HUMAN IMMUNE CELLS DIRECTLY RECOGNIZE XENOGENEIC MHCs

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INTRODUCTION

Over the subsequent half-century, the development of immunosuppressive therapies and refinements in tissue typing has served as a breakthrough in clinical transplantation. The idea of substituting a diseased or damaged organ with a functional organ has emerged as a sound therapeutic treatment for irreversible organ diseases like end-stage organ failure (1). Although transplantation is a lifesaving procedure, the greatest challenge continues to be the lack of an adequate organ supply that can satiate the increasing demand for organ transplants (2). As a result, this has led to a major increase in the number of patients waiting for allograft procedures and the number of patients dying while on the waiting list (2). Efforts have been made to address this problem by expanding the donor pool through: increasing public awareness, improving the efficiency of the donation process, developing standardized donor management protocols, and utilizing less ideal donors; but this has proved to be limiting (3).

Xenotransplantation has been suggested as a possible solution to the chronic shortage of organs (4). However, the preeminent hurdle to this treatment option is the immune response of the recipient against the graft (5). The antibody-mediated rejection has been a major barrier in the transplant of organs/tissues from other species into humans as preformed anti-α-1,3-galactosyltransferase (α-Gal) antibodies binding to α-Gal epitopes in xenografts have caused complement activation, resulting in graft endothelial cell destruction and hyperacute thrombosis (6). Today, research has successfully eliminated the hyperacute rejection in pig xenografts by creating α1,3 GT knockout pigs that lack α-Gal epitopes. However, more studies need to be done to minimize the cell-mediated rejection of xenografts (7, 8).

There is compelling evidence showing that CD4 and CD8 T cells are the key elements in initiating the rejection cascade by direct and indirect recognition of alloantigens, whereas studies on the direct T cell recognition of xenoantigens are conflicting. Several studies reported that direct recognition of murine splenic cells by human T cells were reliant on either exogenous human cytokines or the presence of irradiated autologous PBMCs (9-11). Using human APC-depleted PBMC, porcine stimulator cells, and blocking antibodies, other studies demonstrated direct CD4 but minimal CD8 T cell recognition of porcine xenoantigens in the absence of exogenous human cytokines (12-14). Of note, these studies also demonstrated differences in the magnitude of xenogeneic versus allogeneic T cell responses to murine and porcine stimulator cells (12-14). In this study, we investigated whether the observed discrepancy in the plasticity of human T cells to directly recognize xenoantigens across species depends on the specific type of APC.

METHODS

Media and reagents

Complete culture media (CM) contained RPMI 1640, 1% L-glutamine, 1% penicillin/streptomycin, 50 μM 2β-Mercaptoethanol, 1% sodium-pyruvate, 1% non-essential amino acids, and heat-inactivated 10% FCS. Recombinant human Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and Interleukin-4 (IL-4) were purchased from Peprotech (Rocky Hill, NJ). TLR agonist, LPS, was purchased from Invivogen (San Diego, CA). Media for staining cells contained PBS 1X without Ca^{2+}/Mg^{2+}, heat-inactivated 2% FCS and 2 mM EDTA.

Generation and immunophenotyping of DCs

Human immature monocyte-derived DCs were generated from peripheral blood adhering monocytes cultured in CM with GM-CSF (100 ng/mL) and IL-4 (10 ng/mL). Cultures were fed every 2 days with medium containing cytokines. Day 7 immature DCs were suspended in CM and activated for 4 hours with LPS (1μg/ml) that was removed from the DC suspension prior to coculture with indicated total or isolated human immune cells. Immature and LPS-stimulated mature DCs were stained with the following fluorochrome antibodies: CD1a (HI149), CD80 (L307.4), CD83 (HB15a), CD86 (IT2.2), CD14 (61D3), HLA-DR (LN3), and CD40 (5C3) (BD Pharmingen, BD Bioscience, or eBioscience). Mouse immature bone marrow-derived DCs were generated from isolated bone marrow cultured in a 75 cm^2 flask for 4 days in CM with GM-CSF (10 ng/mL) and IL-4 (10 ng/mL). Day 4 immature DCs were suspended in CM for 4 hours prior to coculture with indicated total or isolated human immune cells. Mature DCs were stained with the following fluorochrome antibodies: CD80 (16-10A1), CD86 (GL1), CD40 (3/23), H-2kb (AF6-88.5), IA/IE (M5/114.15.2), and CD11c (N418) (eBioscience or BD Bioscience). For blocking studies, mouse DCs were treated with indicated monoclonal antibodies H-2d (H-2Kd/H-2Dd) and IA/IE (M5/114.15.2) against MHCs 30 minutes prior to cocultures.

Cell isolation

Human and mouse CD4 T cells, CD8 T cells, B cells, and NK cells were isolated from peripheral blood mononuclear cells (PBMCs) and mouse spleen/lymph nodes, respectively, using the EasySep Cell Enrichment Kit (StemCell Technologies, Vancouver, Canada). The purity of the enriched cell populations were 90-98%.

Immune cells culture

Carboxylfluorescein succinmidyl ester from Molecular Probes (Eugene, OR) labeled immune cells isolated from human PBMCs or mouse splenic and lymphoid tissues were cocultured with human DCs and mouse DCs, respectively at 1:10 ratio (2x105 stimulator: 2x106 responder) for up to 6 days. The immune cell responses to allogeneic and xenogeneic cells were measured by flow cytometry using a panel of indicated antibodies specific for human: CD4 (L200), CD8 (SK1), CD19 (SJ25C1), and CD56 (B159) (BD Bioscience, BD Pharmingen, or eBioscience) and for mouse: CD4 (L200), CD8 (SK1), CD19 (SJ25C1), and CD56 (B159) (BD Bioscience, BD Pharmingen, or eBioscience) immune cell subsets. The proliferation of responding cells was analyzed by flow cytometry based on the decrease in the fluorescence intensity of the CFSE-labeled cells as they divide into daughter cells. In parallel, the supernatants collected from the cocultures were analyzed to further determine cytokine production of responding cells, IL-2, and IFN-γ using ELISA Kits (BD Bioscience, San Diego, CA).

Statistical analysis

Flow cytometry samples were run on the FACSCalibur flow cytometry using Cell QuestPro acquisition software. Data were analyzed using FlowJo analysis software. ELISA plates were read on ELISA reader (Molecular Devices, SpectraMax MS) and analyzed using SoftMax Pro5.4 software. Values are presented as mean ± SEM. Statistical significance was calculated using one-tailed unpaired Student's t-test with a 95% confidence interval (* $p<0.05$) using GraphPad.

RESULTS

Proliferation and cytokine production of human PBMC in response to xenogeneic dendritic cells

In both the allogeneic and xenogeneic settings, the recipient's immune cells enter the transplanted organ via the bloodstream and initiate the rejection cascade. In order to determine the immune cells' impact on xenorejection, we first evaluated the magnitude of the xenoresponse as well as the pro-inflammatory cytokine production of human PBMC to murine DCs. The frequency of proliferating cells within the PBMC, and purified cell subsets in both allogeneic and xenogeneic settings were determined by CFSE-labeling prior to coculture and staining with specific monoclonal antibodies after the coculture (Figure 1, 2). Our data denoted that total PBMC had a stronger response to xenoantigens as compared to alloantigens (Figure 3A). Concurrently, the human CD4 T cells within PBMC had the greatest proliferative response followed by CD8, B, and NK subsets, respectively, to xenogeneic stimulator cells (Figure 3A). The proliferative response was xeno-MHC restricted since blocking antibodies completely suppressed the observed proliferation (Figure 3A). Additionally, the proliferative response of xenoreactive immune cell subsets within PBMC was similar to that of the alloreactive immune cell subsets, except for xenoreactive CD4 T cells and B cells (Figure 3A). Interestingly, the levels of cytokine IL-2 and INF-γ produced by allo- and xenoreactive cells were inversely related to their proliferative response (Figure 3B, C). Collectively, our data demonstrated major differences in human immune cell reactions to xeno- versus alloantigens.

Proliferation and cytokine production of human individual immune cell subsets in response to xenogeneic dendritic cells

Next, we tested the direct contribution of individual immune cell subsets to xeno-MHC recognition by measuring the proliferative responses of CD4, CD8, B, and NK immune cell subsets individually cocultured with murine DCs. Our data showed that both human CD4 and CD8 T cell subsets independently proliferated in response to xenogeneic stimulator cells with CD8 T cells having the highest proliferative response (Figure 4A). The blocking of murine MHC class I and II with specific antibodies inhibited the proliferation, thereby indicating that the xenorecognition by CD4 and CD8 T cells were MHC restricted (Figure 4A). When the proliferative response of xenoreactive T cells were compared to their alloreactive counterparts, we observed a negligible difference between the percentage of xeno- and alloreactive CD4 and CD8 T cells (Figure 4A). However, both xenoreactive CD4 and CD8 T cell subsets secreted much lower amounts of both IL-2 and INF-γ (Figure 4B, C). In addition, the xeno- and alloreactive CD4 T cell subset was the greatest producer of IL-2 and INF-γ cytokines followed by CD8 T cells (Figure 4B, C). Our data also showed that B and NK cell subsets neither proliferated nor produced IL-2 or IFN-γ in response to xenogeneic stimulator cells (Figure 4). However, the B cell subset, and to a lesser extent the NK subset, did moderately proliferate in response to allogeneic stimulator cells and produced IL-2 (Figure 4A, B). The data revealed that human CD4 and CD8 T cell subsets are able to directly and comparably proliferate but producing less cytokines in response to xenogeneic as compared to allogeneic dendritic cells.

Proliferation and cytokine production of human immune cell subset combinations in response to xenogeneic dendritic cells

Next, the activity of various combinations of immune cell subsets were examined to determine whether certain combinations of immune cell subsets enhance their xenoresponses. As depicted in the figure 5,

we cultured different combinations of immune cell subsets with murine DCs and measured their proliferative responses and cytokines production. Our data revealed that the combination of xenoreactive CD4 and CD8 T cells increased the proliferation of CD4 T cells, but not CD8 T cells when compared to individual T cell cultures (Figures 4A and 5A). Concordantly, the proliferative response of xenoreactive B cells increased particularly when combined with CD4 than with CD8 T cells (figures 4A, 5B, C). Moreover, the proliferation of B cells to xenogeneic stimulator cells did not further increase when combined with both CD4 and CD8 T cells (Figure 5D). In contrast, the proliferative response of allogeneic B cells only increased when combined with CD8 T cells (Figure 4A, 5C). Interestingly, the response of B cells to xenogeneic DCs was T-cell dependent since blocking of xeno-MHCs suppressed their proliferative responses (Figure 5D). Higher amounts of IFN- were detected in the supernatants of xenoreactive CD4B and CD8B than in their individual CD4 or CD8 T cell cultures (Figure 4C, 5F). The amount of IFN-γ detected in xenoreactive CD4CD8 culture was greater than the total amount of this cytokine produced in individual xenoreactive CD4 and CD8 T cell cultures (Figure 4C, 5F). In addition, we detected larger amounts of IL-2 and IFN-γ in culture supernatants of alloreactive cells as compared with xenoreactive cells (Figures 3 B-C, 4B-C, 5E-F). An exception to this observation was that CD8B combination produced more IL-2 in response to xenogeneic DCs (figure 5E). The data suggested that B cells can recognize xeno-MHCs and contribute as APCs in xenoreactivity in the presence of T cells.

DISCUSSION

In the present study, we conducted a series of in vitro coculture systems to demonstrate whether human circulating CD4 or CD8 T cells have the flexibility to directly react to murine dendritic cells without exogenous factors. The principal findings of this study are as follows: first, purified human T cell populations were activated and proliferated in response to xenoantigens present on murine dendritic cells in the absence of exogenous cytokines. Second, both CD4 and CD8 T cells responded directly to murine DCs as confirmed by the suppression of their proliferative response in the presence of murine MHC class I and II blocking antibodies. Third, CD8 T cells were not dependent on CD4 T cells to directly recognize xenoantigens present on the murine dendritic cells. Fourth, CD8 T cells had a stronger proliferative response than CD4 T cells when cultured independently with murine DCs. Finally, purified CD4 T cells had a greater proliferative response to allogeneic as compared to xenogeneic DCs. However, the proliferative response of CD4 T cells within total PBMC was higher when cultured with xenogeneic DCs.

Between different species, the xenogeneic MHC is likely to be more disparate than those of allogeneic donor-recipient pairs. In order to determine whether human T cells directly respond to xenogeneic MHCs, it is pertinent to consider the type of xenogeneic stimulator cells. Our data argues there is direct interaction of human T cell receptor molecules with xeno-MHCs expressed on the murine dendritic cells. This interpretation is based on the observation that both CD4 and CD8 T cells independently proliferated by murine DCs since blocking monoclonal antibodies specific for murine class I or II molecules suppressed the proliferative response of these T cells. Several groups have documented that human direct T cell response to murine splenic cells were dependent upon the addition of exogenous cytokines (9, 10). It has been suggested that the requirement for exogenous IL-1 was due to the inability of human T cells to effectively stimulate the release of sufficient amounts of cytokines from murine APCs (10). Batten et al. suggested a defect between the binding of human LFA-1 to mouse ICAM-1 and human CD2 to mouse LFA-3 as a possible mechanism for the insufficient stimulation of IL-1 production (11, 15). Our findings demonstrate direct xenorecognition of human CD4 T cells without exogenous cytokines for optimal proliferation. This inconsistency could be due to a difference in the type and frequency of stimulator cells used in these studies. Indeed, we used a higher number of mature murine DC (DC/T ratio) with enhanced expression of MHC and costimulatory molecules with higher binding affinity (11) in our cocultures, a distinct contrast to low frequency immature murine APCs found within splenocytes used in those studies (9, 10). Our finding is consistent with Lucas et al., who demonstrated that the suboptimal interaction in human CD4 T cells and murine APC was due to insufficient activation of murine APC stimulator cells (16). In addition, Dorling et al. demonstrated the direct recognition of porcine alveolar lavage (AL) cells by human T cells when they used porcine stimulator cells that had been incubated overnight allowing for maturation of AL cells (12).

Prior to this work, studies have shown the ability of total T cell populations to recognize murine APCs in the presence of exogenous cytokines (9, 10). However, these studies were unable to quantify the independent responses of human CD4 or CD8 T cell to murine APCs. Other studies quantified the response of human T cells to porcine stimulator cells, showing that human CD4 and CD8 T cell were able to directly recognize xenoantigens in the absence of cytokines. However, the observed direct CD8 T cell response was dependent on the presence of indirect CD4 T cells (11-14).

In this study, we re-evaluated the contribution of direct CD8 T cell recognition to xenoantigens using murine dendritic cells. In contrast to the studies above, we found a direct recognition of murine MHC by human CD8 T cells. In fact, CD8 T cells elicited the strongest proliferative response to xenoantigens among the other immune cell subsets individually. This suggests that mouse DC provided all the signaling molecules required for CD8 T cell activation and proliferation. Interestingly, the CD8 T cell response was independent of CD4 T cells since the proliferative response of CD8 T cell remained constant when combined with CD4 T cells.

The observed differences might be due to the type of stimulatory APCs used in these studies as DCs are shown to be the most effective antigen-presenting cells (17). In addition, the maturational status of APCs might have also contributed to such discrepancies. Indeed, we observed a reduction in T cell proliferative response when immature murine DCs were used as stimulator cells (data not shown). Moreover, previous studies have shown the impact of APC maturation on magnitude of human T cell xenoresponse (12, 16).

Since the elimination of the hyperacute rejection via the creation of α1,3 GT knockout pigs that lack α-Gal epitopes, the B cells response to xenoantigens has been focused on their role as APCs in cell-mediated chronic rejection. Our data suggest that human B cells specific to xeno-MHCs could potentially act as APCs and contribute to xenoantibody production in the chronic phase of the rejection. Indeed, the proliferation of B cells cocultured with T cells was completely suppressed by murine-MHC blocking antibodies.

A considerable body of evidence exists to suggest that the xenoresponse of human T cells to porcine APCs were greater than the alloresponse (12-14). In the murine system, Alter et al. demonstrated that the alloresponse of human T cells was greater than the xenoresponse to murine APCs (10). In this report, the alloresponse of human CD4 T cells was also greater than the xenoresponse to murine stimulator cells. The discrepancy between the magnitude of the xenogeneic and allogeneic proliferative response in these studies could be attributed to the extent of genetic homology between different species of stimulator cells (15, 16). Collectively, these studies illustrated that TCRs may be more likely to react with intraspecies MHC molecules than distantly related xenogeneic MHC molecules. Interestingly, a minimal proliferative response was observed when murine immune cell subsets were cultured with human dendritic cells (data not shown). It has been suggested that this minimal proliferative response could be due to defects in the murine TCR-human MHC interaction (18, 19). Another possible reason for the weaker response to xenoantigens can be attributed to species specificity of accessory molecule interactions (20, 21).

CONCLUSION

This study has demonstrated that direct recognition of xenogeneic MHCs by human T cells is contingent on the type of stimulatory APC and their maturational status. The greater degree of plasticity of the human TCR observed in this study continues to challenge the assertion that MHC molecules from evolutionary distant species are recognized ineffectively by human T cells.

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FIGURES

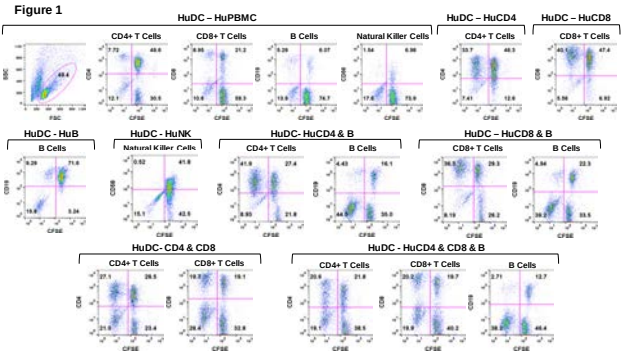

Figure 1. Proliferative response of human immune cells. Carboxylfluorescen succinimidyl ester (CFSE)-labeled PBMC or purified human immune cells were cocultured in various combinations with human dendritic cells for up to 6 days. The flow plots display the frequency of proliferating cells labeled with indicated monoclonal antibodies.

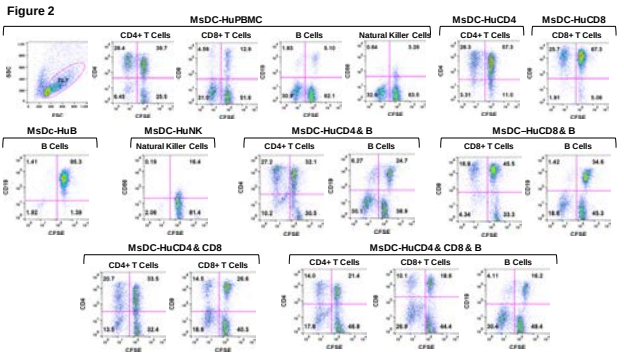

Figure 2. Proliferative response of human immune cells. Carboxylfluorescein succinmidyl ester (CFSE)-labeled PBMC or purified human immune cells were cocultured in various combinations with murine dendritic cells for up to 6 days. The flow plots show the frequency of proliferating cells labeled with indicated monoclonal antibodies.

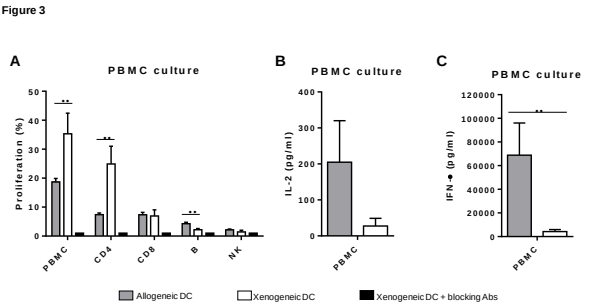

Figure 3. Proliferative response and cytokine production of human PBMC. Carboxylfluorescein succinmidyl ester (CFSE) - labeled PBMC were cocultured with human or murine dendritic cells for up to 6 days. The bar graphs show the percentage of proliferating PBMC and individual cells within PBMC (A). Gray bars represent alloresponse, white bars represent xenoresponse, and black bars represent xenoresponse in the presence of blocking MHC class I and II antibodies. The bar graphs also show the amounts of IL-2 and IFN-γ produced by proliferating PBMC (B, C) (n=3 average of three independent experiments).

FIGURES

Figure 4

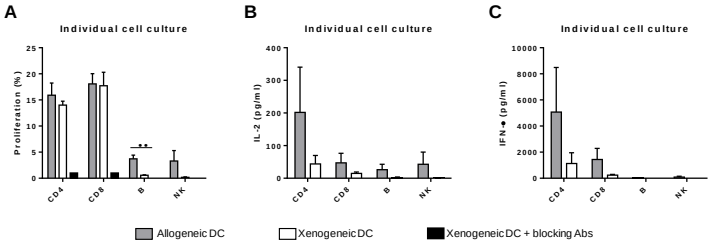

Figure 4. Proliferative response and cytokine production of purified human immune cells. Carboxylfluorescein succinmidyl ester (CFSE) - labeled cells were cocultured with human or murine dendritic cells for up to 6 days. The bar graphs show the percentage of indicated proliferating cells (A). Gray bars represent alloresponse, white bars represent xenoresponse, and black bars represent xenoresponse in the presence of blocking MHC class I and II antibodies. The bar graphs also show the amounts of IL-2 and IFN-γ produced by indicated proliferating cells (B, C) (n=3 average of three independent experiments).

Figure 5

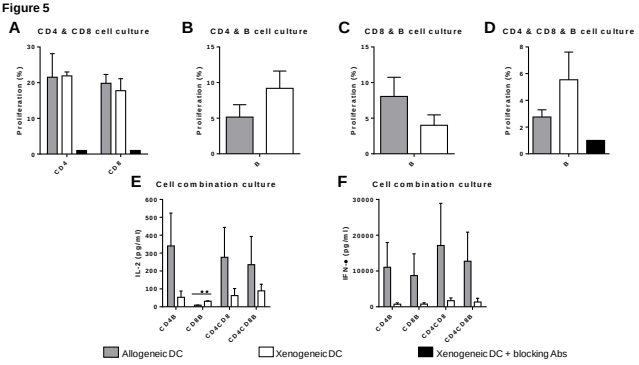

Figure 5. Proliferative response and cytokine production of various combinations of purified human immune cells. Carboxylfluorescein succinmidyl ester (CFSE)-labeled isolated cells were mixed as indicated and cocultured with human or murine dendritic cells for up to 6 days. The bar graphs show the percentage of indicated proliferating cells (A-D). Gray bars represent alloresponse, white bars represent xenoresponse, and black bars represent xenoresponse in the presence of blocking MHC class I and II antibodies. The bar graphs also show the amounts of IL-2 and IFN- produced by indicated proliferating cells (E, F) (n=3 average of three independent experiments).

REFERENCES

1. Ingulli, E. 2010. Mechanism of cellular rejection in transplantation. Pediatr Nephrol 25:61-74.

2. Abouna, G. M. 2008. Organ shortage crisis: problems and possible solutions. Transplant Proc 40:34-38.

3. Saidi, R. F., and S. K. Hejazii Kenari. 2014. Challenges of organ shortage for transplantation: solutions and opportunities. Int J Organ Transplant Med 5:87-96.

4. Dorling, A., K. Riesbeck, A. Warrens, and R. Lechler. 1997. Clinical xenotransplantation of solid organs. Lancet 349:867-871.

5. Samstein, B., and J. L. Platt. 2001. Physiologic and immunologic hurdles to xenotransplantation. J Am Soc Nephrol 12:182-193.

6. Galili, U. 2005. The alpha-gal epitope and the anti-Gal antibody in xenotransplantation and in cancer immunotherapy. Immunol Cell Biol 83:674-686.

7. Higginbotham, L., M. L. Ford, K. A. Newell, and A. B. Adams. 2015. Preventing T cell rejection of pig xenografts. Int J Surg 23:285-290.

8. Vadori, M., and E. Cozzi. 2014. Immunological challenges and therapies in xenotransplantation. Cold Spring Harb Perspect Med 4:a015578.

9. Brunswick, M., and P. Lake. 1986. Functional interactions of human and murine lymphoid cells. I. Analysis of primary and secondary responses. Cell Immunol 103:441-454.

10. Alter, B. J., and F. H. Bach. 1990. Cellular basis of the proliferative response of human T cells to mouse xenoantigens. J Exp Med 171:333-338.

11. Lucas, P. J., G. M. Shearer, S. Neudorf, and R. E. Gress. 1990. The human antimurine xenogeneic cytotoxic response. I. Dependence on responder antigen-presenting cells. J Immunol 144:4548-4554.

12. Dorling, A., G. Lombardi, R. Binns, and R. I. Lechler. 1996. Detection of primary direct and indirect human anti-porcine T cell responses using a porcine dendritic cell population. Eur J Immunol 26:1378-1387.

13. Tahara, H., K. Ide, N. Basnet, Y. Tanaka, and H. Ohdan. 2010. Determination of the precursor frequency and the reaction intensity of xenoreactive human T lymphocytes. Xenotransplantation 17:188-196.

14. Yamada, K., D. H. Sachs, and H. DerSimonian. 1995. Human anti-porcine xenogeneic T cell response. Evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. J Immunol 155:5249-5256.

15. Batten, P., T. Heaton, S. Fuller-Espie, and R. I. Lechler. 1995. Human Anti-Mouse Xenorecognition. Provision of Noncognate Interactions Reveals Plasticity of T Cell Repertoire. The Journal of Immunology 155:1057-1065.

16. Lucas, P. J., C. V. Bare, and R. E. Gress. 1995. The human anti-murine xenogeneic cytotoxic response. II. Activated murine antigen-presenting cells directly stimulate human T helper cells. J Immunol 154:3761-3770.

17. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. Nature 392:245-252.

18. Barzaga-Gilbert, E., D. Grass, S. K. Lawrance, P. A. Peterson, E. Lacy, and V. H. Engelhard. 1992. Species specificity and augmentation of responses to class II major histocompatibility complex molecules in human CD4 transgenic mice. Journal of Experimental Medicine 175:1707-1715.

19. Moses, R. D., H. J. Winn, and H. Auchincloss Jr. 1992. Evidence that Multiple Defects in Cell-Surface Molecule Interactions Across Species Differences are Responsible for Diminished Xenogeneic T Cell Responses. Transplantation 59:203-209.

20. Cunningham, A. C., T. J. Butler, and J. A. Kirby. 1994. Demonstration of direct xenorecognition of porcine cells by human cytotoxic T lymphocytes. Immunology 81:268-172.

21. Engelhard, V., A.-X. Le, and M. Holterman. 1988. Species-specific structural differences in the alpha 1+ alpha 2 domains determine the freqeuncy fo murine cytotoxic T cell precursors stimulated by human and murine class I molecules. The Journal of Immunology 141:1835-1839.