Brief Definitive Report

Two Levels of Help for B Cell Alloantibody Production

By David J. R. Steele,*‡ Terri M. Laufer,§ Stephen T. Smiley,§ Yuichi Ando,* Michael J. Grusby,§ Laurie H. Glimcher,§ and Hugh Auchincloss, Jr.*

*From the *Transplant Unit, Surgical Services, and ‡Renal Unit, Medical Services, Massachusetts General Hospital; and §Department of Cancer Biology, Harvard School of Public Health, and Department of Rheumatology and Immunology, Harvard Medical School, Boston, Massachusetts 02114*

Summary

We have examined whether T cell stimulation by direct or indirect pathways contributes to alloantibody production by B cells after major histocompatibility complex (MHC)-disparate skin graft rejection in mice. Experiments were performed using normal mice, MHC class II–deficient mice, MHC class II–deficient mice with an intact peripheral $CD4^+$ cell population (due to expression of class II antigens only on thymic epithelium), mice lacking the cytoplasmic tail of their MHC class II antigens, and mice depleted of $CD4^+$ cells by anti-CD4 monoclonal antibody treatment. Depletion of recipient $CD4^+$ cells reduced alloantibody production to barely detectable levels. Absence of donor MHC class II antigens did not affect the production of either immunoglobulin (Ig)M or IgG antibodies directed at class I alloantigens. Absence of recipient MHC class II antigens, however, led to production of only IgM but not IgG antibodies, even if the recipients had an intact $CD4^+$ cell population. Absence of the cytoplasmic tail of the recipient's MHC class II antigens led to the production of slightly reduced amounts of IgG antibody. These findings indicate that (*a*) $CD4^+$ cells are essential helper cells for B cell alloantibody production; (*b*) production of IgM alloantibody can occur with help from $CD4^+$ cells, which recognize either donor class II antigens or modified recipient class II antigens; (*c*) isotype switching from IgM to IgG alloantibody requires help from $CD4^+$ cells activated by antigens presented by recipient MHC class II molecules; and (*d*) the cytoplasmic domain of the recipient MHC class II molecules may be involved in the mechanism that leads to isotype switching by B cells. Thus, there are two levels of CD4-mediated help available for B cells responding to alloantigens: one (involving a noncognate interaction) can produce B cell activation, and a second (involving a cognate interaction) is required for differentiation and IgG alloantibody production.

T cells help B cells to produce antibody against T cell–dependent antigens and to switch the isotype of the antibody produced from IgM to other isotypes, including IgG. This T–B cell interaction has been characterized by studies of immune responses to hapten-carrier conjugates and shown to be MHC class II antigen restricted (1, 2). In the case of allogeneic tissue transplantation, helper T cells can be activated directly by class II antigens expressed on donor APC, or indirectly by peptides of donor antigens presented by self class II molecules on recipient APC. Two different schemes have therefore been proposed by which T–B cell collaboration might occur for B cell responses to class I alloantigens expressed on foreign cells (Fig. 1). First, the B cells could be stimulated by class I antigens on donor APC, and the $CD4^+$ helper cells could be stimulated by MHC class II antigens on the same cells. This would bring the T and B cells into physical proximity, but without an interaction between the T cell receptor and the B cell's class II antigens. Thus, this would be a "noncognate" interaction. Second, the B cells could be stimulated by class I antigens on donor cells, and the $CD4^+$ cells could be stimulated by foreign peptides presented by class II antigens on the recipient's own APC. These $CD4^+$ cells could then interact with the B cells in a "cognate" fashion, achieved by the class II–restricted T cell recognition of foreign peptides presented by the B cells.

Using mice that lack MHC class II antigens, as either donors or recipients of MHC-disparate skin grafts, we have examined which of these pathways of interaction leads to the production of alloantibody. The results demonstrate the importance of the indirect pathway in allogeneic responses

This work was presented in part at the American Society of Nephrology meeting in Orlando, FL, October 26–29, 1994.

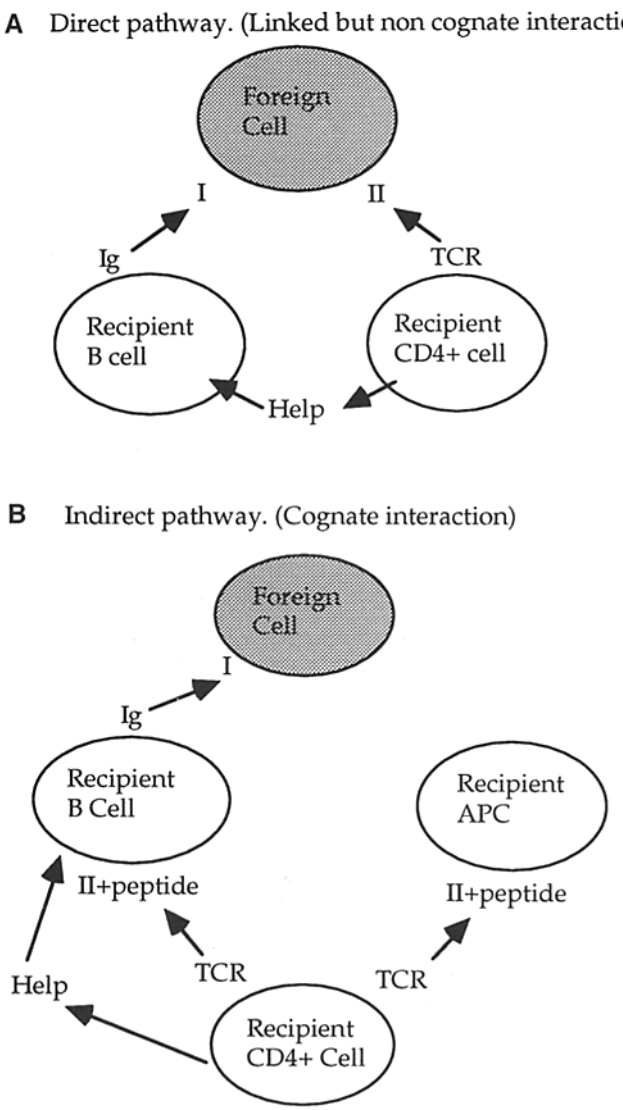

Figure 1. Two possible schemes by which T cells might be stimulated to provide help for B cell anti–class I alloantibody production. (*A*) B cells could be stimulated by class I antigens on the surface of donor APC, and $CD4^+$ helper cells could be stimulated directly by the donor class II antigens on the same APC. This would bring the T and B cells into physical contact but would not allow a cognate interaction between the responding T cell receptor with the MHC class II molecule of the B cell. (*B*) B cells could be stimulated by antigens on donor cells, and $CD4^+$ cells could be stimulated indirectly by foreign peptides presented by the recipient's APC. These $CD4^+$ cells could then provide help for the B cells by a cognate interaction with the B cell's own class II antigens.

in vivo, and they also reveal that $CD4^+$ cells can provide two levels of help for B cells, depending on the nature of the T–B cell interaction.

Materials and Methods

Mice. Male BALB/cByJ (BALB/c) ($H\text{-}2^d$) and C57Bl/6J (B6) ($H\text{-}2^b$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice lacking MHC class II antigens (class II deficient), mice expressing MHC class II antigens only on their thymic epithelium ($CD4^+$ class II deficient), and mice expressing class II antigens with truncated cytoplasmic domains (tailless), were bred in our laboratories.

The genotype and phenotype of mice lacking MHC class II antigens have been previously described (3). For these experiments, mice from the third generation backcross of (B6 × 129)F1 to B6 mice were used. Prior characterization of the class II–deficient mice has shown that no class II antigen expression can be detected. These mice also show a substantial depletion of $CD4^+$ peripheral T cells, although 3–5% of the peripheral $Thy1^+$ cells are $CD4^+$. Class II–deficient mice have normal levels and development of B cells since T cell–independent B cell responses occur in vivo, and T cell–dependent B cell activation can occur in vitro, despite the absence of MHC class II antigens (4).

Mice expressing MHC class II antigens only on their thymic epithelium were generated by breeding class II–deficient mice with a C57B1/6-transgenic strain (gift of Dr. D. Lo, Scripps Research Institute, La Jolla, CA), which expresses the Eα transgene only on thymic epithelium (5). These mice are MHC class II deficient on all cells other than thymic epithelium, but they do have normal numbers of peripheral $CD4^+$ cells, which proliferate in an allo MLR in vitro (Laufer, T., unpublished observations).

Mice that express a class II A^b molecule with a truncated cytoplasmic domain were generated by mating class II–deficient mice with mice expressing an $A_\beta{}^b$ transgene that lacks 13 of its 18 cytoplasmic domain residues. The initial characterization of these mice has been previously reported, demonstrating that they develop normal levels and functions of $CD4^+$ T cells and have normal B cell responses to environmental pathogens (6).

CD4-depleting Therapy. To deplete $CD4^+$ cells in vivo, thymectomized B6 mice were treated with 0.1 ml of GK1.5 ascites on days 6, 3, and 1 before skin grafting. This technique depletes >98% of $CD4^+$ cells for several weeks (7).

Skin Grafting. Trunk skin grafts were placed on mice according to the technique of Billingham and Medawar (8). Mice were anesthetized with chloral hydrate supplemented with ether. After rejecting their grafts, mice were then bled from the retroorbital plexus and subsequently boosted with 10^5 splenocytes of the same donor type by intraperitoneal injection at weekly intervals for 3 wk. Sera used for the experiments shown were all obtained 3 wk after placing the skin grafts.

Antibody-mediated Complement-dependent Cytotoxicity Assay. These assays were performed in two stages with minor modifications from the technique previously described (9). Sera were heat inactivated at 56°C for 30 min and stored at −20°C until tested. 10^5 ^{51}Cr-labeled mouse splenocytes of donor type were incubated with serially diluted sera in a final volume of 50 μl in U-bottomed wells for 15 min at 37°C. Cells were washed with medium, pelleted, resuspended, and incubated with rabbit complement (C-6 Diagnostic, Inc., Mequon, WI) at a final dilution of 1:12 for 30 min at 37°C. Cells were pelleted and 100 μl of the supernatant harvested and counted on a gamma counter (Gamma 4000; Beckman Instruments, Inc., Fullerton, CA). Percentage of lysis was calculated by comparison to the ^{51}Cr release obtained using a rabbit anti–mouse lymphocyte serum made in our laboratory. To inactivate antibody of the IgM isotype, 50 μl of serum was incubated with 5 μl of 1 M 2-ME for 30 min at 37°C before the assay.

Antibody Staining and Flow Cytometric Analysis. Thymic T cells were used as targets for flow cytometric analysis of alloantibody production. FACS® medium contained PBS, 1% BSA, and 0.1% sodium azide. 3 × 10^5 cells were added to each well of a 96-well U-bottomed plate. Mouse FcgIIR was blocked with 20 μl of 2.4 G2, a rat anti–mouse FcgIIR mAb, by incubating with the cells at

4°C for 10 min. The cells were then washed twice with 100 μl of FACS® medium. 25 μl of serum from mice that had rejected skin grafts was then added at 1:2 and 1:4 dilutions to the test wells and incubated at 4°C for 30 min. Cells were again washed twice with 100 μl FACS® solution. FITC-conjugated rat anti-mouse antibodies against IgM, IgG1 (Zymed Laboratories, Inc., South San Francisco, CA), IgG2a, or IgG2b (Baxter Healthcare Corp., Mundelein, IL) were then added and incubated with the cells at 4°C for 30 min. Cells were again washed twice with 100 μl FACS® solution, fixed with 2% paraformaldehyde, and analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Results

To generate alloantibody production, skin grafts from MHC-disparate allogeneic donors were placed on recipient mice. All skin grafts were rejected within 15 d, and neither treatment with anti-CD4 mAb nor the absence of MHC class II antigens on the donor or recipient altered the time to rejection when compared with normal controls. These findings are in keeping with previously reported data (9–11). Sera from recipient mice were tested 3 wk after grafting for the presence of alloantibody, as shown in Fig. 2. Additional sera from later time points were also tested, but the results in all groups were not different in character from those shown.

Normal Mice. Normal recipients of normal MHC-disparate skin grafts produced antidonor cytotoxic alloantibody of IgM isotype and then increasing titers of IgG isotype. The levels at 3 wk are shown in Fig. 2 *A*. The antibodies in these sera were shown to be directed at class I alloantigens by testing them on targets from MHC-congenic strains (data not shown).

CD4-depleted Recipients. Recipients treated with thymectomy and anti-CD4 mAb before skin grafting produced very low or undetectable levels of cytotoxic alloantibody, and there was no switch to IgG isotype, as shown in Fig. 2 *B*. This requirement for $CD4^+$ cells to generate alloantibody production is in agreement with previously reported data (9).

MHC Class II–deficient Mice as Donors. When MHC class II–deficient mice were used as skin graft donors for normal recipients, both IgG and IgM alloantibody production was equivalent to normal controls, as shown in Fig. 2 *C*. Thus, $CD4^+$ cells stimulated only through the indirect pathway,

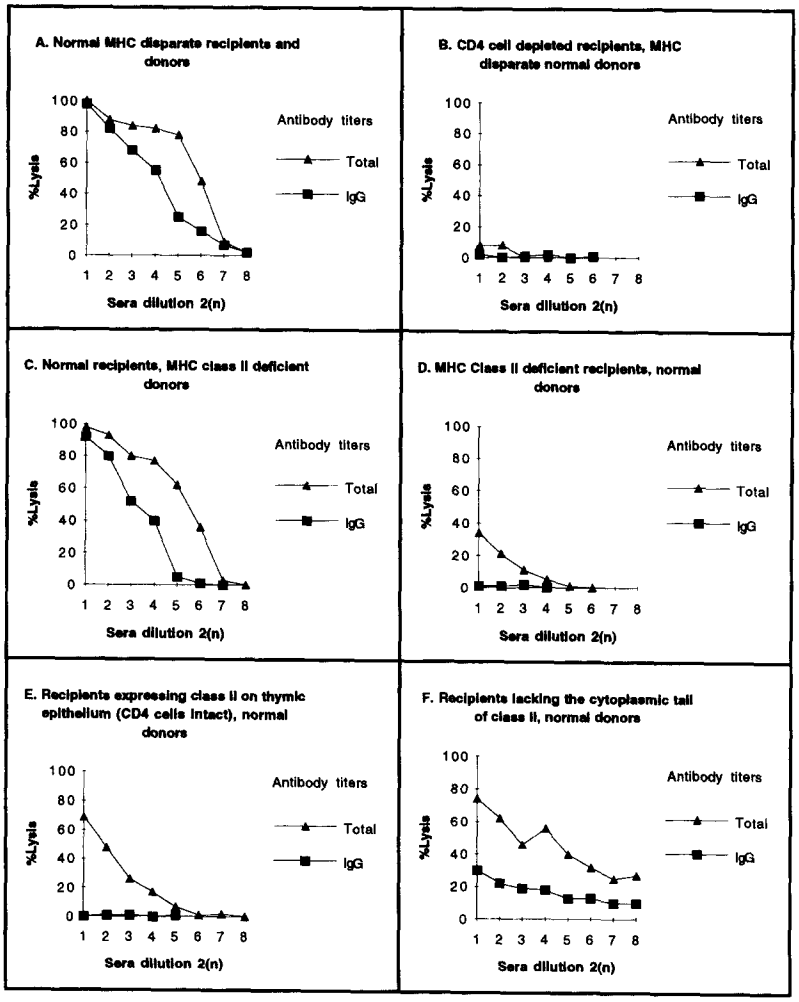

Figure 2. Cytotoxic alloantibody levels in response to skin grafting. Sera were obtained 3 wk after grafting, and alloantibody levels were determined in a ^{51}Cr release antibody-mediated complement-dependent cytotoxicity assay using mouse splenocytes of donor type as targets. IgM combined with IgG levels were measured using untreated sera. IgG levels alone were obtained after treatment of sera with 2-ME. The data presented represent means of lysis by sera from at least four mice in each panel shown. (*A*) B6 mice ($H\text{-}2^b$) received skin grafts from MHC-disparate BALB/c ($H\text{-}2^d$) donors. Almost identical results were obtained when BALB/c recipients received B6 skin grafts (data not shown). (*B*) CD4-depleted B6 mice received skin grafts from BALB/c donors. (*C*) Normal BALB/c recipients received skin grafts from B6 MHC class II–deficient donors. (*D*) B6 MHC class II–deficient recipients received skin grafts from BALB/c donors. (*E*) B6 MHC class II–deficient recipients expressing class II antigens only on their thymic epithelium received grafts from BALB/c donors. (*F*) B6 class II–deficient mice expressing a class II transgene that encodes class II antigens lacking their cytoplasmic tail received BALB/c skin grafts.

responding to modified self class II antigens, provide help efficiently for alloantibody production.

MHC Class II–deficient Mice as Recipients. MHC class II–deficient mice that received normal skin grafts produced cytotoxic alloantibodies, but in reduced titers when compared with normal controls. The antibody produced was of IgM isotype only, and no IgG was found in these recipients, both in assays for antibody-mediated complement-dependent cytotoxicity as shown in Fig. 2 *D* and by FITC anti–antibody staining (data not shown).

Although the donor skin grafts in these experiments expressed class II antigens that could stimulate $CD4^+$ cells directly, the recipients had two defects that could account for their reduced antibody production: they lacked class II antigens on their own cells for use in the indirect pathway, and they also had severely reduced numbers of $CD4^+$ cells to provide help for B cells. Therefore, to test whether direct stimulation, in the absence of the indirect pathway, could provide help for B cells, we needed recipients without class II antigen expression but that had normal numbers of $CD4^+$ cells.

Mice Expressing Class II Antigens Only on Thymic Epithelium as Recipients. Class II–deficient mice that express a class II transgene only on thymic epithelium develop normal numbers of mature $CD4^+$ cells but still lack class II antigens on their peripheral APC. When these mice were recipients of normal skin grafts, they produced only moderate titers of total cytotoxic antibody, all of which was again of IgM isotype, as shown in Fig. 2 *E*. These results were consistent in antibody-mediated complement-dependent assays and in assays using FITC anti-antibody staining (data not shown). Thus, in the presence of direct but in the absence of indirect T cell stimulation, $CD4^+$ cells can provide help for B cells, but only for a limited response.

Mice with Tailless Class II Molecules as Recipients. Finally, recipients lacking the intracytoplasmic domain of their MHC class II antigens were found to produce alloantibody that included both IgM and IgG isotypes. However, in two separate experiments, there was a slight reduction in the IgG component of the total cytotoxic antibody response compared with normal animals (Fig. 2 *F*).

Discussion

The primary purpose of these experiments was to determine the answer to a long-standing question in transplantation immunology: whether donor class II antigens stimulate helper $CD4^+$ cells for alloantibody production by direct recognition, or whether recipient class II molecules, presenting peptides of donor antigens, stimulate a helper response through what has been termed the indirect pathway. We expected that the indirect pathway would be sufficient, since this is the mechanism for a normal immune response. We also expected, based on previous studies, that both IgM and IgG alloantibody responses after skin transplantation would depend on the presence of $CD4^+$ T cells (9). Finally, we were not surprised that stimulation of $CD4^+$ cells only by the nonphysiologic, direct pathway was unable to stimulate IgG antibody production. These experiments, therefore, settle the issue in transplantation immunology, at least as far as the response to class I antigens on skin grafts. In doing so they also provide further evidence that the so-called indirect pathway in transplantation does play a role in the immune response to foreign tissues and that this pathway is actually critical as far as alloantibody production is concerned.

The result we did not expect (which was, therefore, particularly interesting) was that $CD4^+$ cells stimulated by the direct pathway alone would allow an intermediate B cell response, including IgM but not IgG alloantibody production. This result is of interest beyond the field of transplantation immunology since it indicates that B cells are susceptible to two levels of help for antibody responses. When $CD4^+$ cells are stimulated in a way that links them in physical proximity to B cells, their helper factors can apparently activate the B cells partially, allowing the IgM response. However, apparently only when the $CD4^+$ cells are stimulated in a way that provides both linkage and a cognate interaction through the class II molecules of the B cells does the helper response signal the B cells to switch to IgG production. Only a transplantation experiment, especially one involving the recently available class II knock-out mice, would allow us to see this distinction in vivo. In no other way could one bring B cells, recognizing a foreign protein antigen, into a physical but noncognate linkage with $CD4^+$ helper cells than to stimulate the $CD4^+$ cells with allogeneic class II molecules while eliminating the class II molecules of the recipient's own APC. Thus, these transplantation experiments provide new insight into the mechanisms of normal B cell physiology.

Our experiments have not yet proven that a physical linkage is required between the $CD4^+$ cells, stimulated by donor class II antigens, and the B cells that respond to donor class I alloantigens. It seems likely that such linkage is necessary, given the many previous experiments indicating that linkage between T and B cells is a physiologic requirement in vivo (1, 2, 12). However, to demonstrate this requirement in our model we will need to determine whether B cells from mice lacking their own peripheral class II antigen expression can respond to donor alloantigens, such as Thy1, which are not expressed on the same cells that express the class II alloantigens that stimulate $CD4^+$ helper cells.

It is also not clear from our experiments exactly why the cognate interaction between T and B cells, through recognition of the B cells' class II antigens, should be important in the switch to IgG antibody production. One explanation is that such an interaction is simply the best way to achieve the closest possible physical proximity between helper cells and B cells. The other is that the class II molecules of B cells are involved in the signaling that allows conversion to an IgG response. Previous in vitro studies with transfected cell lines have suggested that the cytoplasmic tail of class II molecules is necessary for the expression of costimulatory molecules (13–16), but subsequent studies with the same transgenic mice used in our experiments, which express

only class II molecules with a truncated intracytoplasmic domain, have indicated that they produce normal IgG responses to nominal antigens, although they appear to have a quantitative defect in their ability to present peptides of foreign proteins (6). In our experiments we found that both IgM and IgG alloantibody responses were fundamentally intact in the mice lacking their class II intracytoplasmic domain, although the IgG response appeared to be weaker in several experiments. Thus, our results are consistent either with the interpretation that slightly diminished presentation of peptides by these truncated class II molecules weakens the physical association of T and B cells, or that the absence of the cytoplasmic portion of the B cells' class II molecules impairs the signaling that leads to IgG conversion.

This work was supported in part by U.S. Public Health Service grants HL-36372 and HL-18646 (H. Auchincloss) and AI21569 (L. H. Glimcher) and the Mathers Foundation (L. H. Glimcher).

Address correspondence to Hugh Auchincloss, Jr., White 510B, Massachusetts General Hospital, Fruit Street, Boston, MA 02114.

Received for publication 22 August 1995 and in revised form 3 October 1995.

References

1. Parker, D.C. 1993. T cell-dependent B-cell activation. *Annu. Rev. Immunol.* 11:331–360.
2. Defranco, A.L. 1993. B lymphocyte activation. *In* Fundamental Immunology. W.E. Paul, editor. Raven Press, New York. 505:529.
3. Grusby, M.J., R.S. Johnson, V.E. Papaioannou, and L.H. Glimcher. 1991. Depletion of $CD4^+$ T cells in major histocompatibility complex class II-deficient mice. *Science (Wash. DC).* 253:1417–1420.
4. Markowitz, J.S., P.R. Rogers, M.J. Grusby, D.C. Parker, and L.H. Glimcher. 1993. B lymphocyte development and activation independent of MHC class II expression. *J. Immunol.* 150:1223–1233.
5. Burkly, L.C., S. Degermann, J. Longley, J. Hagman, R.L. Brinster, D. Lo, and R.A. Flavell. 1993. Clonal deletion of V beta 5^+ T cells by transgenic I-E restricted to thymic medullary epithelium. *J. Immunol.* 151:3954–3960.
6. Smiley, S.T., T.M. Laufer, D. Lo, L.M. Glimcher, and M.J. Grusby. 1995. Transgenic mice expressing MHC class II molecules with truncated $\alpha\beta$ cytoplasmic domains reveal signaling-independent defects in antigen presentation. *Int. Immunol.* 7:665–677.
7. Ghobrial, R.R.M., M. Boublik, H.J. Winn, and H.J. Auchincloss. 1989. In vivo use of monoclonal antibodies against murine T cell antigens. *Clin. Immunol. Immunopathol.* 52:486–506.
8. Billingham, R.E., and P.B. Medawar. 1951. Technique of free skin grafting in mammals. *J. Exp. Biol.* 28:385–402.
9. Auchincloss, H.J., R.R.M. Ghobrial, P.S. Russell, and H.J. Winn. 1988. Anti-L3T4 in vivo prevents alloantibody formation after skin grafting without prolonging graft survival. *Transplantation.* 45:1118–1123.
10. Auchincloss, H.J., T. Mayer, R. Ghobrial, and H.J. Winn. 1989. T cell subsets, bm mutants, and the mechanism of allogeneic skin graft rejection. *Immunol. Res.* 8:149–164.
11. Auchincloss, H.J., 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.* 90:3373–3377.
12. Clark, E.A., and J.F. Ledbetter. 1994. How B and T cells talk to each other. *Nature (Lond.).* 367:425–428.
13. Nabavi, N., Z. Ghogawala, A. Myer, I.J. Griffith, W.F. Wade, Z.Z. Chen, D.J. McKean, and L.H. Glimcher. 1989. Antigen presentation abrogated in cells expressing truncated Ia molecules. *J. Immunol.* 142:1444–1447.
14. Nabavi, N., G.J. Freeman, A. Gault, D. Godfrey, L.M. Nadler, and L.H. Glimcher. 1992. Signalling through the MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression. *Nature (Lond.).* 360:266–268.
15. Harton, J.A., and G.A. Bishop. 1993. Length and sequence requirements of the cytoplasmic domain of the A beta molecule for class II-mediated B cell signaling. *J. Immunol.* 151: 5282–5289.
16. St.-Pierre, Y., N. Nabavi, Z. Ghogawala, L.H. Glimcher, and T.H. Watts. 1989. A functional role for signal transduction via the cytoplasmic domains of MHC class II proteins. *J. Immunol.* 143:808.