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Lethal host-versus-graft disease and hypereosinophilia in the absence of MHC I–T-cell interactions

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Introduction

Since the original experiments by Billingham, Brent, and Medawar (1), the model of neonatal tolerance to transplantation antigens has been extensively studied to analyze the different parameters promoting allograft acceptance. Although it has been proposed that in mice neonatally injected with semiallogeneic spleen cells, MHC class I–reactive cytotoxic T cells are tolerized by clonal deletion (2, 3), it is clear that alloantigen-specific, IL-4-secreting CD4 T cells are still present in the periphery (4–7) and are probably responsible for the upregulation of MHC class II molecules on both donor and host B cells (8). In addition, it has been shown that the differentiation of donor-specific CD4 T cells toward the Th2 phenotype is dependent on the early production of IL-4 in vivo (5, 9). Furthermore, blocking endogenous IL-4 was found to restore antidonor cytotoxic T lymphocytes (CTL) activity and abrogated the lymphoid chimerism, thereby resulting in graft rejection (5, 10). Another important feature of this model is the development of autoimmune manifestations, known as host-versus-graft (HVG) disease, due to the polyclonal T–B-cell interactions that occur in this model between host Th2 and donor B cells, resulting in a dramatic increase of serum IgE level and in the production of autoantibodies, some of which are found deposited in the kidneys (11, 12). However, such serological abnormalities are spontaneously downregulated by 10 weeks of age due to the disappearance of lymphoid chimerism (11, 13).

Because IgE production and Th2 priming are critically dependent on the early production of IL-4 (14–16), we have recently analyzed the requirement for NK1.1+ T cells in the generation of alloreactive Th2 responses in this model (17). For this purpose, lymphoid chimerism was induced in both normal and β2-microglobulin–deficient (β2m−) BALB/c mice. We showed that similar Th2 priming and IL-4–dependent IgE production developed in both β2m− or normal BALB/c mice injected at birth with semiallogeneic spleen cells from normal (BALB/c × C57BL/6) F1 mice (CB6F1), indicating that β2m−dependent NK1.1+ host T cells were not required for the generation of alloreactive Th2 responses in vivo (17). Interestingly, when β2m+ CB6F1 spleen cells were injected into BALB/c β2m− mice, the IgE response was even exacerbated and associated with an increased splenomegaly (17). Thus, we hypothesized that MHC class I–T-cell interactions could play a crucial role in this model by regulating Th2 immunity and its associated immunological disorders.

To analyze the role of MHC class I–reactive host T cells interacting with donor cells, we have compared 2 combinations, in which wild-type (WT) or β2m+ newborn BALB/c mice were injected either with normal or with β2m− CB6F1 splenocytes, respectively. Data presented...
here demonstrate that the absence of MHC class I–T-cell interactions leads to spared Th2 immunity that results in accelerated mortality and higher incidence of proteinuria due to antibody-mediated glomerular lesions. The uncontrolled Th2 response of host CD4 T cells is associated with the maintenance of B-cell lymphoid chimerism and with sustained hypereosinophilia in adult mice. Thus, our data support the hypothesis that CD8 T cells might play a more active role than previously anticipated by regulating donor B-cell persistence and/or Th2-cell development during neonatal induction of lymphoid chimerism in mice, thereby preventing chronic Th2 responses and its associated hypereosinophilic syndrome.

Methods
Mice, neonatal injection of semiallogeneic spleen cells. BALB/c (H-2b) and (BALB/c × C57BL/6) F1 (CB6F1) (H-2b+/-) mice were purchased from Centre d’Elevage R. Janvier (Le Genest St. Isle, France). H-2b+/- mice with disrupted β2m genes (18) were back-crossed to BALB/c mice as described previously (19). β2m+/- mice on BALB/c background were used after 9 back-crosses. BALB/c β2m° and CB6F1 β2m° mice were bred and maintained in our own animal facility. BALB/c and BALB/c β2m° mice were injected intraperitoneally within the first 24 hours of life, with 50 × 10^6 spleen cells from 8- to 12-week-old normal or β2m° CB6F1 mice, respectively. Control animals were left untreated.

Quantitation of serum IgE. Serum IgE concentration was quantified by a sandwich ELISA as described previously (17). Serum IgE concentrations measured either in WT or in β2m° control mice were less than 100 pg/mL. We considered that the IgE response was upregulated when IgE concentration was greater than 1 μg/mL.

Albuminuria determination. Urine was collected at 10–16 weeks of age, and the presence of mouse serum albumin (MSA) was determined by competitive ELISA. Briefly, polyvinyl microtiter plates were coated with 2 μg/mL mouse serum albumin (Sigma Chemical Co., St. Louis, Missouri, USA). Urines were first incubated with an appropriate concentration of rabbit anti-mouse albumin antibody (Cappel Laboratories, Cochranville, Pennsylvania, USA), then transferred onto MSA-coated plates. After incubation, bound rabbit anti-MSA IgG were stained with a peroxidase-conjugated sheep anti-rabbit IgG (Serotec, Oxford, United Kingdom), and revealed by 3,3′,5′-tetramethylbenzidine (Fluka Chemical Co., Buchs, Switzerland). Albumin concentration was determined using MSA (Sigma Chemical Co.) to generate a standard curve. The concentration of albumin in WT or β2m° control mice was routinely less than 5 μg/mL. Mice were considered albuminuric when albumin concentration was greater than 15 μg/mL.

Indirect immunofluorescence analysis. Kidney cryostats (4 μm) were performed with a Cryocut 1800 apparatus (Reichert-Jung, Cambridge Instruments GmbH, Nussloch, Germany) after OCT compound (BDH Laboratories, Poole, United Kingdom) embedding. Glomerular IgM and IgG deposits were assessed by indirect immunofluorescence on cryostat sections stained with FITC-labeled rat anti-mouse kappa light chain OX-20 mAb (20), FITC-labeled mouse IgG subclasses–specific (IgG1, IgG2a, IgG2b) antibody (Southern Biotechnology Associates, Birmingham, Alabama, USA), or FITC-labeled rabbit anti-mouse IgM antibody (Meloy Laboratories, Springfield, Virginia, USA).

Histological and cytological analysis. All major organs (liver, heart, intestine, kidney, spleen, and lung) were fixed in 10% formalin and paraffin sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Giemsa. Histological analysis was performed by 2 pathologists who did not have the knowledge of the different experimental groups. Blood white cell counts and spleen cell counts were performed with an automated counter (STKS Coulter, Beckman Coulter, Roissy, France). Differential cell determinations were carried out using spleen cell suspensions that were cytocentrifuged on a Cytospin 3 Shandon apparatus (Life Sciences International, Runcom, United Kingdom), or standard blood smears fixed with methanol and stained with Giemsa. Interpretation was done by 1 of us blinded to the different experimental groups.

Flow cytometric analysis. After red blood cell lysis, spleen cells were incubated with optimal concentrations of FITC-, PE- or biotin-conjugated mAb for 30 minutes at 4°C in PBS containing 5% FCS, 0.1% sodium azide. The following mAb’s were used: FITC-GK1.5 anti-CD4 (TIB 207; American Type Culture Collection [ATCC], Rockville, Maryland, USA), PE-Ly2 anti-CD8α (PharMingen, San Diego, California, USA), PE-RA3-6B2 anti-B220 (PharMingen), FITC-A6-120.1 anti–I–Aβ (PharMingen), FITC-14.4.4S anti–I-E (ATCC, HB 32), PE-Ly24 (Pgp1 anti-CD44 (PharMingen), and biotin anti-Ly22 (MEL-14) anti-CD62L (PharMingen). Cells were then washed and stained with streptavidin-CyChrome (PharMingen). Data were collected on 10,000 living cells as determined by forward and side scatter intensity on a XL Coulter cytometer (Beckman Coulter) and analyzed using the CellQuest software (Becton Dickinson, Mountain View, California, USA).

CD4 T-cell enrichment. Spleen cells were stained with an appropriate concentration of KT1.5 (21), anti-B220 RA3-3A1 (TIB 146; ATCC), anti-MHC class II M5/114 (TIB 120; ATCC), and anti–Mac-1 M1/70 (TIB 128; ATCC) mAb, washed, and incubated with sheep anti-rat IgG M-450 Dynabeads (Dynal, Oslo, Norway). CD4 T cells were negatively selected by magnetic cell separation.

In vitro CD4 T-cell stimulation and cytokine assays. For cytokine production analysis, 96-well culture plates (Costar, Cambridge, Massachusetts, USA) were coated with 145-2C11 anti-CD3ε (CRL-1975; ATCC) at a concentration of 1 μg/mL. CD4-enriched splenic T cells from neonatally injected and control mice were stimulated at 2×10^5 cells per well. Culture medium was HL-1 synthetic medium (Hycor, Irvine, California, USA) supplemented with 2 mM L-glutamine and 50 μg/mL gentamicin (Sigma Chemical Co.). Cultures were incubated for 2 days in a humidified atmosphere of 5% CO2 in air.
 Supernatants from replicate cultures, usually 3–4 wells, were collected after 48 hours and pooled for cytokine analysis. IFN-γ, IL-4, IL-5, and IL-10 were quantified by 2-site sandwich ELISA as described previously (17). Cytokines were quantified from 2–3 titration points using standard curves generated with purified recombinant mouse cytokines, and results were expressed as cytokine concentration in nanograms or picograms per milliliter. Detection limits were 15 pg/mL for IFN-γ and IL-4, 3 pg/mL for IL-5, and 30 pg/mL for IL-10.

Competitive RT-PCR. Total RNA was extracted using TRIzol Reagent (GIBCO-BRL, Cergy-Pontoise, France) according to manufacturer’s recommendations. RT was performed using Moloney Murine Leukemia Virus RT (GIBCO-BRL) and oligo-dT primers. Semi quantitative PCR determination for hypoxanthine-guanine phosphoribosyl transferase (HPRT) and IL-4 genes was performed as described elsewhere (22). Data were expressed as the ratio of IL-4 over HPRT mRNA levels.

Statistical analysis. Statistically significant differences between groups of continuous variables were analyzed using the Mann-Whitney U test. Categorical variables and survival curves were compared, respectively, by the Fisher’s exact test and by the log-rank test.

Results

Effects of β2m deficiency in host versus graft disease. Injection of semiallogeneic spleen cells in newborn BALB/c mice stimulates host-derived alloreactive T-helper cells that react with MHC class II alloantigens on donor B cells. This cognate interaction induces a polyclonal B-cell activation characterized by increased levels of serum IgE and autoantibody production (11, 12). As shown in Figure 1, no difference in serum levels could be observed at 4 weeks of age between the WT (Figure 1a) and the β2m- (Figure 1b) combinations. In striking contrast, whereas serum IgE levels were spontaneously downregulated by 8 weeks of age in the CB6F1-injected WT mice (Figure 1a), induction of lymphoid chimerism in the complete absence of β2m-dependent T cells resulted in uncontrolled IgE responses that remained high until 12 weeks of age (Figure 1b). The incidence of IgE response, at 4 weeks of age, in the CB6F1→BALB/c and CB6F1β2m-→BALB/cβ2m- combinations were 65% (37/57) and 83% (44/53), respectively. Interestingly, whereas most BALB/c mice injected at birth with CB6F1 spleen cells remained healthy and survive, more than 50% of mice died by 15 weeks of age in the β2m- combination (Figure 1c). This increased mortality was associated with an higher incidence of albuminuria (Figure 1d) and a significant augmentation in CD4 T and B cell numbers (data not shown) in the β2m- mice examined (Figure 2d), the 4 other mice displayed only mesangial Ig deposits. Unlike BALB/c mice injected with CB6F1 splenocytes, that exhibited only mild mesangial deposits without any cell proliferation or interstitial changes (Figure 2a), glomeruli from 4 of 6 neonatally injected β2m- mice examined by light microscopy exhibited heavy mesangial and parietal, mainly subendothelial, deposits (Figure 2c). There was also a double contour appearance after silver impregnation (data not shown). In addition, perivascular infiltrates of mononuclear cells were observed in 2 of the 6 β2m- mice (data not shown). Renal function appeared normal in these mice, as urea and creatinine blood levels were unchanged (data not shown). Another striking observation of the β2m- chimeric model was that these mice developed a much larger splenomegaly compared with the WT combination, which still persisted until 10 weeks of age, with variable degrees of fibrosis (not shown). The increased spleen size in neonatally induced β2m- chimeras was associated with a significant augmentation in CD4 T and B cell numbers (data not shown).

Spared Th2 immunity is associated with the maintenance of lymphoid chimerism in β2m-deficient mice. Because splenomegaly in this model is likely to be associated with
uncontrolled Th2 immunity, we next compared the expression of memory/activation markers on T and B cells between these 2 combinations. Results in Figure 3 show that there was a significant increase in antigen-experienced splenic CD4 T cells in both WT and β2m− mice at 6 weeks of age after neonatal induction of lymphoid chimerism. CD44hi/CD62Llow CD4 T-cell numbers were higher in CB6F1β2m−-injected BALB/cβ2m− mice compared with the WT combination. This difference was more evident at 10 weeks of age, where number of memory/effector CD4 T cells were significantly increased in neonatally injected β2m− but not WT mice. Analysis of the splenic B-cell compartment showed that the expression of I-E molecules on B cells was significantly higher in neonatally injected β2m− compared with WT mice at both 6 and 10 weeks of age (Figure 4a). Because MHC class II expression on B cells is upregulated by IL-4 (23, 24), we looked for the presence of IL-4 transcripts in splenic CD4 T cells. Data in Figure 4b demonstrate that IL-4 mRNA were readily detectable in the majority (85%) of 6-week-old neonatally induced β2m− chimeras, compared with 22% in the WT combination. IL-4 is likely to be produced by allospecific Th2 cells interacting with donor F1 B cells, resulting in donor B-cell differentiation to Ig-producing cells. Indeed, the number of plasmocytes was selectively increased in the spleen of 6-week-old neonatally injected β2m− mice (Figure 4c) and was correlated with the persistence of significantly higher levels of B-cell chimerism (Figure 4d). To analyze further the effect of the absence of MHC class I–T-cell interactions in the development of Th2 cells in this model, we measured type-2 cytokine production by splenic CD4 T cells from 6-week-old mice after stimulation with plate bound anti-CD3ε mAb. Data in Figure 5 show that there was a 4-fold upreg-
ulation of IL-4 production in F1-injected WT mice compared with un.injected control animals. In striking contrast, IL-4 secretion was dramatically increased (54-fold) in the neonatally induced $\beta_2 m^0$ chimeras. The lower IL-4 production in control $\beta_2 m^0$ compared with WT BALB/c mice may be explained by the absence of NK T cells that promptly produce this cytokine in response to TCR ligation (25). As for IL-4, IL-10 secretion upon TCR engagement was strongly increased in CD4 T cells from both combinations, but this was more evident in $\beta_2 m^0$ mice. On the other hand, the amounts of IL-5 produced by CD4 T cells was significantly upregulated only in $\beta_2 m^0$ chimeras (Figure 5). IFN-$\gamma$ synthesis was slightly increased (2- to 3-fold) in neonatally injected compared with control mice both in the WT and $\beta_2 m^0$ combinations (data not shown). Thus, the induction of lymphoid chimerism in the absence of MHC class I–T-cell interactions led to the accumulation in the spleen of CD4 T cells with a memory phenotype probably as a consequence of the long-term persistence of donor B cells, resulting in chronic alloantigenic stimulation and increased type-2 cytokine production by CD4 T cells.

**Hypereosinophilic syndrome develops after induction of lymphoid chimerism in the absence of MHC class I–T-cell interactions.** Because the uncontrolled Th2 response that develops in $\beta_2 m^0$ chimeric mice is associated with an increased splenomegaly with signs of fibrosis in most animals, a morphological analysis was performed. Data in Figure 6 show that although neonatally injected WT mice exhibited minimal change in spleen morphology (Figure 6a), a considerable modification of spleen architecture was observed in chimeric $\beta_2 m^0$ BALB/c mice (Figure 6b). Such changes were characterized by a strong hyperplasia of periarteriolar lymphoid sheaths (PALS) (Figure 6b) and by prominent eosinophil-, basophil-, and/or mastocyte-containing infiltrates in the majority (>80%) of $\beta_2 m^0$ chimeras. These modifications were associated with variable degree of fibrosis (Figure 6b and data not shown). Massive perivascular lymphoid infiltration (Figure 6f) and tissue eosinophilia (data not shown) were also occasionally found in the lung (2 of 5 $\beta_2 m^0$ chimeric mice tested) and were correlated with disease severity (data not shown). Histological analysis of others organs such as liver, heart, and intestine did not reveal significant abnormalities (data not shown). Cytological analysis confirmed the increased eosinophilia in the spleen of neonatally induced $\beta_2 m^0$ chimeras compared with F1-injected WT mice (Figure 7a). Furthermore, a significant increase in eosinophil number was detected in the blood of 6- to 10-week-old $\beta_2 m^0$ chimeric mice.

**Figure 4**
Upregulation of MHC class II on B cells, increased number of plasmocytes, and IL4-mRNA are associated with the maintenance of lymphoid chimerism in $\beta_2 m^0$ BALB/c mice. (a) I-E expression level on splenic B lymphocytes from 6- and 10-week-old neonatally injected and control mice was evaluated by flow cytometry. Data are expressed as fold increase versus control (mean ± SEM) of 4–12 mice per group. (b) IL4-mRNA expression in negatively enriched CD4 splenic T cells, from 6-week-old neonatally injected WT and $\beta_2 m^0$ (n = 14) mice, was quantified by semiquantitative RT-PCR. Data are expressed as the ratio of IL-4/HPRT mRNA levels from 9–14 individual mice per group. (c) Cytospins were prepared from spleen cells suspensions from 6-week-old mice; total plasmocyte numbers were calculated from 4–7 individual mice per group. (d) Percentages of I-A$^b$ splenic B220$^+$ cells from 6-week-old WT (n = 16) and $\beta_2 m^0$ (n = 9) mice injected at birth with semiallogeneic splenocytes were evaluated by flow cytometry analysis. Bars represent means of individual mice.

**Figure 5**
Th2-type cytokines production by in vitro stimulated CD4 T lymphocytes. Negatively enriched splenic CD4 T cells from 6-week-old control and neonatally injected mice were stimulated with plate-bound anti-CD3e mAb for 48 hours. IL-4, IL-10, and IL-5 were quantified in culture supernatants by ELISA. Results are expressed as mean ± SEM cytokine concentration (ng/mL) from 4–7 mice per group. Numbers in brackets indicate the fold increase of neonatally injected versus control mean values. $^a$P < 0.05; $^b$P < 0.01.
mice (Figure 7b). Such abnormalities were barely detectable or absent in the majority of F1-injected β2m- sufficient mice. Thus, the uncontrolled type-2 immune response that develops in β2m° BALB/c mice injected at birth with semiallogeneic β2m° splenocytes leads to an hypereosinophilic syndrome.

Discussion
The present report demonstrates that induction of lymphoid chimerism in BALB/c mice in the absence of MHC class I–T-cell interactions results in a lethal syndrome associated with uncontrolled Th2 immunity and sustained hypereosinophilia. In addition, these mice develop a severe form of antibody-mediated glomerulonephritis probably as a consequence of autoreactive F1 donor B-cell activation by host allohelper T cells. However, the autoimmune glomerulonephritis is not, or is not only, responsible for the mortality because biologic signs of renal failure were absent, and some mice died without proteinuria. We postulate that lung damages may contribute to the increased mortality in β2m° chimeric mice. This model markedly contrasts with the mild and self-limited form of HVG disease that occurs in WT BALB/c mice injected with CB6F1 spleen cells (11, 13) and represents the first example of a Th2-dependent lethal chronic inflammatory disease in mice associated with an hypereosinophilic syndrome.

The mechanisms by which β2m deficiency leads to an uncontrolled Th2 response are likely to be related to the lack of interactions between donor MHC class I molecules and host CD8 T cells. In previous experiments, we have shown that the injection of CB6F1 spleen cells into normal or β2m° BALB/c mice induces identical Th2-type responses (17). This could be explained by the fact that in β2m° mice there is a residual population of CD8 T cells that can readily expand after in vivo priming with

Figure 6
Histological changes in the spleen (a–d) and lung (e and f) of 6-week-old WT (a and e) and β2m° BALB/c mice (b, c, d, and f) injected at birth with normal or β2m-deficient CB6F1 splenocytes, respectively. In comparison with the spleen of WT mice (a, ×125), a striking hyperplasia of PALS is observed on H&E staining of β2m° mice (b, ×100). On higher magnification (×800), one sees large numbers of eosinophils (arrows) (c) in a background of activated lymphoid cells, some showing immunoblastic features (arrowheads). Giemsa staining shows that cells with large basophilic granules (arrows) are also present in PALS (d). Note also the presence of scattered plasma cells (arrowheads). (e and f) H&E staining shows perivascular infiltrates (arrows) consisting of activated lymphoid cells associated with eosinophils and basophils in the lung of a β2m° mouse (f, ×100). No significant changes are seen in the lung of a WT mouse (e, ×100).
cells expressing either self- or allogeneic-MHC class I molecules (26, 27). Thus, to avoid the interference of this minor population of CD8 T cells, MHC class I molecules must also be absent on donor-derived CB6F1 cells. The demonstration that MHC class I–T-cell interactions play a crucial role in the control of Th2 immunity and its associated lethal syndrome is rather unexpected. Indeed, it is widely accepted that after neonatal transplantation, tolerance of MHC class I–restricted T cells, expressing the NK1.1 molecule, is absent in 2m° mice (25). NK1.1+ T cells have also been shown to mediate efficient regulatory effector functions. Indeed, as already discussed here, the minor CD8 T-cell population that persist in 2m° mice might have escaped in vivo depletion with anti-CD8 mAb and might play a crucial role in this model. The hypothesis that CD8 T cells might mediate the early administration of this cytokine inhibits the Th2-type response induced by the neonatal injection of allogeneic spleen cells (32). Finally, there is another subset of MHC class I–reactive T cells, expressing the CD8α molecule, that is absent in 2m° mice (25). NK1.1+ T cells have been shown to produce IFN-γ, which is associated with uncontrolled Th2 immunity, which would be exacerbated rather than suppressed by IL-4 production from NK T cells. However, these T-cell subset can also produce IFN-γ, we cannot exclude that they can effectively control type-2 Th cells development in 2m°-sufficient mice.

An important feature of the present study is that the injection of allogeneic splenocytes into newborn BALB/c mice leads, in the absence of MHC class I–T-cell interactions, to a lethal form of HVG disease. Although, the reasons why mice die during the course of the disease remain to be elucidated, it must be stressed that besides the marked glomerulonephritis, these mice also exhibit sustained blood eosinophilia and an important accumulation of eosinophils not only in their spleen but also in their lungs, making this model relevant to human hyper-eosinophilic disease (36). In addition, high numbers of basophils and/or mastocytes were found to be infiltrating the PALS. Eosinophils have been recently shown to mediate rejection of acute and chronic MHC class I–disparate skin allografts in mice (37, 38) and are probably responsible for the generation of fibrotic lesions in some chronic inflammatory diseases (39, 40). Variable degrees of fibrosis were evident in the spleen of chimeric 2m° disease mice and were associated with eosinophil infiltrates.
indicating that such mechanisms could also be involved in our model. Hyper eosinophilia was also associated with an increased capacity of CD4+ T cells to secrete IL-5, a cytokine that plays a central role in the differentiation, proliferation, and recruitment of eosinophils (41). Whether or not eosinophils play a major effector role in the observed histological and clinical manifestations, including mortality, remains to be elucidated.

In conclusion, we have demonstrated that the HVG reaction that develops in the absence of MHC class I–T-cell interactions leads to a lethal leukoproliferative disorder associated with uncontrolled Th2-type immunity and hyper eosinophilic syndrome in mice. These data support the hypothesis that CD8 T cells might play a more active role than previously anticipated by regulating lymphoid chimerism and/or Th2-cell development resulting in a mild and self-limited form of HVG disease. Thus, induction of neonatal lymphoid chimerism in BALB/c mice in the absence of MHC class I–T-cell interactions represents an unique model of Th2-dependent chronic inflammatory disease that may help to understand the mechanisms involved in idiopathic hyper eosinophilic syndrome in humans (36).

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