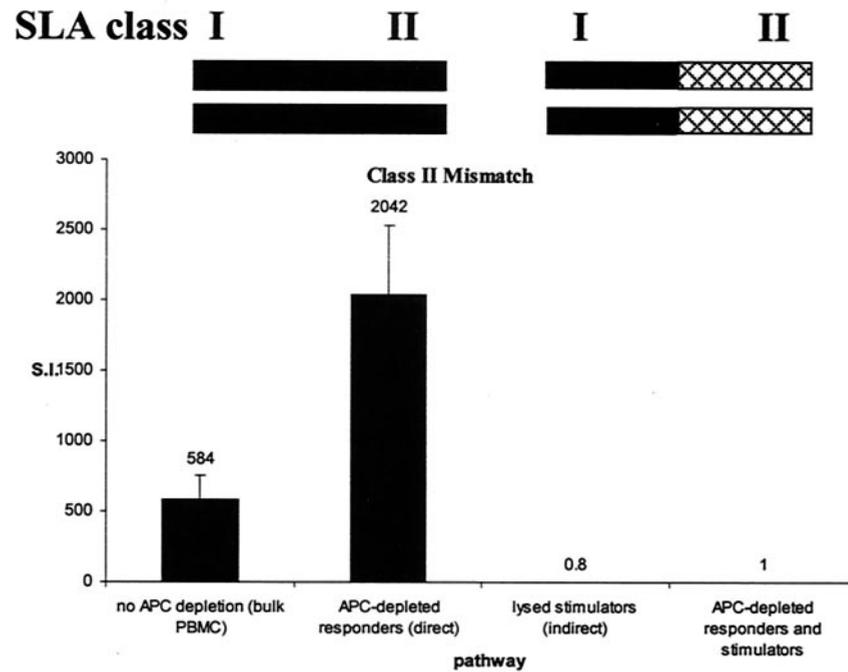
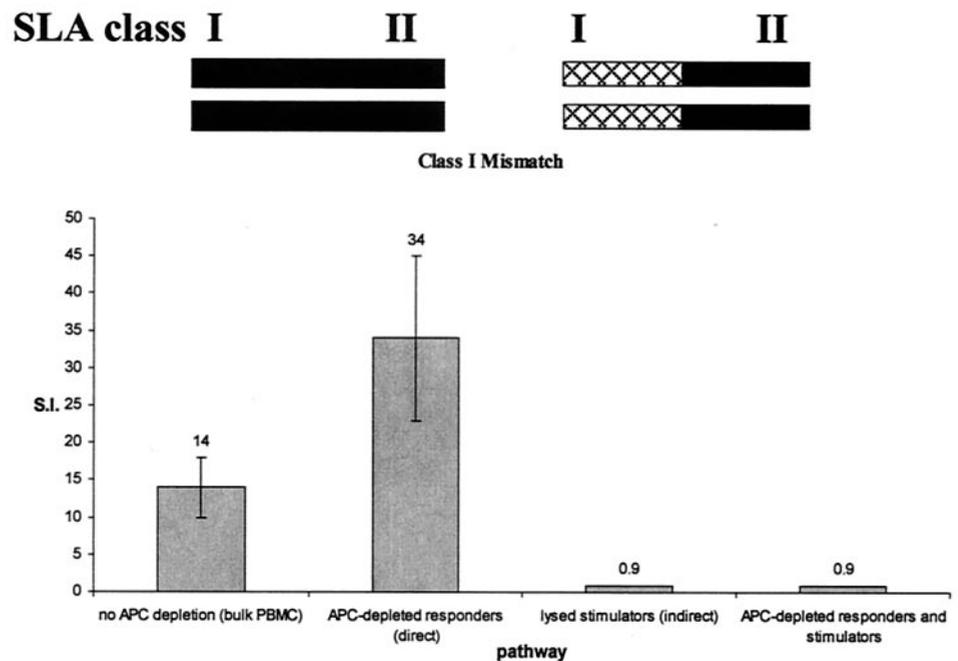


FIGURE 4. Effect of elimination of backstimulation on primary MLR. Proliferative responses across SLA class II (A) and class I (B) mismatch combinations using bulk PBMC, APC-depleted responders, lysed stimulators, and APC-depleted responders and stimulators. No proliferative responses were observed with lysed stimulators or when APCs were eliminated from responders and stimulators.

B



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 backstimu-

lating 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 these nonbackstimulating 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^{ac} anti-SLA^{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^{cc} swine tolerized in vivo to SLA^{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

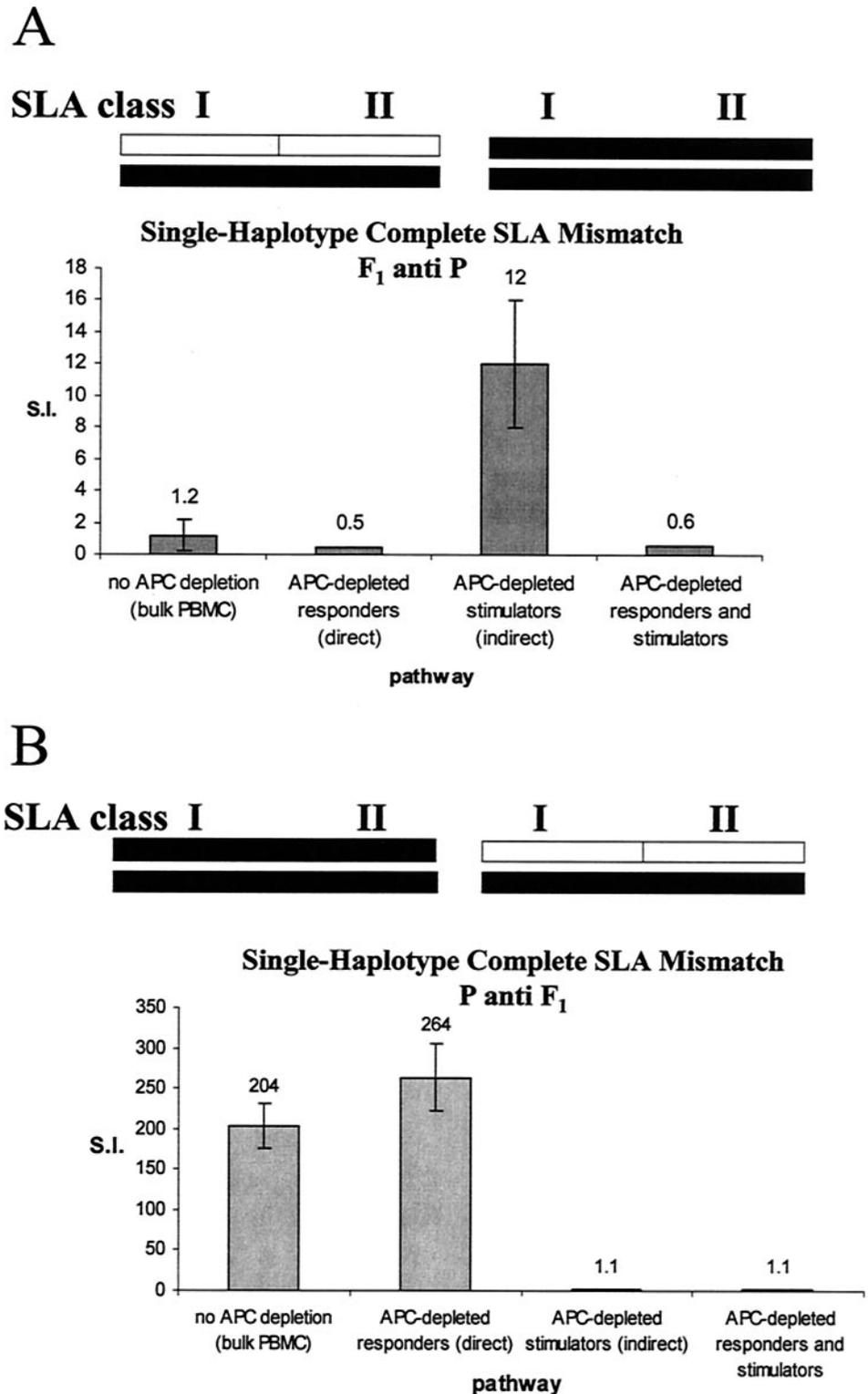


FIGURE 5. Backstimulation accounts for the proliferative response of naive responders to APC-depleted SLA-mismatched stimulators. *A*, MLR using F₁ responders (SLA^{ac}) and parental stimulators (SLA^{cc}). Proliferation in response to APC-depleted stimulators can only be seen when parental stimulators 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.

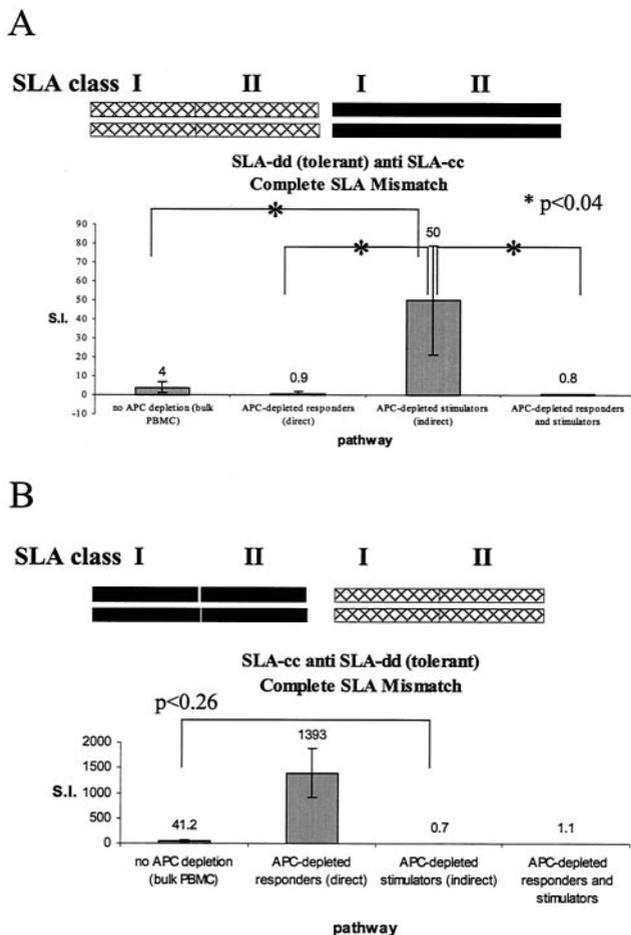


FIGURE 6. Proliferation in response to mismatched APC-depleted stimulators can only be seen when stimulators (naive cc) capable of responding to the responder cells (tolerant dd) are used. *A*, Proliferation of in vivo-tolerized dd responders against fully mismatched nontolerant cc stimulators. Backstimulation 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.

cells and subsequent cytokine release which we have termed backstimulation. 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. 5*B* and 6*B*, 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. 5*B* and 6*B*),

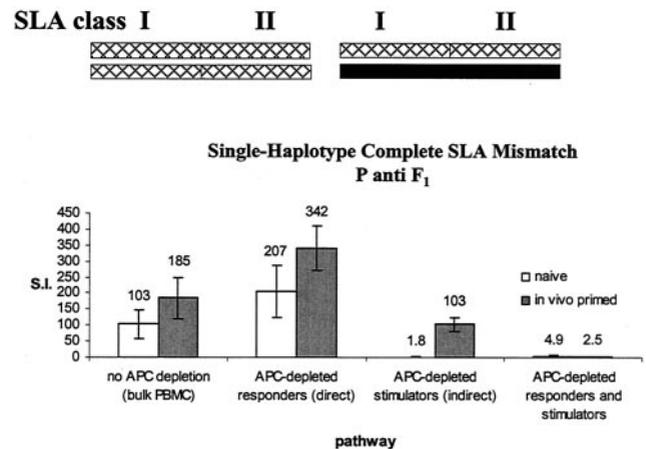


FIGURE 7. Proliferation of naive and primed parental responders (SLA^{CC}) to F₁ stimulators (SLA^{CD}). Parental SLA^{CC} responders were sensitized in vivo to SLA^{CD} by skin grafting as described in *Materials and Methods*.

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.

Only primed T cells responded to SLA-matched PAECs presenting processed allogeneic class II DR

In addition to cytokine production by stimulator cells, backstimulation may also be caused by improved Ag presentation by responder APCs. The following experiments were performed to determine whether the indirect proliferative response of primed cells against tolerant and heterozygous stimulators involved responder T cells rather than enhanced Ag processing/presentation by responder APC. To this end, the PAEC cell line PEDSV.15 (SLA^d) was transduced with a recombinant retrovirus for the pig DR^c allele (Fig. 8*A*) to generate G418-resistant transduced cells named PED-DR. Control transduction involved a retroviral construct only containing the neomycin resistance gene (*NeoR*), which produced the PED-Neo transduced cells (see *Materials and Methods*). As shown in Fig. 8*B*, both the *NeoR* and DR^c proviral genomes were correctly transcribed and led to the two expected genomic (Fig. 8, *A* and *C*) and spliced (Fig. 8, *B* and *D*) transcripts. However, PED-DR cells did not express the transduced DR heterodimer at the cell surface as shown in the flow cytometry analysis presented in Fig. 8*C* (bottom histograms). This result suggested that transduced DR α and - β -chains in PED were fully degraded or presented as peptides on PAEC MHC molecules. The second hypothesis was confirmed by showing that the stimulation of primed, but not naive, T cells with PED-DR led to marked proliferative responses as compared with stimulation with PED-Neo cell controls (Fig. 9). This response was primarily blocked by anti-class II DR, to a lesser extent by anti-DQ, but not by anti-class I mAbs, indicating that class II peptides were mainly presented through the endogenous class II pathways (data not shown).

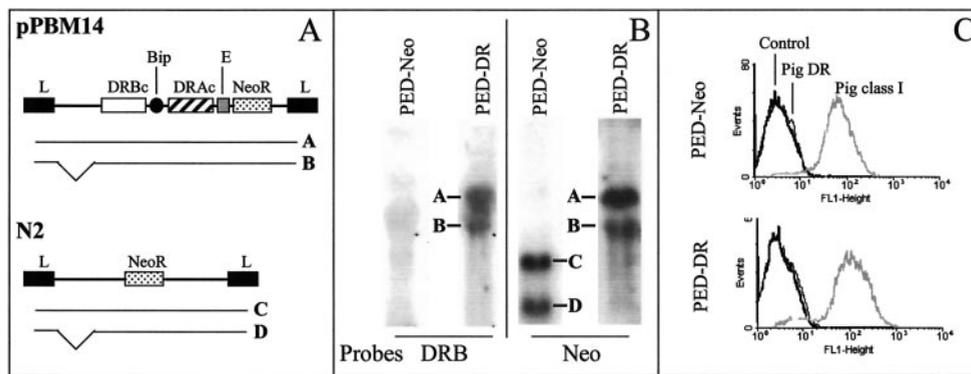


FIGURE 8. Analysis of SLA^d PAEC transduced with pig SLA class II DR^c. *A*, The N2 construct contained the gene for neomycin resistance (*NeoR*) cloned between MuLv Moloney-based long terminal repeat (L). The pPBM14 construct encompassed the sequences for the pig class II DRB^c and DRAc^c cDNA as well as that for *NeoR*. Internal ribosome entry sequences derived from H chain binding protein (Bip) and encephalomyocarditis virus (E) were cloned in between the other cDNAs. Expected spliced and unspliced transcripts (A and C, and B and D, respectively) are depicted under their respective constructs. *B*, Drug-selected PEDSV.15 cells transduced with either N2 (PED-Neo) or pPBM14 (PED-DR) were analyzed by Northern blot for the presence of genomic (A or C) or spliced (B or D) transcripts. *C*, Flow cytometry analysis of transduced PEDSV.15. The mAbs used were: 36-7-5, anti-mouse H-2K^k as control (black line); 40D, anti-pig class II DR (thick gray line); and 2.27.3a, anti-pig class I (thin gray line).

Discussion

The results of these experiments illustrate the requirement of sensitization for detectable *in vitro* proliferative responses to processed allogeneic MHC Ags presented on self APCs. Naive T cells failed to respond to any allogeneic peptides presented through the indirect pathway, including not only peptides derived from minor Ags, but also from SLA class I and II mismatched stimulators. Swine PBMC were deemed well-suited for these studies, given the high levels of MHC class II expression unique to resting swine T cells and the inability of these T cells to stimulate allogeneic responders in the absence of APCs. Therefore, swine T cells could serve as an adequate source of MHC (class II) alloantigen, without directly stimulating responding T cells in the MLR.

Appropriate experimental measures were taken to avoid the phenomenon of backstimulation on the observed proliferative responses. Backstimulation refers to the ability of irradiated stimulator cells to recognize the responder APC and react via release of cytokines into the culture media, leading to a proliferative effect on responder T cells (13, 14, 27). Alternatively, backstimulation may also occur through a mechanism involving activation or maturation of responder APCs, leading to more efficient presentation of processed peptide. The activation or maturation of responder APCs may also cause them to express important secondary signals that could enable direct recognition of stimulator T cells (trans-co-stimulation). The stimulating T cells themselves may also undergo activation-induced expression of secondary signals that allow direct recognition. Preliminary data from our laboratory to be presented elsewhere suggest that activated or PHA-blasted T cells may serve as effective APCs, increasing the likelihood of this latter proposed mechanism of backstimulation.

The use of stimulator cells lysed by repeated F/T effectively abolished backstimulation and failed to evoke any detectable indirect response among native responders (Fig. 4). However, to more closely mimic clinical transplantation, where intact allogeneic cells or tissues are transferred, stimulator cells in the MLR culture assays were kept structurally intact in subsequent experiments. In these experiments, backstimulation was avoided by using stimulators that were unresponsive to the responder, including F₁ progeny and PBMC derived from animals tolerized *in vivo* specifically to the SLA haplotype of the responder. When responders tolerant to the (parental) stimulators were used, a sizable proliferative response was observed in the absence of a direct re-

sponse. 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 allogeneic MHC. 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 by FACS (Fig. 8C). Upon activation with IFN- γ , their class II expression was up-regulated and detectable at high levels on the surfaces of all 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 inability of the transgene to be regulated by IFN- γ (Fig. 9B). 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 II but not class I 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

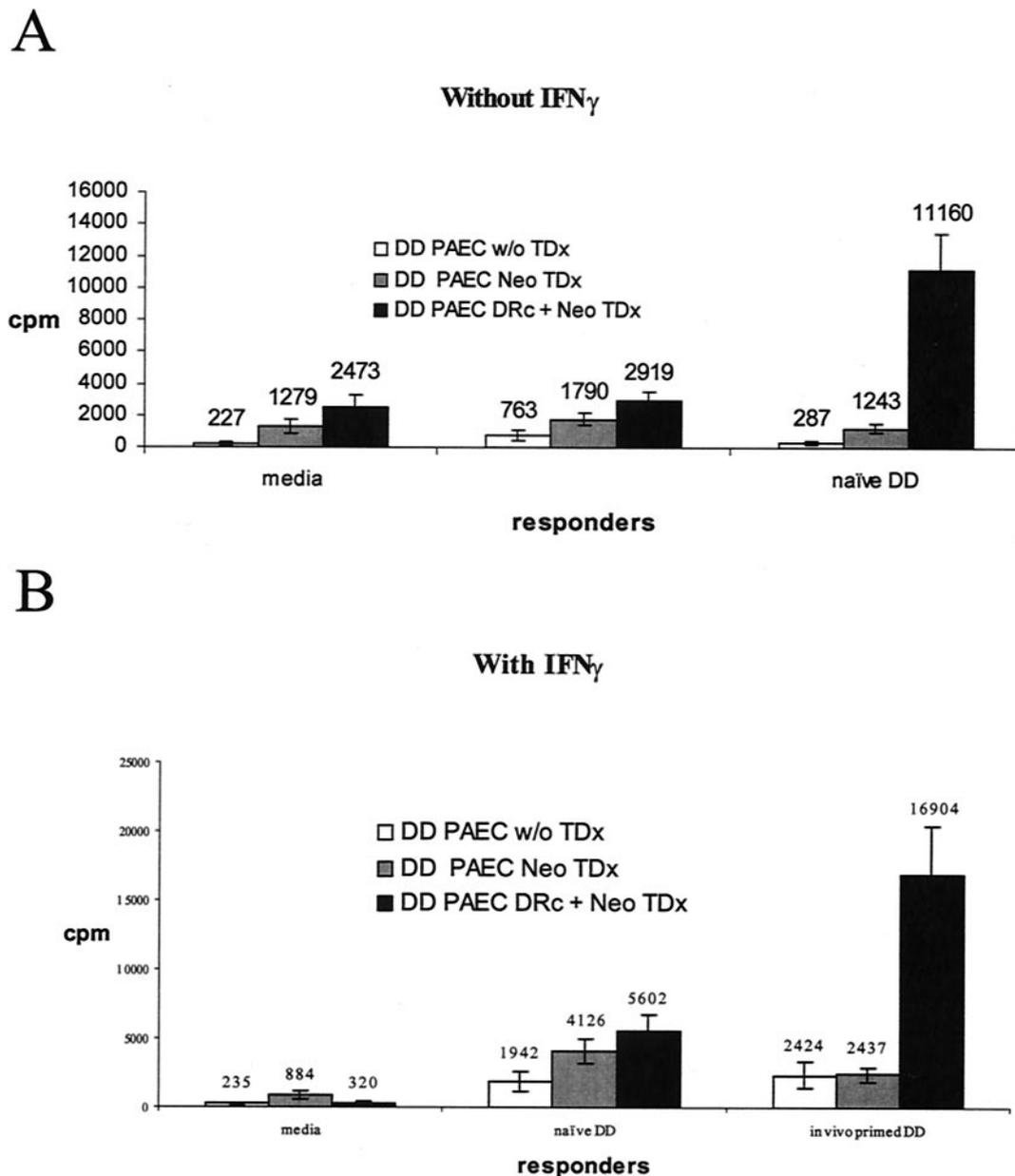


FIGURE 9. Only primed T cells can proliferate in response to matched PAECs transduced with SLA-disparate class II DR β -chain. Proliferation of naive and primed T cells (SLA^{DD}) to SLA-matched PAEC stimulators transduced with SLA^C DR chains. Only in vivo-primed T cells responded to the SLA transgene 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.

pathway; Figs. 3–7). 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 understand-

ing of indirect mechanisms may eventually enable the development of effective therapeutic strategies to prevent or interrupt these immunological responses to foreign tissues.

Acknowledgments

We thank Dr. Kazuhiko Yamada for providing peripheral blood samples from FK506-tolerized MHC-mismatched kidney transplant recipients, Drs. Zachary Gleit and Richard Lee for careful review of the manuscript, and Robin Laber for assistance with preparation of the manuscript.

References

1. Auchincloss, H., Jr., M. Sykes, and D. H. Sachs. 1999. Transplantation immunology. In *Fundamental Immunology*, 4th Ed. W. E. Paul, ed. Raven, New York, p. 1175.
2. Parker, K. E., R. Dalchau, V. J. Fowler, C. A. Priestley, C. A. Carter, and J. W. Fabre. 1992. Stimulation of CD4⁺ T lymphocytes by allogeneic MHC peptides presented on autologous antigen-presenting cells. *Transplantation* 53: 918.

3. Bevan, M. J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross react in the cytotoxic assay. *J. Exp. Med.* 143:1283.
4. Pescovitz, M. D., J. R. Thistlethwaite, Jr., H. Auchincloss, Jr., S. T. Ildstad, T. G. Sharp, R. Terrill, and D. H. Sachs. 1984. Effect of class II antigen matching on renal allograft survival in miniature swine. *J. Exp. Med.* 160:1495.
5. Lechler, R. I., and J. R. Batchelor. 1982. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. *J. Exp. Med.* 155:31.
6. Braun, M. Y., I. Grandjean, P. Feunou, L. Duban, R. Kiss, M. Goldman, and O. Lantz. 2001. Acute rejection in the absence of cognate recognition of allograft by T cells. *J. Immunol.* 166:4879.
7. Lee, R. S., K. Yamada, S. L. Houser, K. L. Womer, M. E. Maloney, H. S. Rose, M. H. Sayegh, and J. C. Madsen. 2001. Indirect recognition of allopeptides promotes the development of cardiac allograft vasculopathy. *Proc. Natl. Acad. Sci. USA* 98:3276.
8. Hornick, P. I., P. D. Mason, R. J. Baker, M. Hernandez-Fuentes, L. Frasca, G. Lombardi, K. Taylor, L. Weng, M. L. Rose, M. H. Yacoub, et al. 2000. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. *Circulation* 101:2405.
9. Auchincloss, H., Jr., R. Lee, S. Shea, J. S. Markowitz, M. J. Grusby, and L. H. Glimcher. 1993. The role of "indirect" recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice. *Proc. Natl. Acad. Sci. USA* 90:3373.
10. Sayegh, M. H., S. J. Khoury, W. W. Hancock, H. L. Weiner, and C. B. Carpenter. 1996. Mechanisms of oral tolerance by MHC peptides. *Ann. NY Acad. Sci.* 778:338.
11. Gould, D. S., and H. Auchincloss, Jr. 1999. Direct and indirect recognition: the role of the MHC antigens in graft rejection. *Immunol. Today* 20:77.
12. Sachs, D. H., G. Leight, J. Cone, S. Schwartz, L. Stuart, and S. Rosenberg. 1976. Transplantation in miniature swine. I. Fixation of the major histocompatibility complex. *Transplantation* 22:559.
13. Schanz, U., D. L. Roelen, J. W. Bruning, M. J. Kardol, J. J. van Rood, and F. H. J. Claas. 1994. The relative radioresistance of interleukin-2 production by human peripheral blood lymphocytes: consequences for the development of a new limiting dilution assay for the enumeration of helper T lymphocyte precursor frequencies. *J. Immunol. Methods* 169:221.
14. Young, N. T., D. L. Roelen, M. J. Dallman, K. J. Wood, P. J. Morris, and K. I. Welsh. 1996. Enumeration of human alloreactive helper T lymphocyte precursor frequencies by limiting dilution analysis of interleukin-2 production. *J. Immunol. Methods* 195:33.
15. Pennington, L. R., J. K. Lunney, and D. H. Sachs. 1981. Transplantation in miniature swine. VIII. Recombination within the major histocompatibility complex of miniature swine. *Transplantation* 31:66.
16. Thistlethwaite J. R., Jr., H. Auchincloss, Jr., M. D. Pescovitz, and D. H. Sachs. 1984. Immunologic characterization of MHC recombinant swine: role of class I and II antigens in in vitro immune responses. *J. Immunogenet.* 11:9.
17. Pescovitz, M. D., J. K. Lunney, and D. H. Sachs. 1985. Murine anti-swine T4 and T8 monoclonal antibodies: distribution and effects on proliferative and cytotoxic T cells. *J. Immunol.* 134:37.
18. Seebach, J. D., M. K. Schneider, C. A. Comrack, A. Leguern, S. A. Kolb, P. A. Knolle, S. Germana, H. DerSimonian, C. LeGuern, and D. H. Sachs. 2001. Immortalized bone-marrow derived pig endothelial cells. *Xenotransplantation* 8:48.
19. Banerjee, P. T., G. C. Kanor, S. Muthukumar, M. Denaro, H. Shimada, S. Zhu, M. D. Rosa, D. H. Sachs, and C. LeGuern. 1997. A polycistronic retrovirus vector for expression of swine MHC class II DR α/β heterodimers. *Xenotransplantation* 4:161.
20. Hantzopoulos, P. A., B. A. Sullenger, G. Ungers, and E. Gilboa. 1989. Improved gene expression upon transfer of the adenosine deaminase minigene outside the transcriptional unit of a retroviral vector. *Proc. Natl. Acad. Sci. USA* 86:3519.
21. Shafer, G. E., D. W. Emery, K. Gustafsson, S. Germana, W. F. Anderson, D. H. Sachs, and C. LeGuern. 1991. Expression of a swine class II gene in murine bone marrow hematopoietic cells by retroviral-mediated gene transfer. *Proc. Natl. Acad. Sci. USA* 88:9760.
22. Utsugi, R., R. N. Barth, R. S. Lee, H. Kitamura, J. C. LaMattina, J. Ambroz, H. Sachs, and K. Yamada. 2001. Induction of transplantation tolerance with a short course of tacrolimus (FK506). I. Rapid and stable tolerance to two-haplotype fully MHC-mismatched kidney allografts in miniature swine. *Transplantation* 71:1368.
23. Sidhu, S., S. Deacock, V. Bal, J. R. Batchelor, G. Lombardi, and R. I. Lechler. 1992. Human T cells cannot act as autonomous antigen presenting cells, but induce tolerance in antigen-specific and alloreactive responder cells. *J. Exp. Med.* 176:875.
24. Summerfield, A., H. J. Rziha, and A. Saalmuller. 1996. Functional characterization of porcine CD4⁺CD8⁺ extrathymic T lymphocytes. *Cell. Immunol.* 168:291.
25. Zuckermann, F. A., and R. J. Husmann. 1996. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. *Immunology* 87:500.
26. Saalmuller, A., F. Weiland, and M. J. Reddehase. 1991. Resting porcine T lymphocytes expressing class II major histocompatibility antigen. *Immunobiology* 183:102.
27. Harrison, M. R., and W. E. Paul. 1973. Stimulus-response in the mixed lymphocyte reaction. *J. Exp. Med.* 138:1602.
28. Sprent, J., and M. Schaefer. 1985. Properties of purified T cell subsets. I. In vitro responses to class I vs. class II H-2 alloantigens. *J. Exp. Med.* 162:2068.
29. Pichler, W. J., and T. Wyss-Coray. 1994. T cells as antigen-presenting cells. *Immunol. Today* 15:312.