IMMUNOHEMATOLOGY

Immune responsiveness against allogeneic platelet transfusions is determined by the recipient's major histocompatibility complex class II phenotype

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BACKGROUND: Immunoglobulin G (IgG) anti-platelet (PLT) immunity has been shown to be initiated by indirect allorecognition where recipient T cells recognize donor PLT antigens presented by class II molecules encoded by the major histocompatibility complex (MHC) on recipient antigen-presenting cells. To understand how the recipient's MHC class II molecules may influence PLT alloimmunity, immune responsiveness against transfused PLTs was tested in different mouse strains.

STUDY DESIGN AND METHODS: Various inbred and mutant mouse strains were transfused with allogeneic PLTs and IgG donor antibodies were measured by flow cytometry.

RESULTS: When recipient mice, expressing both MHC class II I-A and MHC class II I-E molecules, were transfused weekly with allogeneic PLTs, high titers of IgG donor antibodies were generated. In comparison, however, recipient mice expressing only MHC class II I-A molecules had significantly (p < 0.001) reduced IgG antibody responsiveness against PLT transfusions. The low IgG responder status against allogeneic PLT transfusions was rescued in transgenic mice expressing I-E molecules and in mice genetically deficient in either β_2-microglobulin or CD8+ T cells.

CONCLUSION: IgG immune responsiveness against allogeneic PLT transfusions is dependent on recipient expression of I-E MHC class II molecules, whereas I-A expression is linked with CD8-mediated suppression of PLT immunity. The data suggest that strategies to modify recipient MHC class II presentation of donor PLT antigens would be effective in eliminating PLT alloimmunity.

The development of immunoglobulin G (IgG) donor antibodies in patients exposed to allogeneic platelet (PLT) transfusions can lead to a state of clinical refractoriness where the patient destroys transfused donor PLTs and thus derives no benefit from subsequent transfusions.[1] The antibodies are usually IgG and directed against molecules encoded by the major histocompatibility complex (MHC) on the PLT surface. Although leukoreduction of PLTs has significantly reduced the incidence of these antibodies, the largest randomized prospective clinical trial demonstrated that approximately 19 percent of recipients still became alloimmunized.[2] Understanding the nature of these antibodies, particularly with respect to the immune mechanisms responsible for their production, is fundamental to designing specific immunotherapies to further reduce their incidence.

The production of IgG alloantibodies can be initiated by two recipient T-cell recognition mechanisms. The first is termed the direct pathway and occurs when the T-cell

receptors of recipient CD4+ T cells directly interact with MHC class II molecules on donor antigen-presenting cells (APCs).[3] The second pathway is analogous to the normal immune response and is termed the indirect pathway.[4-10] This mechanism occurs when allogeneic non-APCs are administered to the recipient and it involves the processing and presentation of allelic donor antigens (e.g., MHC class I molecules) by APCs to Th cells of the recipient.[4-10] With respect to PLT alloimmunization, although direct allorecognition is primarily removed by leukoreduction strategies, the indirect pathway is still available for a recipient to mount an alloantibody response against donor PLT antigens.

With a murine model of PLT alloimmunization, Kao[11] demonstrated that transfusions of allogeneic leukoreduced PLTs generated anti-donor MHC class I alloantibodies by the third transfusion. Subsequently, it was demonstrated that the IgG alloantibody production was due to immune mechanisms consistent with indirect allorecognition.[12-16] For example, it was found that MHC class II antigen processing pathways within recipient APCs were important in not only stimulating PLT alloimmunity, but also in regulating the alloantibody response against the donor PLTs.[16] Nevertheless, the nature of how these processing pathways may interact with and make PLT antigens available for MHC class II molecules and presentation is unknown.

In mice, MHC class II molecules are encoded by genes within the I region of the MHC on chromosome 17.[19] They are expressed on such APCs as dendritic cells, epidermal Langerhans cells, and macrophages and are responsible for presenting antigenic peptides to CD4+ Th cells.[17] MHC class II molecules are heterodimeric structures, consisting of noncovalently assembled α and β chains. Two sets of MHC class II molecules exist in mice and are designated I-A and I-E.[17] Based on crystal structure, murine I-A molecules are considered the counterparts of human HLA-DQ molecules whereas I-E molecules are analogous to HLA-DR molecules.[18] Most murine strains express both MHC class II I-A and I-E molecules; however, strains of the $H2^b$, $H2^s$, and $H2^q$ haplotypes do not express I-E class II molecules because of a I-E α chain gene deletion.[17] These mice have allowed the mapping of immune responsiveness against a number of antigens and suggest that MHC class II molecules are critical elements that determine the immune response of a host against foreign protein antigens.[17]

The objective of this study was to examine the influence of the recipient's MHC class II haplotype on PLT alloimmunization. The results suggest that IgG immune responsiveness against trans-fused PLTs is dependent on recipient expression of MHC class II I-E molecules, whereas recipient I-A molecules are linked with CD8+ T-cell-mediated regulation of the response. The data suggest that manipulating recipient PLT antigen presentation events on MHC class II molecules could be an effective therapy to eliminate PLT alloimmunization.

MATERIALS AND METHODS

Animals

Inbred female BALB/c ($H2^d$), CBA ($H2^k$), A ($H2^a$), C57BL/6 ($H2^b$), 129P3 ($H2^b$), SJL ($H2^s$), and H2 congenic C.B10-$H2^b$/LilMcdJ (BALB.B, $H2^b$) mice, 8 to 10 weeks of age, were used as either PLT donors or recipients and purchased from Jackson Laboratories (Bar Harbor, ME). B6.129S2-Cd8a^{tm1Mak} CD8-knockout (KO, $H2^b$) and B6.129P2-B2m^{tm1Unc} β_2-microglobulin (β_2M, $H2^b$)-KO mice were used as PLT recipients and purchased from Jackson Laboratories. Breeding pairs of 107-1 I-E transgenic (Tg, $H2^b$) mice (obtained from R. Flavell, Yale University, New Haven, CT) were derived from B10 mice and back-crossed onto the C57BL/6 background[19] and bred in the St. Michael's Hospital Research Vivarium. The 107-1 Tg mouse is a C57BL/6 mouse that carries an Eα transgene that allows the expression of MHC class II I-E molecules, along with I-A^b, on all thymic epithelial cells, B cells, macrophages, and dendritic cells[19] at levels equivalent to wild-type MHC class II I-E-expressing mouse strains.[20] The MHC haplotype of each mouse strain is shown in Table 1.

PLT preparation

The indicated donor mice were bled and PLTs were prepared as previously described.[15] Briefly, mice were bled under isofluorane anesthesia via the tail vein and cardiac puncture into ethylenediaminetetraacetate microvettes (Sarstedt, St. Laurent, Canada). The blood was pooled,

TABLE 1. H2 haplotypes of the murine strains and their PLT WBC levels									
		H2 loci							Number of
Strain	Haplotype	K	I-Aa	I-Ab	I-Ea	I-Eb	L	D	WBCs/μL*
BALB/c	d	d	d	d	d	d	d	d	1.3 ± 0.4
CBA	k	k	k	k	k	k	k	k	1.1 ± 0.3
A	a	k	k	k	k	k	d	d	1.4 ± 0.5
C57BL/6	b	b	b	b	−†	b	b	b	0.9 ± 0.3
129	b	b	b	b	−	b	b	b	
SJL	s	s	s	s	−	s	s	s	
BALB.B	b	b	b	b	−	b	b	b	
β₂M KO	b	b	b	b	−	b	b	b	
CD8 KO	b	b	b	b	−	b	b	b	
107-1 I-E Tg	b	b	b	b	d	b	b	b	

* WBCs were enumerated in 10 different PLT preparations from the indicated donor strains of mice by flow cytometry. Results are expressed as the mean ± SD of the calculated WBC/μL values.
† Indicates the strains that are I-Ea null.

centrifuged at $250 \times g$, and the PLT-rich plasma collected. The PLT-rich plasma was centrifuged at $1000 \times g$ for 18 minutes and PLTs were adjusted to a stock concentration of 10^9 per mL in phosphate-buffered saline for transfusion. White blood cells (WBCs) were enumerated by flow cytometry as previously described[21] and all were determined to be leukoreduced (Table 1).

Transfusion protocol and blood preparation

Recipient mice were bled 24 hours before the first transfusion and injected with 100 μL of stock PLTs weekly via the tail vein. Each week, blood was collected into red top microvettes (Sarstedt) and allowed 1 hour at 22°C to clot. Part of the collected fresh sera was used to determine antibody titers, whereas the remainder was frozen at –20°C and used for IgG isotype determinations. At the end of the 5-week protocol, mice were killed.

Flow cytometric detection of donor antibodies

For the detection of IgG donor antibodies, 10^6 donor spleen cells were incubated with titrations of fresh recipient sera for 45 minutes at 4°C, washed once, and labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Fc-specific, Cedarlane Laboratories, Hornby, Ontation, Canada) for 30 minutes at 4°C in the dark. Cells were analyzed by flow cytometry; 10,000 events were acquired using an electronic cellular (lymphocyte) gate based on forward and side scatter and were analyzed using computer software (CELLQUEST, Becton Dickinson,
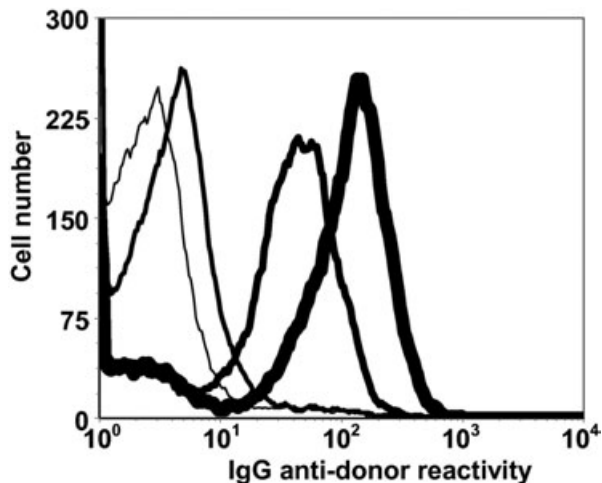
San Jose, CA; Fig. 1). Matched serum obtained before the first transfusion was used as the negative control in all experiments. Isotype characterization of the donor antibodies was performed using FITC-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 antibodies (Cedarlane Laboratories). The values for total IgG or IgG isotype levels were calculated as a fold increase ratio and calculated by the following formula:

$$\text{Mean channel fluorescence (MCF) of test serum} \div \\ \text{MCF of prebleed serum.}$$

Statistical analysis

The significance between means was determined by a t test.

RESULTS

When strains expressing both I-A and I-E molecules (BALB/c, CBA, or A mice) were transfused weekly with allogeneic PLTs from C57BL/6 mice, detectable IgG donor antibodies developed by the second transfusion (Fig. 2). IgG titers were determined after the fifth transfusion and found to be between 1:800 and 1:1600 in the recipient strains of mice. Characterization of IgG isotypes in the serum of BALB/c recipients showed that the T helper (Th)1-associated IgG2a isotype was predominantly produced but detectable levels of IgG1, IgG2b, and IgG3 were also present (Fig. 3). In contrast, as also shown in Fig. 3, CBA recipients produced predominantly IgG2b donor antibodies against transfused PLTs although the IgG1,

Fig. 1. Flow cytometric histogram analysis of IgG donor antibody production in a C57BL/6 (—), CBA (━), and BALB/c (▬) mouse transfused weekly for 5 weeks. Typical examples are shown. Donor WBCs were incubated with a 1:100 dilution of sera obtained from the recipient mice after the fifth transfusion, labeled with FITC-goat anti-mouse IgG, and analyzed by flow cytometry. The prebleed sera (—) from a BALB/c mouse is shown.
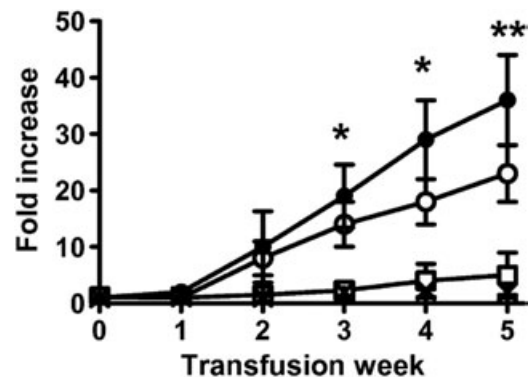

Fig. 2. IgG donor antibody development in BALB/c (●), CBA (○), C57BL/6 (□), 129 (×), and BALB.B (+) mice during five weekly allogeneic PLT transfusions. BALB/c and CBA mice were transfused with C57BL/6 PLTs and the C57BL/6, 129 and BALB.B mice were transfused with BALB/c PLTs. Fold increase was calculated by the formula MCF of test serum + MCF of prebleed serum. The results are expressed as the mean (± SD) of the fold increases in 15 mice from each group. Significance of the IgG response between BALB/c mice and C57BL/6 mice is shown: *p < 0.01; **p < 0.001.
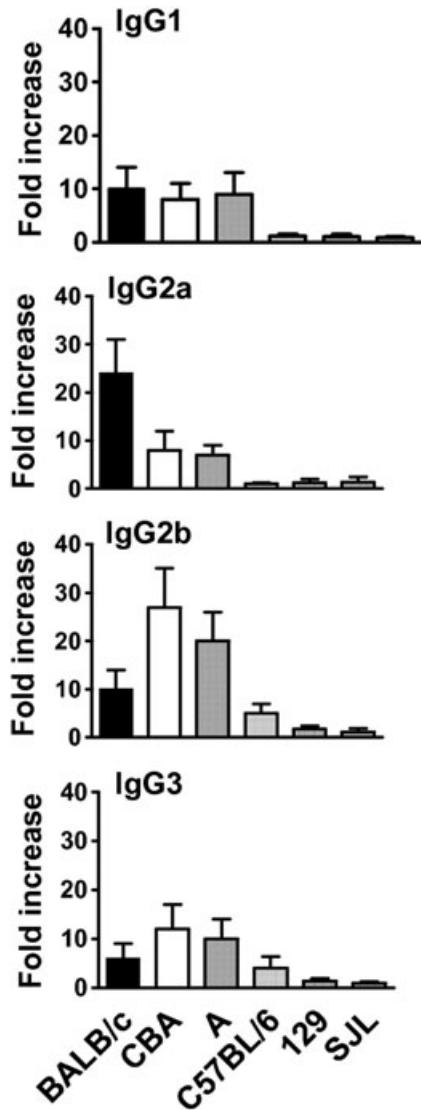
Fig. 3. Analysis of IgG1, IgG2a, IgG2b, and IgG3 isotypes in BALB/c, CBA, A, C57B46, 129, and SJL recipient mice after the fifth PLT transfusion. Fold increase was calculated by the formula MCF of test serum ÷ MCF of prebleed serum. The results are expressed as the mean (± SD) of the fold increases in 15 mice from each group.

Fig. 4. IgG donor antibody development in β_2M KO (●), CD8 KO (○), C57BL/6 (□), and 107 I-E Tg (◇) mice during five weekly allogeneic PLT transfusions. All the mice were transfused with BALB/c PLTs. Fold increase was calculated by the formula MCF of test serum ÷ MCF of prebleed serum. The results are expressed as the mean (± SD) of the fold increases in 15 mice from each group. Significance of the IgG response between CD8 KO mice and C57BL/6 mice is shown: *p < 0.01.

To determine if the low antibody response may be due to CD8+ T-cell-mediated suppression, CD8–/– and β_2M–/– KO mice on the C57BL/6 background were transfused with PLTs from BALB/c mice. Both KO mice produced a significant (p < 0.01) IgG donor antibody response against the PLT transfusions by the second transfusion (Fig. 4) week, which was composed primarily of the IgG2a isotype (Fig. 5).

The role of I-E MHC class II expression on IgG alloantibody production was assessed by transfusing 107-I-E Tg mice with allogeneic PLTs from CBA mice. I-E expression in the Tg mice rescued the IgG low responsiveness of the C57BL/6 background; the Tg mice produced an IgG donor antibody response in a similar fashion to that of BALB/c mice. The Tg mice produced high-titered (1:800) IgG donor antibodies by the third transfusion (Fig. 4) composed predominantly of the IgG2a isotype, but detectable IgG1, IgG2b, and IgG3 isotypes were also observed (Fig. 5). A summary of the recipient IgG anti-donor isotype responses against all donor PLTs is shown in Table 2.

DISCUSSION

Indirect allorecognition occurs when allogeneic donor proteins are processed and presented by recipient APC to recipient Th cells.[3-10] This pathway has been previously shown to be responsible for PLT alloimmunization in murine models.[12-16] Because of this, the present study was designed to address the influence of recipient MHC class II molecules on PLT immunity. Our results show that one set of recipient MHC class II molecules (I-E) are responsible for stimulating IgG immune responsiveness against

IgG2a, and IgG3 isotypes were also observed. Similar results were observed in A strain recipient mice.

To determine the effects of I-A MHC class II molecules on anti-PLT immune responsiveness, C57B46, 129, or SJL mice were transfused with either BALB/c or CBA mouse PLTs for 5 weeks and tested for the presence of IgG donor antibodies. C57BL/6 recipient mice mounted a low antibody response against only BALB/c PLTs detectable by the fifth transfusion (Fig. 2) and characterized by the production of Ig2b and IgG3 isotypes (Fig. 3). In contrast, BALB.B, SJL, and 129 recipient mice were IgG nonresponders against all allogenic PLT transfusions (Figs. 2, 3).
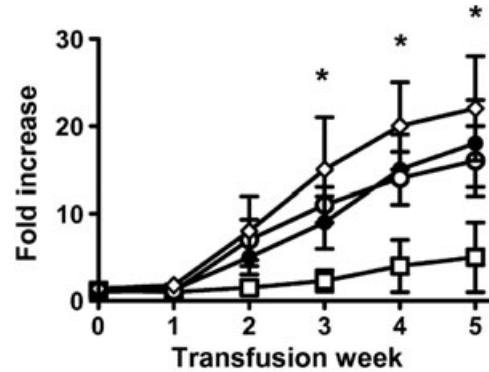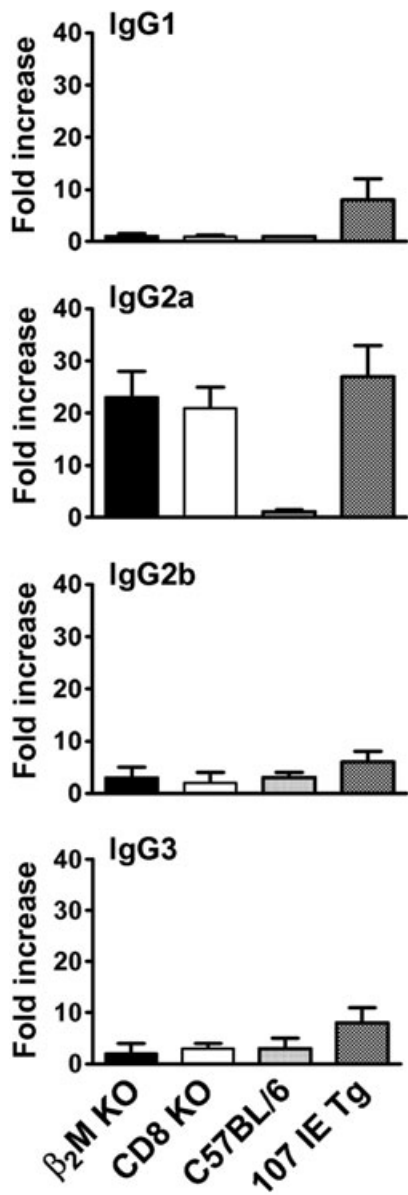
Fig. 5. Analysis of IgG1, IgG2a, IgG2b, and IgG3 isotypes in β_2M KO, CD8 KO, C57BL/6, and 107 I-E Tg mice after the fifth PLT transfusion. Fold increase was calculated by the formula MCF of test serum ÷ MCF of prebleed serum. The results are expressed as the mean (± SD) of the fold increases in 15 mice from each group.

TABLE 2. Summary of recipient IgG anti-donor isotype responses against the indicated donor PLT transfusions

Recipient mice	Donor PLT source			
	BALB/c	CBA	C57BL/6	A
I-A+/I-E+				
BALB/c	○○○○*	◐●●●	◐●●●	◐●●●
CBA	○○●◐	○○○○	○○●◐	○○●◐
A	○○●◐	○○●◐	○○●◐	○○○○
107-1 I-E Tg	◐●●●	◐●●●	○○○○	ND†
I-A+/I-E−				
C57BL/6	○○○○	○○○○	○○○○	○○○○
129	○○○○	○○○○	ND	○○○○
BALB.B	○○○○	○○○○	ND	○○○○
SJL	○○○○	○○○○	ND	○○○○
β_2M KO	○●○◐	○●○◐	○○○○	○●○◐
CD8 KO	○●○◐	○●○◐	○○○○	○●○◐

* The circles represent from left to right IgG1, IgG2a, IgG2b, and IgG3 anti-donor isotypes and the shading represents the strength of the response: (○) not detected; (◐) 2- to 10-fold increase; (◖) 10- to 20-fold increase; (●) > 20-fold increase.
† ND = not determined.

predominately IgG2b responses (Fig. 3). The reasons for this are unclear, but others have demonstrated that various inbred strains of mice can produce different isotypes of donor antibodies against both allo- and xenoantigens.[22-25] Alternatively, the MHC-dependent selection of different populations of T cells able to respond against alloantigens may be different in the two strains.[26] In addition, it has been shown that epitope presentation by different MHC class II molecules can significantly influence the outcome of Th1/Th2 responses.[27,28] Furthermore, I-E expression has been shown to alter the T-cell repertoire through clonal selection in the thymus. For example, Huber and colleagues[29] demonstrated that the T-cell repertoires in the spleens of C57BL/6 (I-A+I-E–) and 107-1 I-E Tg (I-A+/I-E+) mice differ substantially. Perhaps the way allogeneic PLT antigens are displayed on the recipient's MHC class II molecules during antigen presentation determines the outcome of the IgG isotype response against allogeneic PLT transfusions. These results support this notion in that the I-A+/I-E+ strains could produce Th2-associated IgG1 anti-donor isotypes, whereas the I-A+/I-E– KO mice, devoid of CD8+ T cells, produced primarily a Th1-associated IgG2a response (Fig. 3). These results suggest that expression of I-E MHC class II molecules is necessary for IgG immune responsiveness against allogeneic PLTs and this was confirmed with the 107-1 I-E Tg mice as PLT recipients; expression of I-E MHC class II molecules on the IgG low responder C57BL/6 strain enabled the recipient to mount a significant IgG immune response against PLTs. These mice produced an IgG antibody response in a similar manner to BALB/c recipients (Table 2) and indicate that I-E MHC class II molecules are the permissive restriction element that allow recipients to fully respond

PLTs, whereas the other MHC class II set of molecules (I-A) may significantly suppress the anti-PLT immune response via CD8+ T cells.

The I-A/I-E+ strains of mice (BALB/c, CBA, and A) responded against PLTs with a vigorous IgG anti-donor immune response (Figs. 2, 3). The major difference between the IgG responses was the differential production of IgG isotypes where BALB/c mice produced predominantly IgG2a, whereas CBA and A mice generated

against allogeneic PLTs. Taken together, it appears that the outcome of the IgG anti-donor response against allogeneic PLT transfusions is dictated by the particular MHC class II haplotype of the recipient.

When C57BL/6, 129, SJL, or BALB.B mice (all I-A+/I-E–) were transfused with allogeneic PLTs, a weak but detectable IgG immune response was generated only in the C57BL/6 recipients. Other studies have also demonstrated that C57BL/6 mice are low antibody responders to a number of different antigens.[30-32] Of interest was the observation that mice of the C57BL/6 background and knocked out for either β_2M or CD8+ T cells responded strongly to PLT transfusions but only by producing predominantly Th1-associated IgG2a isotypes. This suggests that recipient MHC class II I-A molecules have the ability to present PLT alloantigens to CD4+ T cells for antibody production, but these events are somehow linked to the generation of CD8+ T-cell-mediated suppression. The nature of this suppression is unknown, although Cosmi and coworkers[33] have recently demonstrated that CD8+CD25+ T cells can mediate suppression via production of soluble mediators such as transforming growth factor-β. Perhaps PLT-induced CD8+ T cells mediate suppression via mediators in I-A+/I-E– mouse strains. We are currently studying this.

With respect to the cellular regulation of PLT immunity, our previous results have shown that removal of CD8+ T cells from BALB/c I-A+/I-E+ mice enhances their ability to respond against PLTs but only by producing primarily IgG2a isotypes.[16] This, together with the present results in C57BL/6 mice, suggests that CD8+ T cells are responsible for modulating PLT immunity but that the degree of suppression is dictated by the MHC class II haplotype of the recipient. Thus, strains positive only for I-A MHC class II molecules present PLT peptides in a manner to set up a fully suppressive state mediated by CD8+ T cells. In contrast, the presence of I-E molecules in BALB/c mice lessens the CD8+ T-cell suppression and allows these cells to actually help generate a full IgG isotype (i.e., all isotypes produced) response against PLTs. It is not clear, however, how the CD8+ T cells actively down regulate the IgG anti-donor isotype production, but it may be due to activation of different CD4+ T cells selected on the recipient's MHC class II molecules.

In summary, the results suggest that I-A+/I-E+ mouse strains can mount a significant immune response against allogeneic PLTs, but in murine strains lacking I-E MHC class II molecules, the ability to generate an IgG immune response against PLTs is actively suppressed by CD8+ T cells. These data not only confirm that PLTs mediate immunity via indirect T-cell allorecognition on recipient MHC class II molecules but suggest that immunotherapies aimed at the recipient's MHC class II molecules can eliminate PLT-induced alloimmunization.

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