Allograft rejection is associated with development of functional IgE specific for donor MHC antigens

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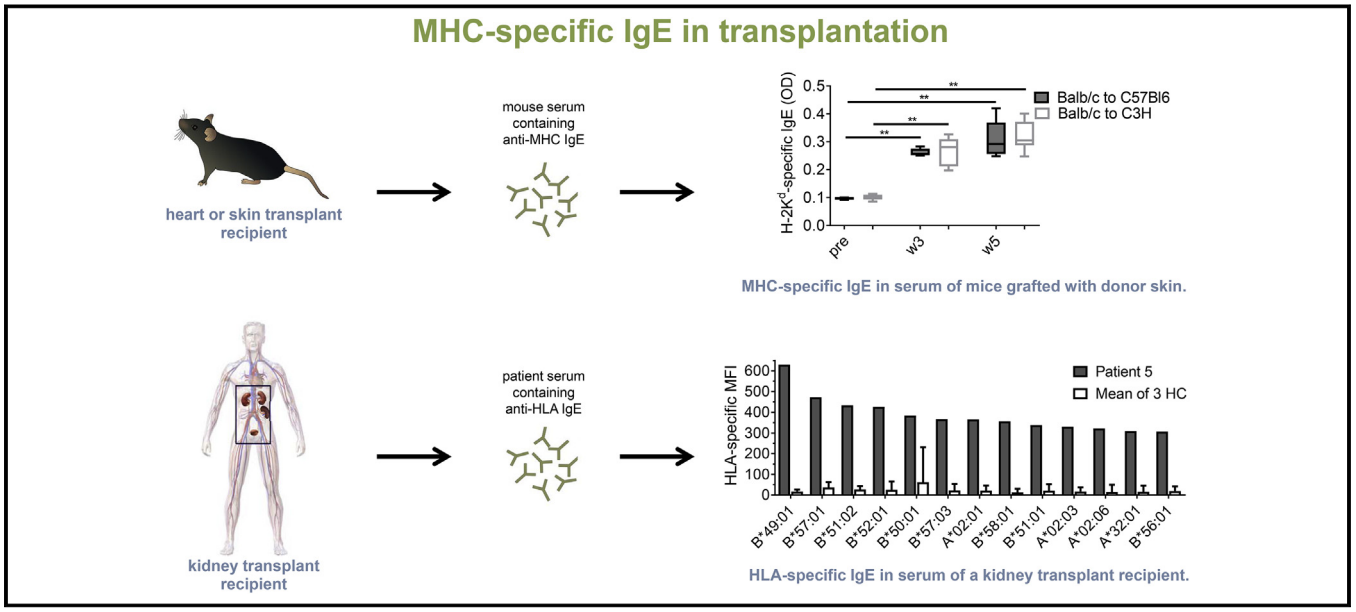
GRAPHICAL ABSTRACT



Background: Donor-specific antibodies of the IgG isotype are measured routinely for diagnostic purposes in renal transplant recipients and are associated with antibody-mediated rejection and long-term graft loss.

Objective: This study aimed to investigate whether MHC-specific antibodies of the IgE isotype are induced during allograft rejection.

Methods: Anti-MHC/HLA IgE levels were measured in sera of mice grafted with skin or heart transplants from various donor strains and in sera of kidney transplant patients with high levels of HLA IgG. Mediator release was triggered *in vitro* by stimulating basophils that were coated with murine or human IgE-positive serum, respectively, with specific recombinant MHC/HLA antigens. Kidney tissue samples obtained from organ donors were analyzed by using flow cytometry for cells expressing the high-affinity receptor for IgE (FcεRI).

Results: Donor MHC class I– and MHC class II–specific IgE was found on acute rejection of skin and heart grafts in several

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murine strain combinations, as well as during chronic antibody-mediated heart graft rejection. Anti-HLA IgE, including donor HLA class I and II specificities, was identified in a group of sensitized transplant recipients. Murine and human anti-MHC/HLA IgE triggered mediator release in coated basophils on stimulation with specific MHC/HLA antigens. HLA-specific IgE was not linked to atopy, and allergen-specific IgE present in allergic patients did not cross-react with HLA antigens. FcεRI$^+$ cells were found in the human renal cortex and medulla and provide targets for HLA-specific IgE. Conclusion: These results demonstrate that MHC/HLA-specific IgE develops during an alloresponse and is functional in mediating effector mechanisms. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: *Transplantation, immunology, alloreactivity, humoral rejection, IgE, donor-specific antibodies*

Abbreviations used
ABMR: Antibody-mediated rejection
DSA: Donor-specific antibody
HRP: Horseradish peroxidase
MFI: Median fluorescence intensity
PE: Phycoerythrin
RBL: Rat basophil leukemia
SAB: Single-antigen bead

Despite considerable improvements in 1-year graft survival over the last decades, long-term kidney graft survival has largely stagnated and remains suboptimal.[1,2] Immunologic graft injury is still the leading cause of late graft loss, and antibody-mediated rejection (ABMR) is widely considered the most important predictor and mediator of late graft injury.[3,4] Consequently, recent research has focused on investigating the occurrence of donor-specific antibodies (DSAs) and in particular anti-HLA DSAs and their pathophysiologic role in renal graft damage.[5] Increasingly, DSAs are recognized as important in liver, heart, and lung transplantation as well.[6-9]

Among HLA DSAs, both preformed (ie, present at baseline before transplantation)[10,11] and *de novo* (ie, newly developing after transplantation)[11-14] antibodies are recognized to affect graft survival. Approximately 30% of transplant candidates show preformed HLA-specific antibodies, and roughly 20% to 30% have *de novo* DSAs after transplantation.[5]

DSAs are predominantly of the IgG isotype (ie, antibody subtype), with IgM and IgA DSAs also described.[15] Recently, distinct effects for the IgG subclasses IgG_1 to IgG_4 have been reported.[16,17] However, little is known about the role of other isotypes, and to the best of our knowledge, anti-HLA IgE has not been reported to date. Solid-phase assays measuring antigen-specific antibodies in patient sera have been developed for diagnostic purposes. In particular, Luminex-based measurement of antibodies of the IgG isotype against a high number of individual HLA antigens (approximately 100 for class I and approximately 100 for class II) is used routinely in many transplantation centers.

Importantly, however, not all DSAs have an immediate negative effects on outcome because a proportion of DSA-positive recipients maintain unimpaired graft function for a considerable period of time. For instance, although approximately 90% of patients with histologic features of ABMR in late failing heart allografts had detectable DSAs, a substantial proportion (approximately 35%) of patients devoid of histologic ABMR features also had detectable DSAs.[18] Thus, measurement of DSAs in transplant candidates and recipients is a valuable diagnostic tool but is typically insufficient on its own to allow individualized clinical decision making. Therefore, it is important to better understand the biology of the humoral allograft response and its mechanisms of affecting the graft. In this respect, efforts are ongoing to define DSA subsets with higher potency of causing graft injury. The complement-binding fraction of IgG DSAs is a step in this direction,[13,19-23] but the clinical utility of this parameter is still under debate.

IgE is an antibody isotype with unique nonredundant functional properties and effector mechanisms that are not shared by other isotypes.[24] In contrast to other isotypes that are most abundant in serum, IgE is mostly found in tissue and is present in serum in only very low amounts (approximately 10,000-fold lower than IgG).[24] In tissue IgE binds tightly to mast cells and basophils through the specific high-affinity receptor FcεRI. Mast cell degranulation is triggered on cross-linking of IgE through binding of specific antigen, releasing potent proinflammatory mediators (including TNF-α and IL-6). IgE plays a prominent role in T_H2-type immune disorders, in particular type I allergy and parasite infection. However, additional IgE-mediated mechanisms are also increasingly recognized. Notably, autoantibodies of the IgE isotypes develop in T_H2-driven immunity in patients with lupus and atopic dermatitis,[25-27] and increased IgE levels seem to play a role in atherosclerosis.[28] The cellular immune response to allografts is recognized to include features of T_H2 immunity in some rejection settings.[29-31] However, the occurrence and potential relevance of IgE in graft-directed immunity in transplantation has not been investigated thus far.

In the present study we reveal that IgE antibodies specific for donor MHC class I and MHC class II antigens developed during graft rejection in several mouse transplantation models. The induced MHC-specific IgE was functional and mediated effector cell degranulation *in vitro* and *in vivo* on specific antigen stimulation. Moreover, we identified donor HLA-specific IgE in sera of sensitized kidney transplant patients, which was also functional *in vitro* and demonstrates that FcεRI$^+$ target cells are present in human kidneys.

METHODS

The Methods are available in this article's Online Repository at www.jacionline.org.

RESULTS

MHC-specific IgE is induced on rejection of allogeneic skin and heart allografts

BALB/c (H-2^d) tail skin was grafted onto naive fully mismatched C57BL/6 (H-2^b) mice and recipient serum was analyzed for IgE specific for K^d and D^d (ie, MHC class I) and I-E^d (ie, MHC class II) through an ELISA using recombinant MHC monomers to investigate whether anti-donor IgE develops

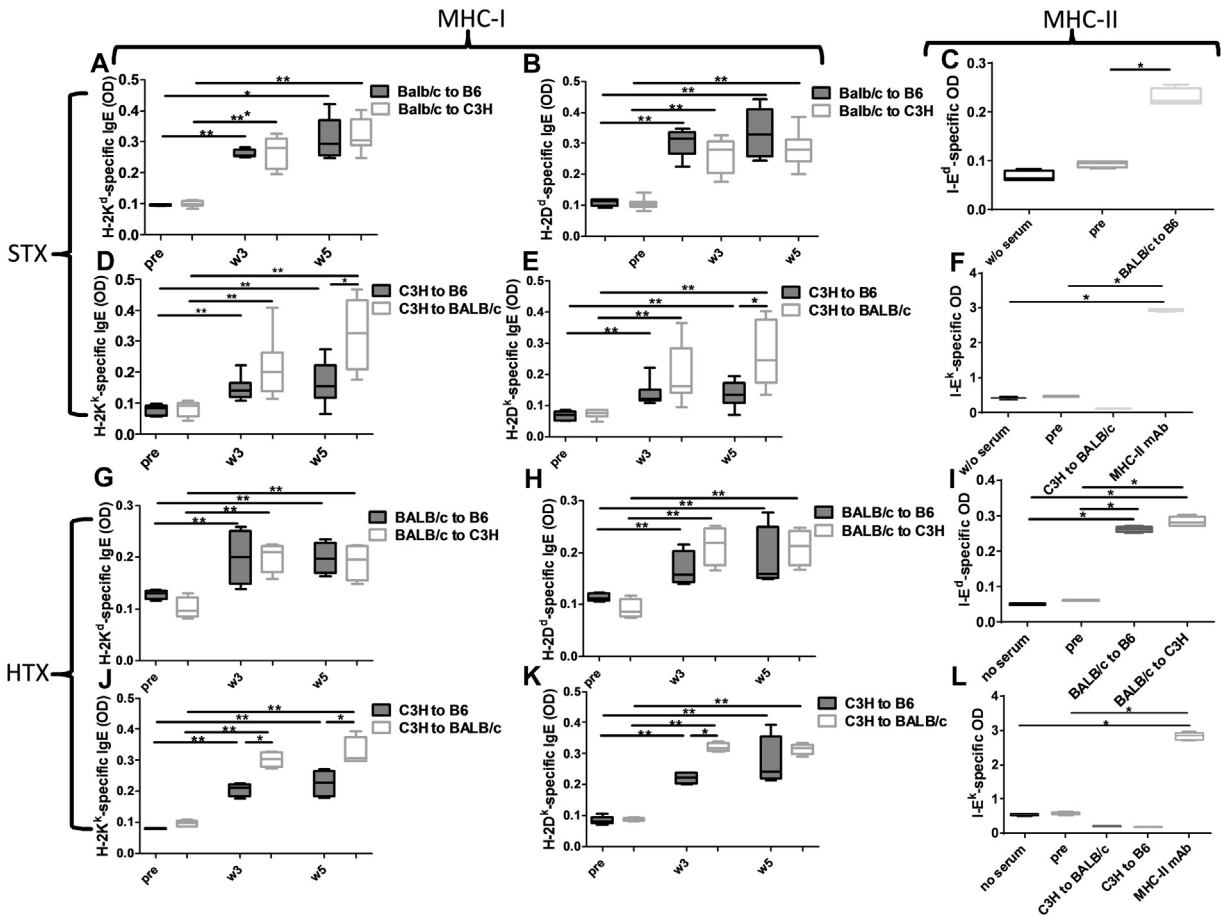


FIG 1. Donor MHC–specific IgE rapidly develops after allogeneic murine skin and heart transplantation. **A-C** and **G-I,** Analysis of IgE specific for MHC class I (ie, H-2K^d and H-2D^d; Fig 1, *A* and *B*, skin; Fig 1, *G* and *H*, heart) 3 and 5 weeks *(w)* after transplantation and MHC class II (ie, I-E^d; Fig 1, *C*, skin; Fig 1, *I*, heart) 5 weeks after transplantation in the setting of BALB/c transplants to C57BL/6 or C3H recipients. **D-F** and **J-L,** The same analysis was done for H-2K^k, H-2D^k (Fig 1, *D* and *E*, skin; Fig 1, *J* and *K*, heart) and I-E^k (Fig 1, *F*, skin; Fig 1, *L*, heart) in the setting of C3H transplants to C57BL/6 or BALB/C recipients. Serum was pooled for analysis of I-E^d– and I-E^k–specific IgE to accommodate the high amount of serum required. Results pooled from 2 experiments are shown (n = 6 for all skin and n = 4 for all heart transplant experiments). *P* values were calculated with the Mann-Whitney *U* test. $*P < .05$ and $P > .01$, $**P \leq .01$ and $P > .001$, and $***P \leq .001$, respectively. *HTX,* Heart transplantation; *pre,* pre-transplantation; *STX,* skin transplantation.

during allograft rejection. Additionally, BALB/c tail skin was grafted onto C3H (H-2^k) mice to control for strain-specific effects. All grafts were rejected within 12 days.

In both recipient strains K^d- and D^d-specific IgE was consistently detectable in sera of all recipients at 3 and 5 weeks after transplantation (Fig 1). MHC class I–specific IgE serum levels remained stable until at least 6 months after transplantation and were detectable for at least 12 months (see Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org). IgE was specific for donor MHC antigens because IgE did not develop to unrelated MHC class I antigens (see Fig E2 in this article's Online Repository at www.jacionline.org). In addition, I-E^d–specific IgE was detectable 5 weeks after transplantation (Fig 1, *C*). Moreover, C3H (H-2^k) mice were used as alternative skin graft donors, and BALB/c mice were used as an alternative recipient in addition to C57BL/6 mice. Again, anti-MHC class I IgE (ie, K^k- and D^k-specific IgE) developed on rejection, with BALB/c recipients showing significantly greater levels of MHC-specific IgE than C57BL/6 recipients (Fig 1, *D* and *E*). I-E^k–specific IgE was detectable in neither BALB/c nor C57BL/6 recipients (Fig 1, *F*).

In parallel, the more abundant IgG subclasses were analyzed. Significantly increased levels of K^d- and D^d-specific IgG_1, $IgG_{2a/c}$, IgG_{2b}, and IgG_3 were detected compared with pretransplantation baseline levels on skin rejection in the BALB/c to C57BL/6 combination (see Fig E1, *C-J*). Thus both T_H1- and T_H2-associated IgG subclasses were present in sera of recipient mice.[32]

Because IgE, in contrast to IgG, is not heat stable,[33-36] serum was heat inactivated to rule out that the detection antibody potentially cross-reacts with the more abundant MHC-specific IgG and to confirm that the IgE signal detected in the ELISA was specific for IgE. Heat inactivation eliminated the H-2K/D–specific IgE signal, whereas the H-2K/D–specific IgG signal remained unaltered (see Fig E3 in this article's Online Repository at www.jacionline.org), demonstrating the specificity of the IgE signal.

Next, we evaluated donor-specific IgE in a primarily vascularized heterotopic heart transplant model. The same

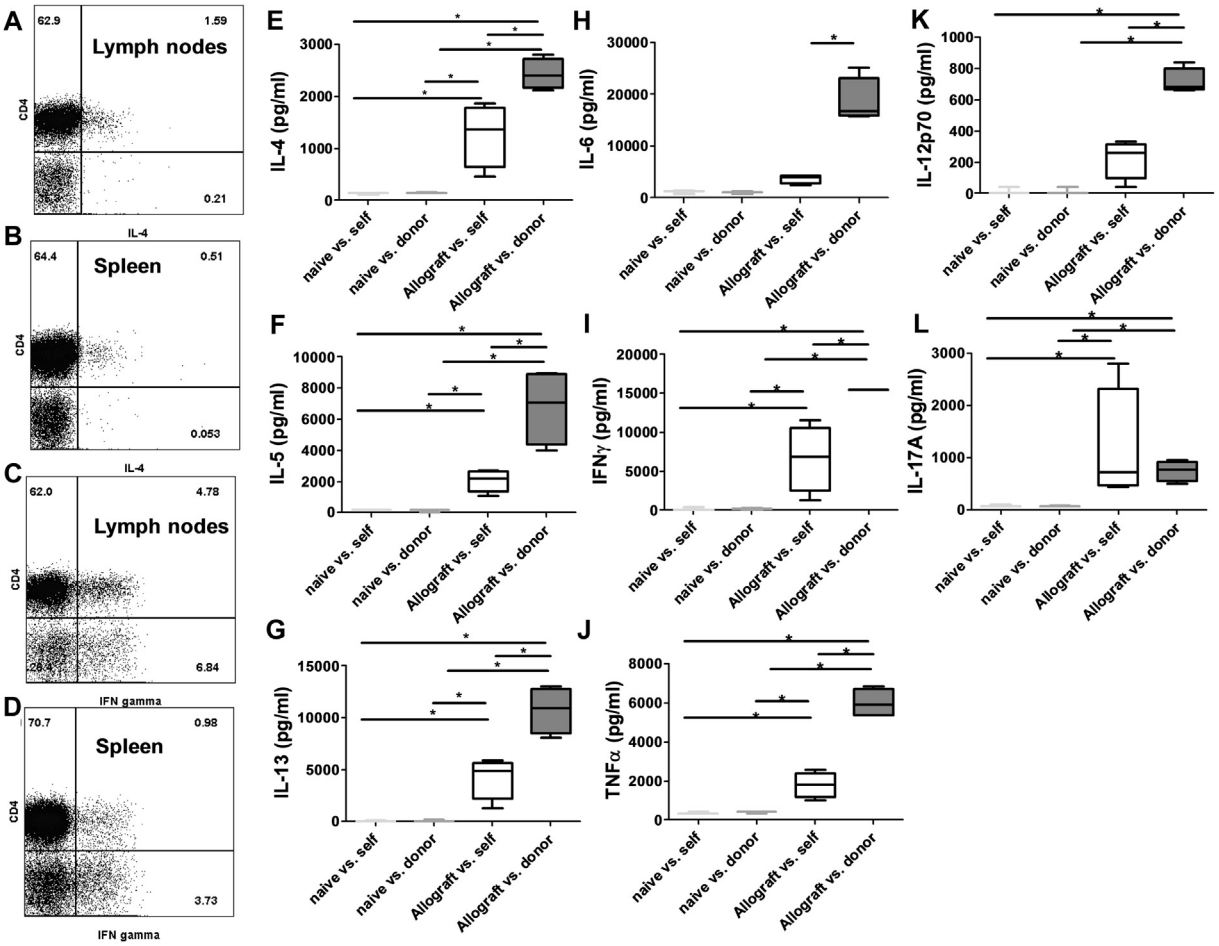


FIG 2. Rejection of fully mismatched skin grafts also induces a T_H2 T-cell response. Splenocytes *(Spleen)* or lymph node cells *(Lymph nodes)* of BALB/c mice that rejected C3H skin grafts were isolated 3 weeks after transplantation. **A-D,** Cells were stimulated for 6 hours with phorbol 12-myristate 13-acetate and ionomycin and analyzed by using flow cytometry by gating on $CD3^+CD4^+$ cells and intracellular staining for IL-4 (Fig 2, *A* and *B*, showing one representative picture of 6) and IFN-γ (Fig 2, *C* and *D*, showing one representative plot of 6). Additionally, BALB/c splenocytes from naive or C3H skin recipients (allograft) were stimulated for 4 days with irradiated C3H (donor) or BALB/c (self) splenocytes. **E-L,** Subsequently, supernatants were analyzed with the Luminex ProcartaPlex to determine the indicated cytokines (n = 6 for allografts and n = 4 for naive). *P* values were calculated with the Mann-Whitney *U* test. $*P < .05$.

4 donor-recipient combinations as for the skin transplantation experiments were tested, with all heart grafts being rejected within 11 days. Again, all recipient mice had detectable levels of donor MHC class I–specific IgE (Fig 1, *G*, *H*, *J*, and *K*), which were greater in BALB/c than in C57BL/6 mice. With regard to MHC class II, again I-E^d– but not I-E^k–specific IgE was induced (Fig 1, *I* and *L*). Taken together, these results show that IgE specific for donor MHC class I and II antigens develops during rejection of skin and heart allografts.

Rejection of allogeneic skin grafts is associated with a T_H2 T-cell response

Because IgE induction is dependent on T_H2 cytokines,[32] we assessed whether skin graft rejection in the models used would elicit a systemic T_H2 response, as was shown in other transplantation settings.[31,37,38] A small but clearly distinguishable $CD4^+$ cell population positive for IL-4 was detected (Fig 2, *A* and *B*) in addition to T_H1-associated $CD4^+IFN\text{-}\gamma^+$ cells in spleens and lymph nodes of BALB/c mice that had rejected C3H skin (Fig 2, *C* and *D*). Further recipient splenocytes secreted IL-4, IL-5, IL-6, IL-13, IFN-γ, TNF-α, and IL-12p70 on cocultivation with irradiated donor splenocytes (Fig 2, *E-L*). Hence a donor-specific and most likely polyclonal systemic T_H2 response accompanies the development of donor-specific IgE.

Functional activity of donor-specific IgE

IgE is the only isotype capable of efficiently binding through FcεRI to basophils and mast cells and to trigger degranulation on antigen-specific cross-linking. Therefore the functional activity of MHC-specific IgE was assessed by incubating sera of BALB/c mice that had rejected C3H skin with a basophil cell line that expresses FcεRI. Subsequently, respective donor MHC antigens (ie, recombinant monomers K^k or D^k, respectively) were added, and degranulation was measured through a surrogate marker

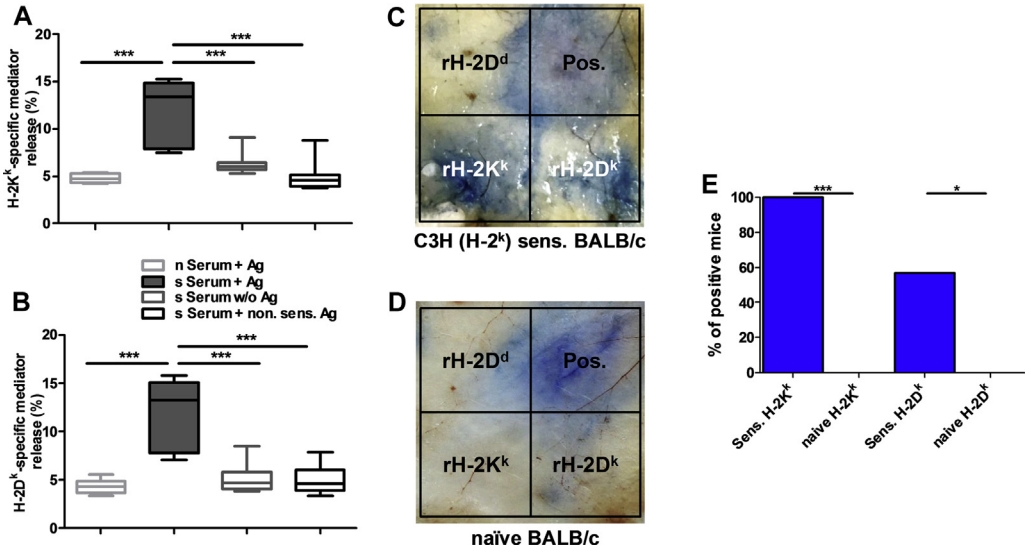


FIG 3. Functional activity of donor-specific IgE developing after allogeneic murine allotransplantation. **A** and **B,** IgE-containing serum from C3H skin-grafted BALB/c mice (*n Serum*, pre-transplantation; *s Serum*, post-transplantation) loaded onto basophils leads to mediator release (β-hexosaminidase release as a surrogate marker for histamine) on stimulation with recombinant donor (H-2K^k and H-2D^k) but not with nonsensitized (H-2K^d and H-2D^d) MHC antigens *(non. sens. Ag)* or without antigen *(w/o Ag) in vitro*. Pooled data of 3 independent experiments are shown. **C-E,** *In vivo* degranulation shown by injecting Evan blue intravenously into naive or C3H skin-grafted BALB/c mice and subsequently challenging the mice intradermally with recombinant donor MHC antigens (rH-2K^k and rH-2D^k), recombinant self MHC class I antigens (rH-2D^d), and a mast cell degranulator (Pos. 48/80) or H-2D^d (ie, negative control). Mast cell degranulation leads to vascular leakiness shown by means of blue intradermal staining. Fig 3, *C*, shows a representative photo of one of 8 sensitized mice in 2 independent experiments. Fig 3, *D*, shows a representative photo of one of 6 naive mice in 2 independent experiments. Fig 3, *E*, shows the percentage of positive mice on H-2K^k and H-2D^k stimulation in sensitized and naive mice. *P* values were calculated with the Mann-Whitney *U* (Fig 3, *A* and *B*) or χ^2 (Fig 3, *E*) tests. $^*P < .05$ and $^{***}P \leq .001$, respectively.

(ie, β-hexosaminidase). Both K^k and D^k triggered mediator release, but unrelated nonsensitized MHC antigens did not (Fig 3, *A* and *B*).

To assess whether the MHC-specific IgE was also functional *in vivo*, we performed a cutaneous type I hypersensitivity reaction assay whereby BALB/c mice were sensitized with C3H skin grafts. Three to 5 weeks after transplantation, the mice received an intravenous injection of Evans blue and were challenged intradermally with recombinant K^k and D^k. After challenge with donor MHC class I antigen, mast cell degranulation was visible in most skin-grafted mice (Fig 3, *C-E*). Taken together, MHC-specific IgE is functionally active *in vitro* and *in vivo* and mediates mast cell/basophil degranulation on stimulation with the specific sensitized MHC class I antigens.

MHC-specific IgE is also induced in a murine model of humoral heart allograft rejection

Although acute skin and heart graft rejection in untreated recipients leads to a donor-specific humoral response, the observed acute rejection-induced graft injury is largely cell mediated.[39] Recently, a heart transplant model eliciting a predominantly humoral rejection associated with IgG DSAs was developed by transplanting Bm12.Kd.IE donor hearts into B6 recipients.[40,41] In this setting donor hearts are not rejected acutely, and chronic graft injury is associated with germinal center development, anti-class I plus II alloantibody responses, and graft vasculopathy. The Bm12.Kd.IE mouse differs from the C57BL/6 mouse by 1 MHC class I antigen (ie, K^d) and 2 MHC class II antigens (ie, I-E^d and I-A^{bm12}).[40]

To test whether donor MHC-specific IgE also develops during humoral graft rejection, we analyzed the sera of B6 recipients undergoing transplantation with a Bm12.Kd.IE heart for donor-specific (ie, K^d and I-E^d) IgG$_1$ and IgE. Most recipients had detectable levels of K^d– and I-E^d–specific IgE (and IgG$_1$; Fig 4, *A*, *B*, *D*, and *E*). No antibodies against the unrelated (nondonor) D^d antigen were detected (Fig 4, *C* and *F*). Histopathology of the explanted hearts was characterized by high-grade intimal arteritis in larger-caliber vessels (ie, coronary arteries), whereas small arteries and capillaries were not affected. The edematous myocardium showed very little inflammation but focal necrosis of the ischemic type, most likely resulting from occlusive intimal arteritis (Fig 4, *G*). The transplanted hearts were also stained immunohistochemically for C4d deposition. All analyzed hearts demonstrated C4d$^+$ staining of endothelial cells, primarily in arterioles and capillaries, which is comparable with the staining pattern in clinical ABMR (Fig 4, *H*). Thus donor-specific IgE develops during chronic ABMR of a primarily vascularized heart graft.

HLA class I and II antigen–specific IgE is detectable in sensitized kidney transplant patients

Next, we investigated whether MHC-specific IgE occurs in the clinical setting. Therefore we analyzed sera of a pilot cohort of sensitized kidney transplant recipients who had rejected a kidney

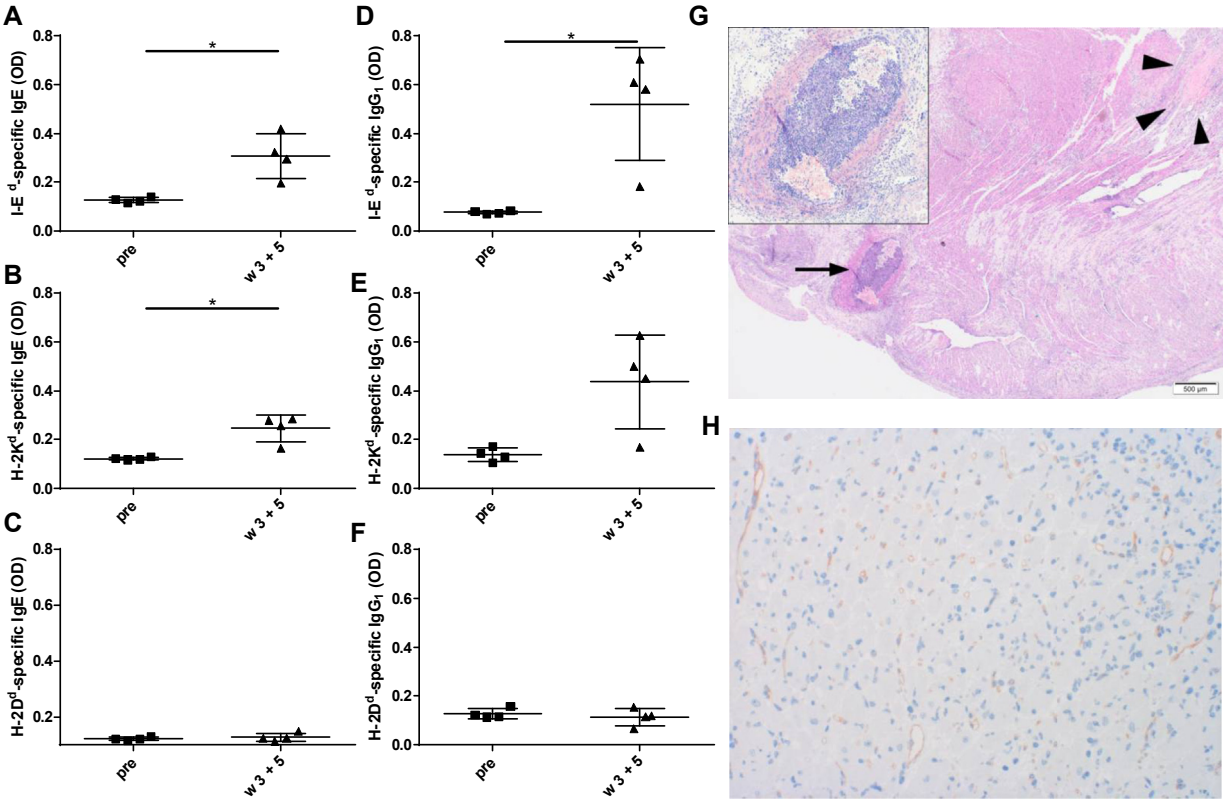


FIG 4. MHC-specific IgE is induced in a murine model of humoral heart allograft rejection. **A-F,** Analysis of IgE (Fig 4, *A-C*) or IgG_1 (Fig 4, *D-F*) specific for MHC class I (ie, H-$2K^d$ = donor and H-$2D^d$ = nondonor) or MHC class II (ie, I-E^d = donor) antigens in sera of C57BL/6 recipients grafted with Bm12.Kd.IE hearts at weeks *(w)* 3 or 5 after transplantation (n = 4). *P* values were calculated with the Mann-Whitney *U* test. **P* < .05. **G,** Representative hematoxylin and eosin staining showing high-grade intimal arteritis (*arrow* and *insert*) in larger-caliber vessels (coronary arteries). Small arteries and capillaries are not affected. The edematous myocardium shows very little inflammation but does show focal necrosis of the ischemic type *(triangles)*, most likely resulting from occlusive intimal arteritis. **H,** Representative C4d staining (brown chromogen) showing the typical staining pattern for ABMR (ie, endothelial C4d deposition in arterioles and capillaries).

TABLE I. Number of positive HLA class I and II reactions for IgE and IgG

IgE	HLA class I	HLA class II	IgG	HLA class I	HLA class II
Positive = MFI > mean MFI of 3 healthy control subjects + 2*SD and MFI > 25	**No. of positive reactions**	**No. of positive reactions**	**Positive MFI > 1000**	**No. of positive reactions**	**No. of positive reactions**
Patient 1	34	14	Patient 1	89	68
Patient 2	16	22	Patient 2	52	36
Patient 3	0	7	Patient 3	2	24
Patient 4	37	21	Patient 4	53	35
Patient 5	45	21	Patient 5	67	50

graft. All 5 patients were broadly sensitized against HLA class I (see Fig E4 in this article's Online Repository at www.jacionline.org) and/or HLA class II (see Fig E5 in this article's Online Repository at www.jacionline.org) antigens, as assessed by using a standard HLA-specific IgG single-antigen bead (SAB) assay (Table I).

The same patient samples were then analyzed for HLA-specific IgE by using a modified SAB assay with a non–cross-reacting anti-IgE–detecting antibody (clone MHE-18). Because of the drastically lower physiologic levels of IgE compared with IgG, the expected fluorescence intensity was much lower, and the threshold for positive bead reactivity was adjusted accordingly in that the mean median fluorescence intensity (MFI) of 3 healthy control subjects plus 2*SDs was defined as a threshold for each antigen (with an MFI > 25 as the lower limit). In 4 of 5 tested patients, HLA class I–specific IgE was detected (Fig 5). Among

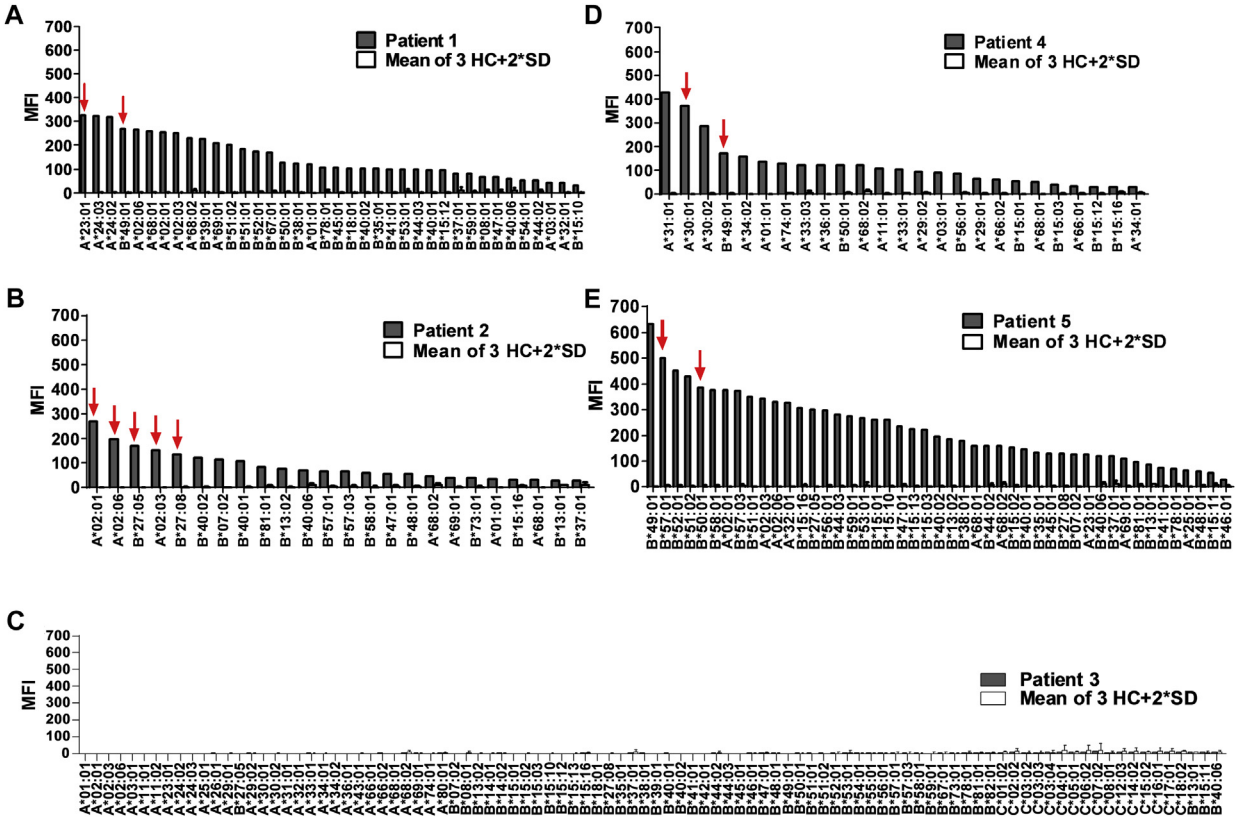


FIG 5. Anti–HLA class I IgE in kidney transplant recipients. **A-E,** HLA-specific IgE levels were measured in sera of kidney transplant recipients with high levels of IgG DSAs with One Lambda LABScreen Single Antigen HLA Class I kits. IgE binding to several HLA class I antigens was detectable in 4 of 5 analyzed patients. Each panel shows the MFI representing a single patient compared with the mean of 3 healthy control subjects *(HC)* plus 2*SDs. *Red arrows* indicate kidney donor-specific bead reactivity. For patients with HLA-specific IgE, only positive beads are shown.

the positive IgE reactivities, several were specific for donor HLA antigens (indicated with red arrows in Fig 5). Furthermore, HLA class II–specific IgE was also detectable in the sera of all 5 patients (see Fig E6, *A-E*, in this article's Online Repository at www.jacionline.org).

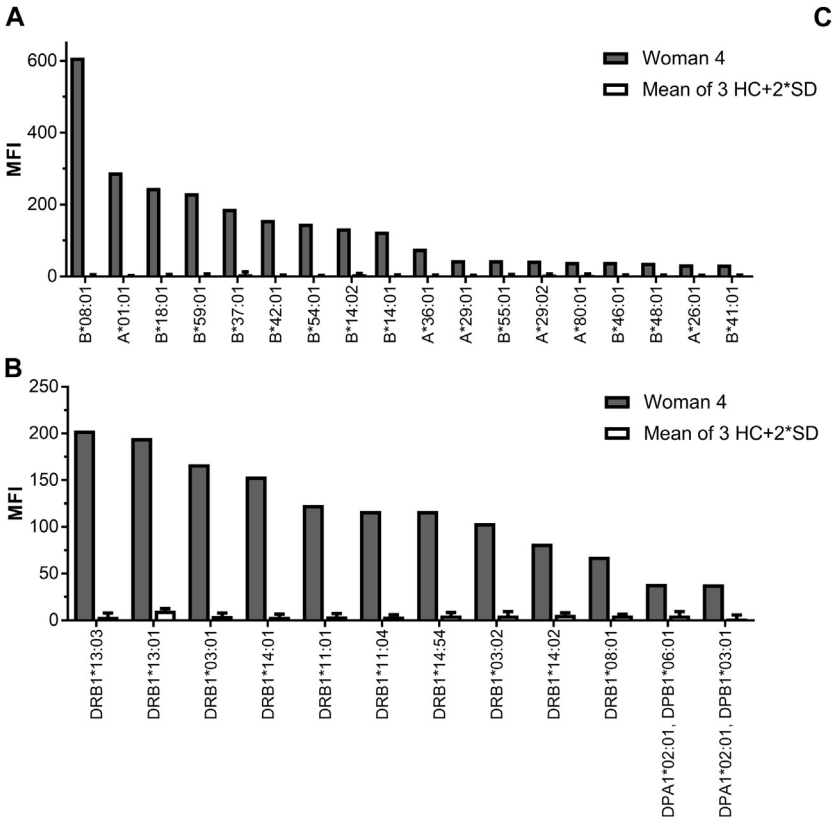
Serum was heat inactivated to denature IgE antibodies (analogous to the murine studies described above) to assess the specificity of the assay for IgE. IgG in heat-inactivated serum is known to be stable.[42] IgE signals were decreased for both HLA class I and HLA class II, supporting a high specificity for IgE in the assay (data not shown). No cross-reactivity with other isotypes and high specificity of the anti-human IgE antibody (MHE-18) for IgE was confirmed by using ELISA MHE-18 binding to a panel of coated human isotypes, as previously described (see Fig E6, *F*).[43] The number of donor-specific HLA reactivities (IgG and IgE) for each tested patient is summarized in Table I. These results demonstrate that IgE antibodies specific for HLA class I and class II antigens are detectable in sensitized kidney transplant patients.

In western countries type I allergy is prevalent in approximately 25% of subjects and is thus the main disorder associated with increased serum IgE levels. Therefore we tested sera of patients undergoing transplantation for the presence of allergen-specific IgE by using a comprehensive multiallergen test containing the most common allergens.[44] Only 1 patient (patient 5) had detectable levels of allergen-specific IgE in the allergen array chip, with reactivities against Ara h 1 (ie, major peanut allergen), Der p 4 (ie, house dust mite allergen), Phl p 1, Phl p 4, and Phl p 5b (ie, 3 major grass pollen allergens of *Phleum pratense*; see Fig E7, *A* and *B*, in this article's Online Repository at www.jacionline.org). Hence 4 of 5 patients had positive results for HLA-specific IgE without being atopic and without having allergen-specific IgE.

To address the reverse question of whether IgE that develops during the course of type I allergy cross-reacts with HLA antigens, we tested whether sera of allergic patients known to have very high levels of total IgE contain HLA-specific IgE. No HLA class I– or HLA class II–specific IgE was detectable in a group of allergic patients, indicating that HLA-specific IgE develops *de novo* after transplantation because of antigen-specific sensitization (see Fig E7, *C-H*).

A previous publication has shown frequent development of HLA-specific IgG during pregnancy.[45] To address the question of whether anti-HLA IgE can develop as a consequence of sensitizing events other than transplant rejection, we analyzed sera from 10 pregnant women known to have high levels of HLA-specific IgG. Eight of 10 women had detectable anti–HLA class I IgE antibodies, and 7 of 10 had anti–HLA class II antibodies (Fig 6, *A-C*).

Collectively, these results demonstrate that IgE specific for donor HLA class I and II antigens occurs in selected kidney transplant patients as part of the antigen-specific alloresponse.



C

IgE:	HLA I	HLA II
	Number of pos. reactions:	Number of pos. reactions:
Woman 1	43	0
Woman 2	0	1
Woman 3	28	5
Woman 4	17	12
Woman 5	0	10
Woman 6	43	5
Woman 7	61	1
Woman 8	23	0
Woman 9	16	18
Woman 10	45	0

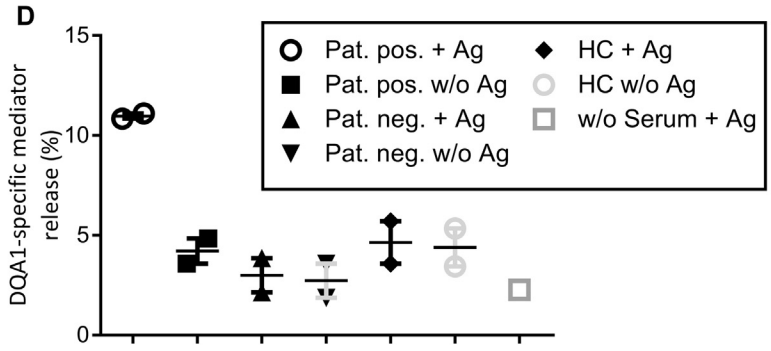


FIG 6. HLA-specific IgE also occurs during pregnancy, and donor HLA-specific IgE found in sera of kidney transplant patients shows functional activity. **A-C,** HLA-specific IgE levels were measured in sera of pregnant women known to have positive HLA IgG results. Results obtained with One Lambda LABScreen Single Antigen HLA Class I (Fig 6, *A*) and Class II (Fig 6, *B*) kits are shown. Each panel (Fig 6, *A* and *B*) shows the MFI of a representative woman compared with the mean of 3 healthy control subjects *(HC)* plus 2*SDs. Only the positive reactivities are shown. In Fig 6, *C*, reactivities of the 10 analyzed women are summarized. **D,** IgE-containing sera from kidney transplant patients were used to load a rat basophil cell line transfected to express human FcεRI. We tested patients that were either IgE positive (ie, *Pat. pos.* = patients 2 and 4) or IgE negative (ie, *Pat. neg.* = patients 1 and 3) for the respective HLA antigen (ie, DQA1). Cells were then stimulated with or without *(w/o)* beads bearing the respective HLA antigen *(Ag)*. Mediator release (ie, β-hexosaminidase) was measured and compared with healthy volunteer control sera *(HC)*, sera and cells without antigen beads, or only beads and cells without serum.

HLA-specific IgE from renal transplant recipients is functional *in vitro* and triggers basophil degranulation

In a next step the functionality of HLA-specific IgE was assessed. Basophils transfected to express human FcεRI were incubated with patient sera and stimulated with selected beads expressing single HLA antigens (namely A3, A24, A32, and DQA1). Basophil degranulation occurred on stimulation with specific class I (see Fig E8 in this article's Online Repository at www.jacionline.org) or class II (Fig 6, *D*) antigens, to which the patient had a positive IgE signal but not on stimulation with antigen, to which no positive IgE signal was detectable. Furthermore, heat inactivation of serum abrogated basophil degranulation (see Fig E9 in this article's Online Repository at www.jacionline.org).

Therefore renal transplant recipients had HLA-specific IgE that is functional in mediating basophil degranulation.

Human kidney tissue contains FcεRI$^+$ cells

The major functionality of IgE depends on tissue-resident cells expressing the IgE high-affinity receptor FcεRI. To determine whether anti-HLA IgE developing during transplant rejection has a target in the kidney, we analyzed tissue homogenates generated from human kidneys obtained from organ donors for FcεRI-expressing cell subsets. CD45$^+$FcεRI$^+$ cells were detectable in the medulla and cortex, including basophils (CD45$^+$CD123$^+$c-Kit$^+$FcεRI$^+$) and mast cells (CD45$^+$c-Kit$^+$FcεRI$^+$), which were present in 7 of 7 kidney tissue samples derived from different donors (see Fig E10 in this article's Online Repository at www.jacionline.org). These data demonstrate that the human kidney contains FcεRI-expressing cell populations that present a local intragraft target for anti-HLA IgE that develop during kidney transplant rejection.

DISCUSSION

This study demonstrates that functional IgE specific for donor MHC class I and class II antigens develops in murine and human recipients of solid allografts undergoing rejection. To our knowledge, this is the first report that DSAs of the IgE isotype develop during graft rejection.

Donor-specific IgE developed in 4 different mouse strain combinations using distinct MHC haplotypes and on rejection of 2 different types of grafts (ie, heart and skin). IgE DSAs were rapidly induced on acute rejection and persisted long-term (up to 12 months). Notably, MHC-specific IgE developed during humoral heart graft rejection, as characterized by the vasculopathy reminiscent of clinical ABMR. The functional capacity of MHC-specific IgE is indicated by induction of effector cell degranulation *in vitro* and *in vivo* on antigen stimulation. Importantly, preclinical mouse data were corroborated in sensitized patients experiencing kidney graft rejection, in which IgE specific for donor HLA class I and II antigens was measurable in serum. The other HLA specificities (ie, nondonor HLA antigens), which are also commonly observed for IgG,[46] might be due to epitope sharing, which was already shown for several HLA antigens.[47-49] Alternatively, they might be due to previous sensitization events, such as pregnancies, blood transfusions, or previous transplantations. Indeed, our data show that anti-HLA IgE can also develop during pregnancy. As in murine models, MHC-specific IgE was functional in inducing basophil degranulation on antigen stimulation. Most of the kidney transplant patients with anti-HLA IgE did not have detectable IgE to an array containing most of clinically relevant allergens.[44] Likewise, selected allergic patients with very high levels of total IgE had no detectable levels of HLA-specific IgE within the broad range of HLA molecules tested. This supports the assumption that induction of HLA-specific IgE is due to antigen-specific humoral sensitization occurring during alloreactivity.

The observed MFIs for anti-HLA IgE were low, which was expected, given the fact that IgE is the least abundant isotype in serum and might be bound to donor HLA antigens in the graft. Therefore an MFI cutoff *sui generis* had to be defined and was used for determining IgE positivity. The validity of the signal was tested in a series of control experiments, including heat inactivation and selection of an anti-IgE–detecting antibody that does not bind other antibody isotypes. Notably, positivity for HLA-specific IgE in the Luminex assay correlated with mediator release, providing strong evidence for the validity of the HLA IgE Luminex assay.

For alloantibody generation, B cells are required to present HLA antigens to T cells through the indirect pathway of allorecognition.[50] In turn, interaction of B cells with CD4$^+$ T cells leads to secretion of cytokines and generation of class-switched alloantibodies.[51-53] IgE induction is dependent on IL-4 and other T_H2-associated cytokines,[32] which were evident in the murine setting of graft rejection. A T_H2-skewed alloresponse was associated with graft acceptance in some studies, but accumulating evidence suggests that prolonged exposure to T_H2-associated cytokines promotes graft rejection.[37,54] Previous preclinical reports described T_H2-associated graft rejection linked to eosinophilic and mast cell infiltrates,[29,55,56] and eosinophils and mast cells were also noted in the process of graft rejection in patients undergoing heart, lung, kidney, and liver transplantation.[57-59] The present study reveals that MHC-specific IgE occurs during allograft rejection, thereby providing a possible humoral effector mechanism of T_H2-associated alloimmunity.

Recent studies in patients with autoimmune diseases have revealed a novel link between IgE and tissue damage. Self-reactive IgE was capable of enhancing the inflammatory response by IgG and thereby directly contributing to tissue damage in a murine systemic lupus erythematosus model.[27] Here we demonstrate the presence of FcεRI-expressing cells in the medulla and cortex of human kidneys, providing the target cells necessary for local IgE-mediated effector functions. Thus anti-donor IgE developing after transplantation might bind to tissue-resident mast cells/basophils in the allograft. Cell-bound IgE can then be cross-linked by either soluble donor HLA antigen, likely in form of HLA-containing exosomes,[60,61] or by HLA expressed on donor parenchymal or hematopoietic cells, triggering degranulation and mediator release. The ensuing proinflammatory response could directly inflict local damage and reinforce the anti-donor T-cell response through various mediators, including TNF-α and IL-6.[62,63]

We gratefully acknowledge the National Institutes of Health's Tetramer Core Facility for provision of MHC class I H-2K^k, MHC class I H-2D^k, MHC class I H-2K^d, MHC class I H-2D^d, MHC class II I-E^k, and MHC class II I-E^d monomers.

Key messages

- **Anti-donor MHC IgE develops in several mouse models of heart and skin transplantation, including a model of antibody-mediated heart graft rejection.**
- **Anti-donor HLA IgE is detected in a cohort of sensitized kidney transplant recipients.**
- **Functionality of both mouse and human anti-MHC/HLA IgE is shown in *in vitro* and *in vivo* mediator release assays.**

REFERENCES

1. Wekerle T, Segev D, Lechler R, Oberbauer R. Strategies for long-term preservation of kidney graft function. Lancet 2017;389:2152-62.

2. Lamb KE, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: a critical reappraisal. Am J Transplant 2011;11:450-62.
3. Sellares J, de Freitas DG, Mengel M, Reeve J, Einecke G, Sis B, et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. Am J Transplant 2012;12:388-99.
4. Valenzuela NM, Reed EF. Antibody-mediated rejection across solid organ transplants: manifestations, mechanisms, and therapies. J Clin Invest 2017;127:2492-504.
5. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. Nat Rev Nephrol 2012;8:348-57.
6. O'Leary JG, Demetris AJ, Friedman LS, Gebel HM, Halloran PF, Kirk AD, et al. The role of donor-specific HLA alloantibodies in liver transplantation. Am J Transplant 2014;14:779-87.
7. Smith JD, Banner NR, Hamour IM, Ozawa M, Goh A, Robinson D, et al. De novo donor HLA-specific antibodies after heart transplantation are an independent predictor of poor patient survival. Am J Transplant 2011;11:312-9.
8. Laffer S, Hogbom E, Roux KH, Sperr WR, Valent P, Bankl HC, et al. A molecular model of type I allergy: Identification and characterization of a nonanaphylactic anti-human IgE antibody fragment that blocks the IgE-FcεRI interaction and reacts with receptor-bound IgE. J Allergy Clin Immunol 2001;108:409-16.
9. Levine DJ, Glanville AR, Aboyoun C, Belperio J, Benden C, Berry GJ, et al. Antibody-mediated rejection of the lung: a consensus report of the International Society for Heart and Lung Transplantation. J Heart Lung Transplant 2016;35:397-406.
10. Lefaucheur C, Loupy A, Hill GS, Andrade J, Nochy D, Antoine C, et al. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. J Am Soc Nephrol 2010;21:1398-406.
11. Caillard S, Becmeur C, Gautier-Vargas G, Olagne J, Muller C, Cognard N, et al. Pre-existing donor-specific antibodies are detrimental to kidney allograft only when persistent after transplantation. Transpl Int 2017;30:29-40.
12. Wiebe C, Gibson IW, Blydt-Hansen TD, Pochinco D, Birk PE, Ho J, et al. Rates and determinants of progression to graft failure in kidney allograft recipients with de novo donor-specific antibody. Am J Transplant 2015;15:2921-30.
13. Guidicelli G, Guerville F, Lepreux S, Wiebe C, Thaunat O, Dubois V, et al. Non-complement-binding de novo donor-specific anti-HLA antibodies and kidney allograft survival. J Am Soc Nephrol 2016;27:615-25.
14. Aubert O, Loupy A, Hidalgo L, van Huyen J-PD, Higgins S, Viglietti D, et al. Antibody-mediated rejection due to preexisting versus de novo donor-specific antibodies in kidney allograft recipients. J Am Soc Nephrol 2017;28:1912-23.
15. Doran TJ, Susal C, Opelz G, Geczy AF. IgA class antibodies and flow cytometric cross-matching in renal transplantation. Transplantation 1999;67:309-14.
16. Khovanova N, Daga S, Shaikhina T, Krishnan N, Jones J, Zehnder D, et al. Subclass analysis of donor HLA-specific IgG in antibody-incompatible renal transplantation reveals a significant association of IgG4 with rejection and graft failure. Transpl Int 2015;28:1405-15.
17. Lefaucheur C, Viglietti D, Bentlejewski C, Duong van Huyen JP, Vernerey D, Aubert O, et al. IgG donor-specific anti-human HLA antibody subclasses and kidney allograft antibody-mediated injury. J Am Soc Nephrol 2016;27:293-304.
18. Loupy A, Toquet C, Rouvier P, Beuscart T, Bories MC, Varnous S, et al. Late failing heart allografts: pathology of cardiac allograft vasculopathy and association with antibody-mediated rejection. Am J Transplant 2016;16:111-20.
19. Loupy A, Lefaucheur C, Vernerey D, Prugger C, Duong van Huyen JP, Mooney N, et al. Complement-binding anti-HLA antibodies and kidney-allograft survival. N Engl J Med 2013;369:1215-26.
20. Lefaucheur C, Loupy A, Vernerey D, Duong-Van-Huyen JP, Suberbielle C, Anglicheau D, et al. Antibody-mediated vascular rejection of kidney allografts: a population-based study. Lancet 2013;381:313-9.
21. Bartel G, Wahrmann M, Schwaiger E, Kikic Z, Winzer C, Horl WH, et al. Solid phase detection of C4d-fixing HLA antibodies to predict rejection in high immunological risk kidney transplant recipients. Transpl Int 2013;26:121-30.
22. Sicard A, Ducreux S, Rabeyrin M, Couzi L, McGregor B, Badet L, et al. Detection of C3d-binding donor-specific anti-HLA antibodies at diagnosis of humoral rejection predicts renal graft loss. J Am Soc Nephrol 2015;26:457-67.
23. Stegall MD, Chedid MF, Cornell LD. The role of complement in antibody-mediated rejection in kidney transplantation. Nat Rev Nephrol 2012;8:670-8.
24. Wu LC, Zarrin AA. The production and regulation of IgE by the immune system. Nat Rev Immunol 2014;14:247-59.
25. Valenta R, Mittermann I, Werfel T, Garn H, Renz H. Linking allergy to autoimmune disease. Trends Immunol 2009;30:109-16.
26. Charles N, Hardwick D, Daugas E, Illei GG, Rivera J. Basophils and the T helper 2 environment can promote the development of lupus nephritis. Nat Med 2010;16:701-7.
27. Henault J, Riggs JM, Karnell JL, Liarski VM, Li J, Shirinian L, et al. Self-reactive IgE exacerbates interferon responses associated with autoimmunity. Nat Immunol 2016;17:196-203.
28. Tsiantoulas D, Bot I, Ozsvar-Kozma M, Göderle L, Perkmann T, Hartvigsen K, et al. Increased plasma IgE accelerate atherosclerosis in secreted IgM deficiency: novelty and significance. Circ Res 2017;120:78-84.
29. Goldman M, Le Moine A, Braun M, Flamand V, Abramowicz D. A role for eosinophils in transplant rejection. Trends Immunol 2001;22:247-51.
30. Sadeghi M, Daniel V, Weimer R, Wiesel M, Hergesell O, Opelz G. Pre-transplant Th1 and post-transplant Th2 cytokine patterns are associated with early acute rejection in renal transplant recipients. Clin Transplant 2003;17:151-7.
31. Kishimoto K, Dong VM, Issazadeh S, Fedoseyeva EV, Waaga AM, Yamada A, et al. The role of CD154-CD40 versus CD28-B7 costimulatory pathways in regulating allogeneic Th1 and Th2 responses in vivo. J Clin Invest 2000;106:63-72.
32. Finkelman FD, Holmes J, Katona IM, Urban JF Jr, Beckmann MP, Park LS, et al. Lymphokine control of in vivo immunoglobulin isotype selection. Annu Rev Immunol 1990;8:303-33.
33. Loveless M. Immunological studies of pollinosis IV: the relationship between thermostable antibody in the circulation and clinical immunity. J Immunol 1943;47:165.
34. Prouvost-Danon A, Abadie A, da Ponte JV. Heat inactivation of rat and mouse IgE. Mol Immunol 1980;17:247-53.
35. Prouvost-Danon A, Binaghi RA, Abadie A. Effect of heating at 56°C on mouse IgE antibodies. Immunochemistry 1977;14:81-4.
36. Demeulemester C, Weyer A, Peltre G, Laurent M, Marchand F, David B. Thermoinactivation of human IgE: antigenic and functional modifications. Immunology 1986;57:617-20.
37. Wang H, Hosiawa KA, Min W, Yang J, Zhang X, Garcia B, et al. Cytokines regulate the pattern of rejection and susceptibility to cyclosporine therapy in different mouse recipient strains after cardiac allografting. J Immunol 2003;171:3823-36.
38. Haeryfar SM, Lan Z, Leon-Ponte M, Duffy KR, Ge W, Liu W, et al. Prolongation of cardiac allograft survival by rapamycin and the invariant natural killer T cell glycolipid agonist OCH. Transplantation 2008;86:460-8.
39. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. Transplantation 2012;93:1-10.
40. Ali Jason M, Negus Margaret C, Conlon Thomas M, Harper Ines G, Qureshi MS, Motallebzadeh R, et al. Diversity of the CD4T cell alloresponse: the short and the long of it. Cell Rep 2016;14:1232-45.
41. Harper IG, Ali JM, Harper SJ, Wlodek E, Alsughayyir J, Negus MC, et al. Augmentation of recipient adaptive alloimmunity by donor passenger lymphocytes within the transplant. Cell Rep 2016;15:1214-27.
42. Schwaiger E, Wahrmann M, Bond G, Eskandary F, Bohmig GA. Complement component C3 activation: the leading cause of the prozone phenomenon affecting HLA antibody detection on single-antigen beads. Transplantation 2014;97:1279-85.
43. Lupinek C, Roux KH, Laffer S, Rauter I, Reginald K, Kneidinger M, et al. Trimolecular complex formation of IgE, FcεRI, and a recombinant nonanaphylactic single-chain antibody fragment with high affinity for IgE. J Immunol 2009;182:4817-29.
44. Lupinek C, Wollmann E, Baar A, Banerjee S, Breiteneder H, Broecker BM, et al. Advances in allergen-microarray technology for diagnosis and monitoring of allergy: the MeDALL allergen-chip. Methods 2014;66:106-19.
45. Küssel L, Herkner H, Wahrmann M, Eskandary F, Doberer K, Binder J, et al. Longitudinal assessment of HLA and MIC-A antibodies in uneventful pregnancies and pregnancies complicated by preeclampsia or gestational diabetes. Sci Rep 2017;7:13524.
46. Richter R, Susal C, Kohler S, Qidan S, Schodel A, Holschuh L, et al. Pretransplant human leukocyte antigen antibodies detected by single-antigen bead assay are a risk factor for long-term kidney graft loss even in the absence of donor-specific antibodies. Transpl Int 2016;29:988-98.
47. Wade JA, Hurley CK, Takemoto SK, Thompson J, Davies SM, Fuller TC, et al. HLA mismatching within or outside of cross-reactive groups (CREGs) is associated with similar outcomes after unrelated hematopoietic stem cell transplantation. Blood 2007;109:4064-70.
48. Marrari M, Mostecki J, Mulder A, Claas F, Balazs I, Duquesnoy RJ. Human monoclonal antibody reactivity with human leukocyte antigen class I epitopes defined by pairs of mismatched eplets and self-eplets. Transplantation 2010;90:1468-72.
49. Lomago J, Jelenik L, Zern D, Howe J, Martell J, Zeevi A, et al. How did a patient who types for HLA-B*4403 develop antibodies that react with HLA-B*4402? Hum Immunol 2010;71:176-8.
50. Shiu KY, McLaughlin L, Rebollo-Mesa I, Zhao J, Burton H, Douthwaite H, et al. Graft dysfunction in chronic antibody-mediated rejection correlates with B-cell-

dependent indirect antidonor alloresponses and autocrine regulation of interferon-gamma production by Th1 cells. Kidney Int 2017;91:477-92.
51. Conlon TM, Saeb-Parsy K, Cole JL, Motallebzadeh R, Qureshi MS, Rehakova S, et al. Germinal center alloantibody responses are mediated exclusively by indirect-pathway CD4 T follicular helper cells. J Immunol 2012;188:2643-52.
52. Hickey MJ, Valenzuela NM, Reed EF. Alloantibody generation and effector function following Sensitization to human leukocyte antigen. Front Immunol 2016;7.
53. Dijke EI, Platt JL, Blair P, Clatworthy MR, Patel JK, Kfoury AG, et al. B cells in transplantation. J Heart Lung Transplant 2016;35:704-10.
54. Tay SS, Plain KM, Bishop GA. Role of IL-4 and Th2 responses in allograft rejection and tolerance. Curr Opin Organ Transplant 2009;14:16-22.
55. Surquin M, Le Moine A, Flamand V, Rombaut K, Demoor FX, Salmon I, et al. IL-4 deficiency prevents eosinophilic rejection and uncovers a role for neutrophils in the rejection of MHC class II disparate skin grafts. Transplantation 2005;80:1485-92.
56. Surquin M, Le Moine A, Flamand V, Nagy N, Rombaut K, Demoor FX, et al. Skin graft rejection elicited by beta 2-microglobulin as a minor transplantation antigen involves multiple effector pathways: role of Fas-Fas ligand interactions and Th2-dependent graft eosinophil infiltrates. J Immunol 2002;169:500-6.
57. Yousem SA. Graft eosinophilia in lung transplantation. Hum Pathol 1992;23:1172-7.
58. Ishida T, Hyodo Y, Ishimura T, Takeda M, Hara I, Fujisawa M. Mast cell numbers and protease expression patterns in biopsy specimens following renal transplantation from living-related donors predict long-term graft function. Clin Transplant 2005;19:817-24.
59. El-Refaie AM, Burt AD. Mast cells and c-Kit expression in liver allograft rejection. Histopathology 2005;47:375-81.
60. Liu Q, Rojas-Canales DM, Divito SJ, Shufesky WJ, Stolz DB, Erdos G, et al. Donor dendritic cell–derived exosomes promote allograft-targeting immune response. J Clin Invest 2016;126:2805-20.
61. Marino J, Babiker-Mohamed MH, Crosby-Bertorini P, Paster JT, LeGuern C, Germana S, et al. Donor exosomes rather than passenger leukocytes initiate alloreactive T cell responses after transplantation. Sci Immunol 2016;1:aaf8759.
62. Theoharides TC, Valent P, Akin C. Mast cells, mastocytosis, and related disorders. N Engl J Med 2015;373:163-72.
63. McLachlan JB, Hart JP, Pizzo SV, Shelburne CP, Staats HF, Gunn MD, et al. Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection. Nat Immunol 2003;4:1199-205.

METHODS

Animals

Female C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H/HeNCrl (H-2^k) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Bm12.Kd.IE[E1] mice were generated and bred in the laboratory of G. Pettigrew. Mice were housed under specific pathogen-free conditions in individually ventilated filter cages (up to 5 mice) on sterile standard bedding; sterile water and a standard pellet diet were administered *ad libitum*. Housing rooms were equipped with a 12-hour light cycle. Mice were used at 6 to 8 weeks of age, with an average weight of 18 to 22 g.

Skin transplantation

Full-thickness tail skin from fully mismatched BALB/c (H-2^d) and C3H (H-2^k) mice was grafted and visually inspected thereafter at short intervals. Grafts were considered to be rejected, when less than 10% remained viable.

Cardiac transplantation

Cervical heterotopic heart transplantation was performed with a modified cuff technique for revascularization, as described previously.[E2] Briefly, after preparation of the right external jugular vein and common carotid artery cuffs, the donor heart was harvested and transferred to the recipient's neck. The main pulmonary artery and aorta were anastomosed to the recipient's external jugular vein and carotid artery, respectively. Cardiac graft survival was determined by means of daily palpation and observation under the microscope, with complete cessation of heart beats indicating the end of graft survival. Bm12.Kd.IE hearts were transplanted intra-abdominally, as described by Ali Jason et al.[E3]

Recombinant H-2K, H-2D, and I-E monomers

MHC class I H-$2K^k$, MHC class I H-$2D^k$, MHC class I H-$2K^d$, MHC class I H-$2D^d$, MHC class II I-E^k, and MHC class II I-E^d monomers were kindly provided by the National Institutes of Health's Tetramer Core Facility.

H-2K–, H-2D–, and I-E–specific ELISA

To measure MHC-specific antibodies in the sera of transplanted mice, we modified an ELISA protocol originally developed to measure allergen-specific IgE levels in serum.[E4] Plates were coated with rH-$2K^k$, rH-$2K^d$, rH-$2D^k$, rH-$2D^d$, I-E^d, or I-E^k (5 μg/mL), for I-E^k anti-I-A/I-E mAb clone M5/114.15.2, BioLegend, San Diego, Calif 1:1000, served as a positive control. Sera were diluted 1:2.5 for IgE and 1:125 for IgG_1, $IgG_{2a/b}$, and IgG_3, respectively. Where indicated, sera were heat inactivated at 56°C for 60 minutes. Bound antibodies were detected with rat anti-mouse IgE (clone R35-72), IgG_1 (clone A85-1), $IgG_{2a/c}$ (clone R19-15), IgG_{2b} (clone R9-91), or IgG_3 (clone R2-38) mAbs (BD, San Diego, Calif) diluted 1:1000, and a horseradish peroxidase (HRP)–coupled goat anti-rat antiserum (Amersham Biosciences, Little Chalfont, United Kingdom) was diluted 1:2000. The substrate for HRP was ABTS (60 mmol/L citric acid, 77 mmol/L $Na_2HPO_4 \times 2\ H_2O$, 1.7 mmol/L ABTS [Sigma, St Louis, Mo], and 3 mmol/L H_2O_2). Absorption was measured with a Victor microplate reader (PerkinElmer, Waltham, Mass).

Intracellular cytokine staining

One million splenocytes or lymph node cells were incubated for 5 hours with phorbol 12-myristate 13-acetate/ionomycin (at 50 ng/mL and 1 μg/mL, respectively) in the presence of BD GolgiStop Protein Transport Inhibitor. Subsequently, cells were stained according to the protocol of the mouse $T_H1/T_H2/T_H17$ Phenotyping Kit (BD Bioscience, Franklin Lakes, NJ), with additional phycoerythrin (PE)-Cy7–conjugated anti-CD3 staining (clone 17A2; BioLegend, San Diego, Calif). Flow cytometric analysis was performed with the BD FACSCanto II and FlowJo software (TreeStar, Ashland, Ore).

Detection of secreted cytokines in MLR supernatants

Aliquots of 4×10^5 responder cells were incubated in triplicates with 4×10^5 irradiated (30 Gy) stimulator cells (either allogeneic or syngeneic as a control) from BALB/c, C3H, and C57BL/6 mice for 4 days. Subsequently, supernatants were analyzed with the ProcartaPlex Mouse Essential Th1/Th2 Cytokine Panel (6-plex; eBioscience, San Diego, Calif), according to the manufacturer's protocol. Samples were measured with a MAGPIX instrument (Luminex, Austin, Tex) and xPONENT software (Luminex).

Rat basophil leukemia cell degranulation assay

The rat basophil leukemia (RBL)−2H3 cell subline was cultured, as described previously,[E5] in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% FCS. Cells (4×10^4) were plated in 96-well tissue-culture plates (Greiner Bio-One, Kremsmünster, Austria), loaded with 1:1.5 diluted mouse sera, and incubated for 2 hours at 37°C in a 5% CO_2 atmosphere. Supernatants were removed, and the cell layer was washed with 2× Tyrode buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 0.4 mmol/L NaH_2PO_4, 5.6 mmol/L D-glucose, 12 mmol/L $NaHCO_3$, 10 mmol/L HEPES, and 0.1% [wt/vol] BSA [pH 7.2]). Preloaded cells were stimulated with rH-$2K^k$, rH-$2K^d$, rH-$2D^k$, or rH-$2D^d$ (0.03 μg/well) for 30 minutes at 37°C. Supernatants were analyzed for β-hexosaminidase activity by means of incubation with the substrate 80 μmol/L 4-methylumbelliferyl-N-acetyl-β-D-glucosamide (Sigma-Aldrich) in citrate buffer (0.1 mol/L, pH 4.5) for 1 hour at 37°C. The reaction was stopped by addition of 100 μL of glycine buffer (0.2 mol/L glycine and 0.2 mol/L NaCl, pH 10.7), and fluorescence was measured at an excitation/emission of 360/465 nm by using a fluorescence microplate reader (PerkinElmer). Results are reported as a percentage of total β-hexosaminidase released after addition of 1% Triton X-100. Determinations were done in triplicates.

Mediator release assay with humanized RBL cells transfected with human FcεRI

RBL-2H3 cells transfected[E6,E7] with cDNA coding for the human high-affinity IgE receptor chains α, β, and γ were maintained in RPMI 1640 medium supplemented with 5% FBS, 4 mmol/L l-glutamine and 1 mg/mL gentamicin sulfate.[E6] Cells were incubated with sera from patients or healthy control subjects and incubated with selected single HLA antigens coupled to single-antigen beads (One Lambda, Canoga Park, Calif). β-Hexosaminidase release from activated RBL cells was measured, as previously described.[E8]

Cutaneous type I hypersensitivity reaction

An adapted protocol previously described by Wiedermann et al[E7] was used. Briefly, mice were injected intravenously with 100 μL of 0.5% Evans blue (Sigma-Aldrich). Subsequently, 30 μL of rH2-K^k and rH-$2D^k$ (2 μg/mL diluted in PBS) was injected intradermally into the shaved abdominal skin, as described previously. As a positive control, the mast cell–degranulating compound 48/80 (20 μg/mL; Sigma-Aldrich) was injected intradermally, whereas PBS or rH-$2D^d$ (2 μg/mL diluted in PBS) was injected as a negative control. Fifteen minutes after injection, mice were killed, and the blue color intensity of a positive skin reaction caused by vascular permeability was compared with the individual positive and negative control on inverted skin.

Histologic analysis

Four-micrometer sections of heart tissues were cut from paraffin-embedded tissue fixed in 4.5% formalin (with a buffered pH of 7.5), stained with hematoxylin and eosin according to standard protocols, analyzed, and graded by an experienced pathologist. For C4d, antigen retrieval was done with Pascal/Tris EDTA, pH 9, and subsequent blocking in 3% H_2O_2/PBS, followed by avidin-biotin block (Vector Laboratories, Burlingame, Calif) and 10% goat serum (Sigma-Aldrich). Polyclonal rabbit anti-mouse C4d (Hycult Biotech, Uden, The Netherlands) was diluted 1:50 in PBS and incubated overnight at 4°C PBS, and biotinylated polyclonal goat anti-rabbit serum (Vector Laboratories) was diluted 1:300 in PBS and incubated at room temperature for 30 minutes. AEC-Chromogen (3-amino-9-ethylcarbazole) was added for 10 minutes prior to hematoxylin counterstaining performed for 1 minute, before embedding the samples.

Sera from kidney transplant patients

Sera were obtained from patients undergoing kidney transplantation who were known to be broadly sensitized to high levels of HLA-specific antibodies and were waiting for retransplantation at the Vienna Transplantation Unit. All patients experienced at least 1 episode of histopathologically proven rejection (in 4 of 5 there were clear signs of an antibody-mediated or mixed rejection type). Samples were placed in aliquots and frozen at −80°C.

Assessing isotype specificity of anti-human IgE by using ELISA

As described by Lupinek et al,[E9] ELISA plates (Costar; Corning, Corning, NY) were coated (coating buffer, 0.1 mol/L $NaHCO_3$ [pH 9.6]) overnight at 4°C with 300 ng of human IgM, IgG_1, IgG_2, IgG_3, and IgG_4 (Sigma-Aldrich) and IgE purified from serum of an allergic patient by using affinity chromatography.[E10] After washing with PBST (PBS [pH 7.5] with 0.05% [vol/vol] Tween 20) and blocking with PBST/1% (wt/vol) BSA for 2.5 hours at 37°C, 0.2 μg/mL biotin-conjugated anti-human IgE mAb (clone MHE-18; BioLegend) diluted in PBST/0.5% (wt/vol) BSA was applied and incubated overnight at 4°C. After washing with PBST (PBS and 0.5% [vol/vol] Tween 20), Streptavidin-HRP conjugates (BD Biosciences) diluted 1:10,000 in PBST/0.5% (wt/vol) BSA were applied, and after incubation at room temperature for 30 minutes, unbound detection antibody was removed by washing with PBST. The substrate for HRP was ABTS (60 mmol/L citric acid, 77 mmol/L $Na_2HPO_4 \times 2H_2O$, 1.7 mmol/L ABTS [Sigma], and 3 mmol/L H_2O_2). All samples were measured in triplicates, and results show mean values with SDs.

Detection of HLA-specific IgE

Deposition of IgE to single-antigen beads (SABs) were assessed by using a modified protocol for IgG detection by using One Lambda SAB kits (LABScreen Single Antigen HLA class I and HLA class II Antibody Detection Tests). In brief, 1 μL of SABs was first incubated with 4 μL of patient sera, and beads were then washed and incubated with biotin-conjugated anti-human IgE mAb (dilution 1:20, clone MHE-18; BioLegend). In a second step PE-conjugated streptavidin (dilution 1:200; eBioscience, San Diego, Calif) was added. Samples were measured on a Luminex flow analyzer. The presence of HLA-specific IgE was determined based on baseline SAB reactivity patterns of 3 healthy volunteers. SAB reactivities with an MFI greater than the corresponding mean MFI of 3 healthy volunteers plus 2*SDs were considered positive. We decided to have a minimum MFI of 25 because of the observation that in the basophil release assay the lowest MFI for an HLA-specific IgE antibody that triggered a release was 40, whereas another bead value with an MFI of 21.5 did not. Additionally, because some mean MFIs of the healthy control subjects exceeded 30, we concluded that some beads have a stronger background signal. Therefore each bead value also had to be greater than the mean of the 3 healthy control subjects plus 2*SDs to make our assay more stringent. As a control, in selected samples IgE reactivities were destroyed by means of heat inactivation before bead incubation.

Detection of allergen-specific IgE in patient sera

Patient sera were analyzed with the MeDALL allergen chip for allergen-specific IgE, as described by Lupinek et al.[E11]

Detection of total IgE in patient sera

Patient sera were analyzed with ImmunoCAP total IgE kits (Thermo Fisher Scientific, Waltham, Mass), according to the manufacturer's manual.

Cell isolation from human renal tissue

Human tissue (30 g) was obtained from organ donors in whom consent for research was present. Kidney tissue was minced, incubated in digest mix for 30 minutes at 37°C, dissociated with a gentleMACS machine (Miltenyi Biotech, Bisley, United Kingdom) and then passed sequentially through 100-, 50-, and 30-μm cell strainers. Addition of 44% Percoll followed by centrifugation was used to remove debris. Cells were blocked with human FcR block (Miltenyi Biotech) and incubated with antibodies for 1 hour at 4°C, followed by Live/Dead cell staining (Zombie Aqua Fixable Viability Kit; BioLegend) for 20 minutes at room temperature. Cell-surface receptor staining was undertaken at room temperature. Samples were processed on a Fortessa flow cytometer (Becton Dickinson, Basel, Switzerland), and data were analyzed with FlowJo software (TreeStar). Antibodies were used at 1:200 as follows: mouse anti-human CD45 (PE; clone 2D1; eBioscience), mouse anti-human CD3 (A700, clone UCHT1; eBioscience), mouse anti-human CD15 (A700, clone U63D; BioLegend), mouse anti-human CD19 (A700, clone HIB19; eBioscience), mouse anti-human MHC class II (BV785, clone L243; eBioscience), mouse anti-human CD123 (fluorescein isothiocyanate; clone 6H6; eBioscience), mouse anti-human c-Kit (allophycocyanin; clone YB5B8; eBioscience), and mouse anti-human IgεR (peridinin-chlorophyll-protein complex; clone AER-37; BioLegend).

Study approval

All human samples were collected on written informed consent. The studies were approved by the ethics review board of the Medical University of Vienna (EK no. 267/2011). Ethical approval for the kidney tissues was granted by the local ethics committee of the University of Cambridge (REC12/EE/0446 and REC15/EE/0152), and the studies were also approved by NHS Blood and Transplant. We received sera from pregnant women with high HLA IgG levels from the Biobank for Pregnancies at the Department of Obstetrics and Feto-maternal Medicine, Medical University of Vienna, Vienna General Hospital ("Biobank"; EK nos. 619/2006 and 559/2015, respectively). All murine experiments were approved by the Ethics and Animal Welfare Committee of the Medical University of Vienna and were performed in accordance with national and international guidelines of laboratory animal care. All animals received humane care in compliance with FELASA and ARRIVE and were approved by the Austrian Federal Ministry of Science, Research and Economy BMWF GZ: 66.009/0295-1I/3b/2011 and GZ 66.009/0230- II/3b/2011.

Statistical analysis

The reported P values are results of 2-sided Mann–Whitney U or χ^2 tests. P values of less than .05 were considered statistically significant. Whiskers in box plots indicate the minimum to maximum range of the values, and error bars indicate SDs.

REFERENCES

E1. El-Refaie AM, Burt AD. Mast cells and c-Kit expression in liver allograft rejection. Histopathology 2005;47:375-81.
E2. Brandacher G, Zou Y, Margreiter R, Steurer W. Cervical heterotopic heart transplantation in the mouse with an improved cuff technique. Eur Surg Res 1998;30:52.
E3. Ali Jason M, Negus Margaret C, Conlon Thomas M, Harper Ines G, Qureshi MS, Motallebzadeh R, et al. Diversity of the CD4T cell alloresponse: the short and the long of it. Cell Rep 2016;14:1232-45.
E4. Vrtala S, Sperr WR, Reimitzer I, van Ree R, Laffer S, Muller WD, et al. cDNA cloning of a major allergen from timothy grass (Phleum pratense) pollen; characterization of the recombinant Phl pV allergen. J Immunol 1993;151:4773-81.
E5. Rauter I, Krauth MT, Flicker S, Gieras A, Westritschnig K, Vrtala S, et al. Allergen cleavage by effector cell-derived proteases regulates allergic inflammation. FASEB J 2006;20:967-9.
E6. Vogel L, Luttkopf D, Hatahet L, Haustein D, Vieths S. Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. Allergy 2005;60:1021-8.
E7. Wiedermann U, Jahn-Schmid B, Bohle B, Repa A, Renz H, Kraft D, et al. Suppression of antigen-specific T- and B-cell responses by intranasal or oral administration of recombinant bet v 1, the major birch pollen allergen, in a murine model of type I allergy. J Allergy Clin Immunol 1999;103:1202-10.
E8. Hartl A, Hochreiter R, Stepanoska T, Ferreira F, Thalhamer J. Characterization of the protective and therapeutic efficiency of a DNA vaccine encoding the major birch pollen allergen Bet v 1a. Allergy 2004;59:65-73.
E9. Lupinek C, Roux KH, Laffer S, Rauter I, Reginald K, Kneidinger M, et al. Trimolecular complex formation of IgE, FcεRI, and a recombinant nonanaphylactic single-chain antibody fragment with high affinity for IgE. J Immunol 2009;182:4817-29.
E10. Laffer S, Hogbom E, Roux KH, Sperr WR, Valent P, Bankl HC, et al. A molecular model of type I allergy: identification and characterization of a nonanaphylactic anti-human IgE antibody fragment that blocks the IgE-FcεRI interaction and reacts with receptor-bound IgE. J Allergy Clin Immunol 2001;108:409-16.
E11. Lupinek C, Wollmann E, Baar A, Banerjee S, Breiteneder H, Broecker BM, et al. Advances in allergen-microarray technology for diagnosis and monitoring of allergy: the MeDALL allergen-chip. Methods 2014;66:106-19.

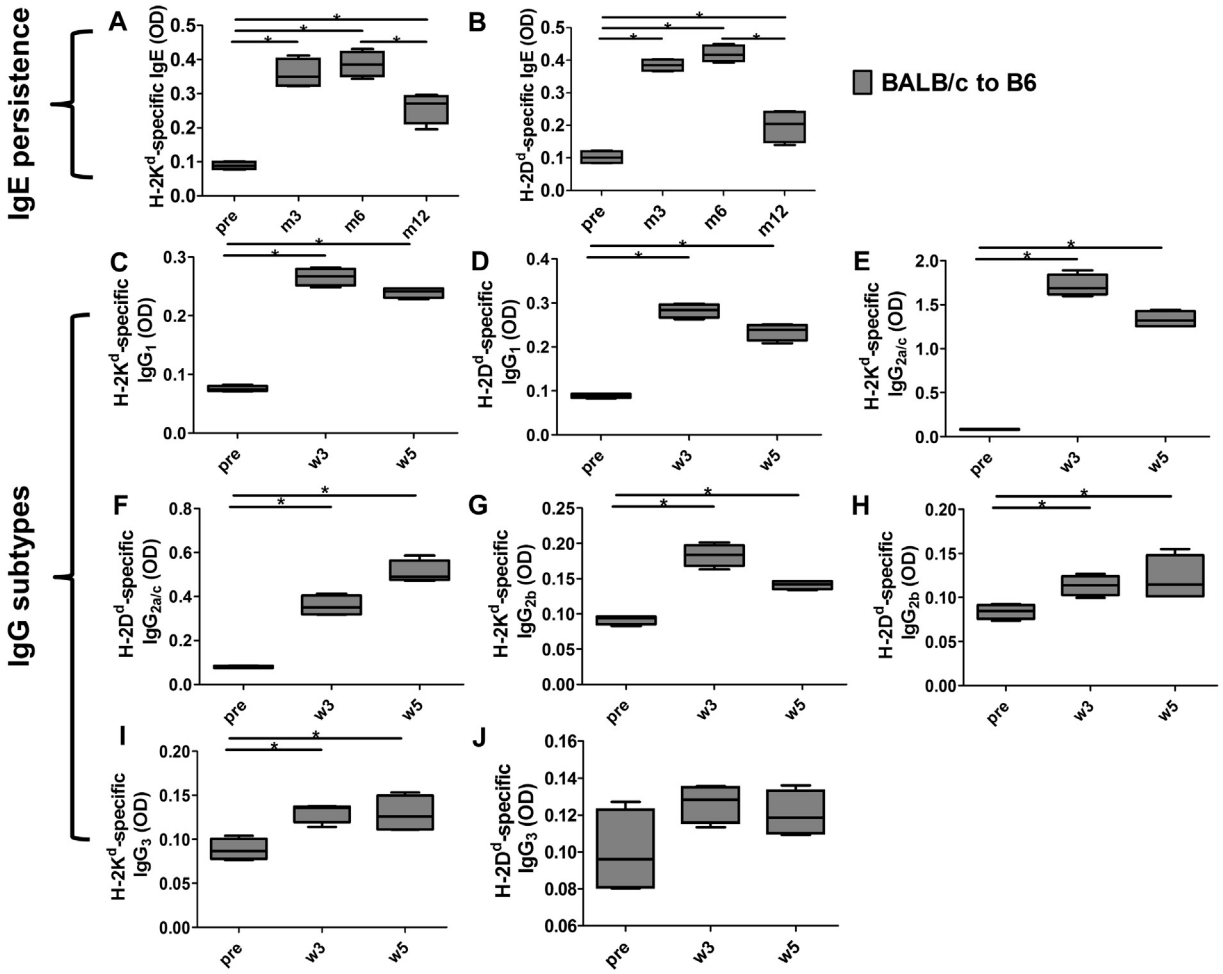


FIG E1. MHC-specific IgG subtypes and IgE persistence after skin transplantation. Antibody isotypes (indicated in the respective *y-axis legend*) specific for MHC class I (ie, H-2K^d [**A, C, E, G,** and **I**] and H-2D^d [**B, D, F, H,** and **J**]) in the setting of BALB/c to C57BL/6J skin transplants. Antibody levels were measured in sera of recipient mice at indicated time points (n = 6). *pre*, Pre-transplantation; *w*, weeks after transplantation. *P* values were calculated with the Mann-Whitney *U* test. **P* < .05.

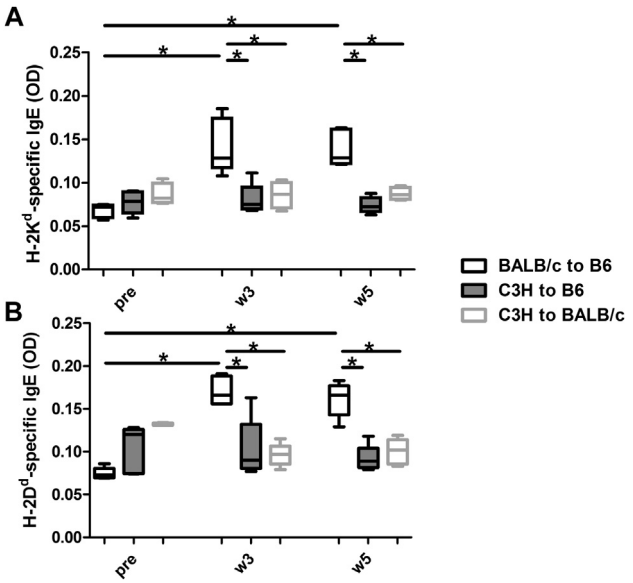


FIG E2. Developing IgE is specific for donor MHC antigens. IgE specific for MHC class I H-2K^d **(A)** and H-2D^d **(B)** antigens in the setting of the indicated skin transplant combinations (ie, donor antigens for BALB/c to B6 and nondonor antigens for C3H to B6 and C3H to BALB/c combinations). Antibody levels were measured in sera of recipient mice (n = 5 per group) at indicated time points. *pre,* Pre-transplantation; *w,* weeks after transplantation. *P* values were calculated with the Mann-Whitney *U* test. **P* < .05.

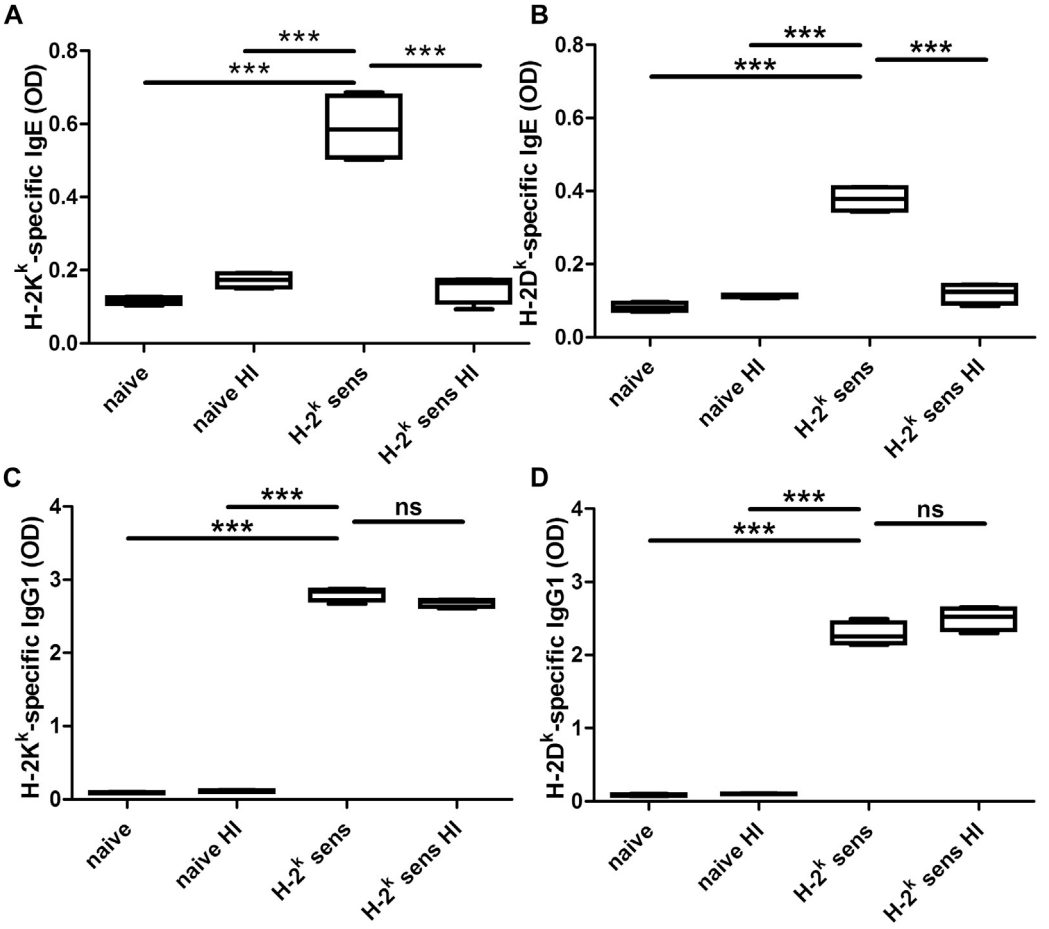


FIG E3. Heat inactivation abrogates the MHC-specific IgE signal but not the signal for IgG_1. IgE (**A** and **B**) or IgG_1 (**C** and **D**) specific for MHC class I (H-$2K^k$ [Fig E3, *A* and *C*] and H-$2D^k$ [Fig E3, *B* and *D*]) in naive mice or in the setting of BALB/c to C57BL/6 skin transplants. Antibody levels were measured in pooled serum of recipient mice with *(HI)* or without heat inactivation, as indicated. *P* values were calculated with the Mann-Whitney *U* test. ***$P \leq .001$. *ns*, Not significant.

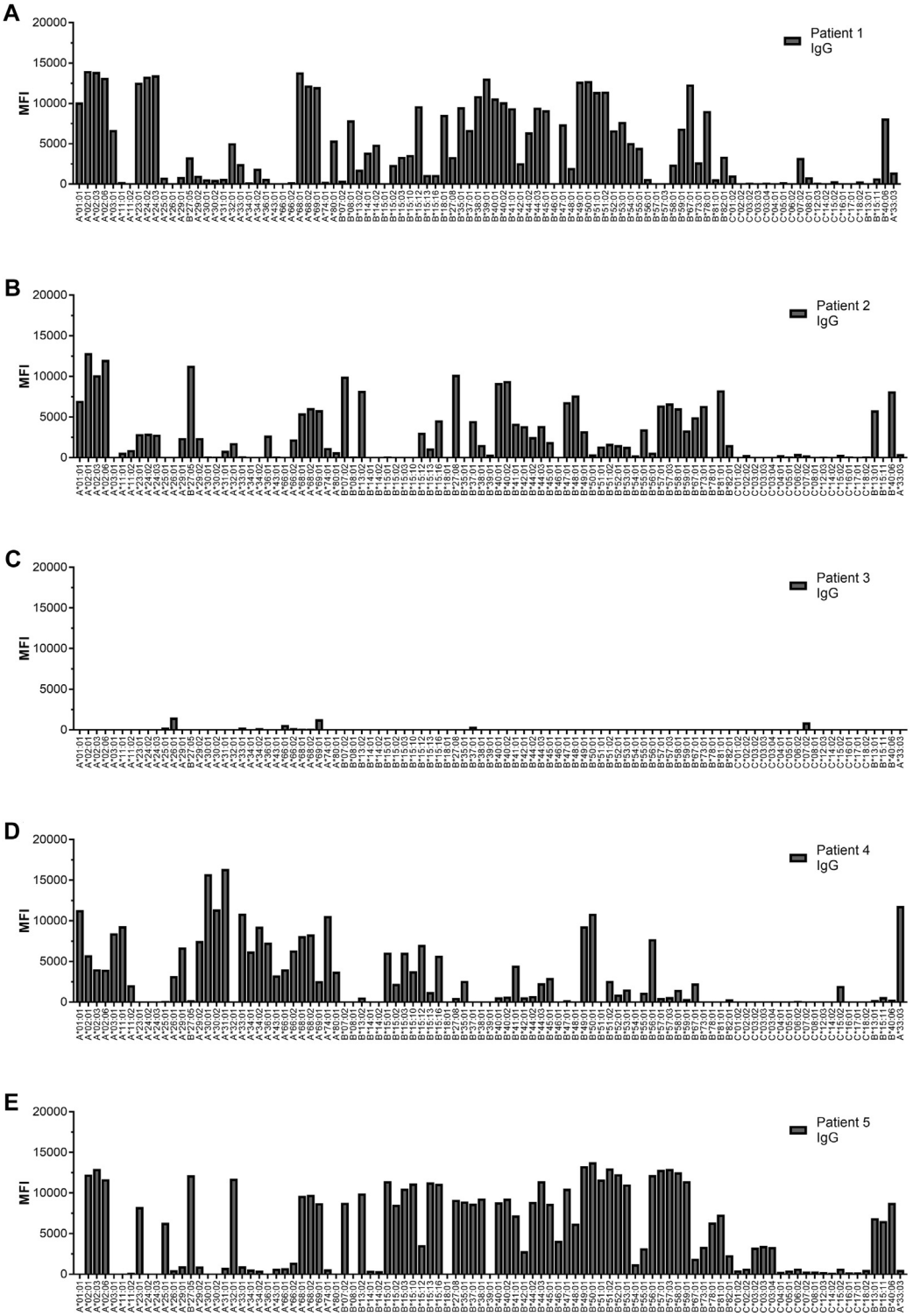


FIG E4. Anti−HLA class I IgG levels measured with One Lambda LABScreen Single Antigen HLA Class II kits in patients used for HLA-specific IgE analysis. **A-E,** Each panel shows the MFI for the panel of SABs.

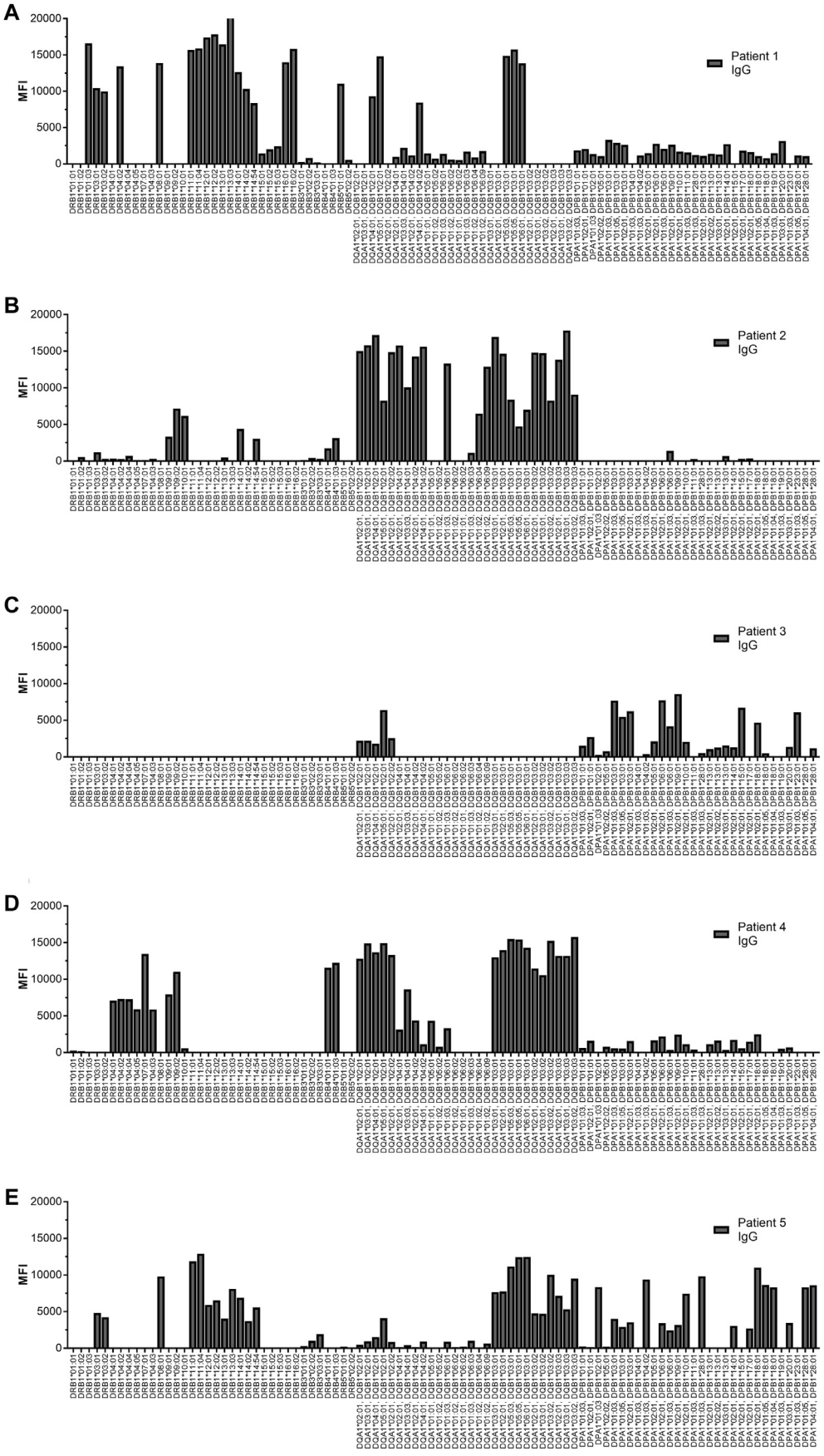


FIG E5. Anti−HLA class II IgG levels measured with One Lambda LABScreen Single Antigen HLA Class II kits in the patients used for HLA-specific IgE analysis. **A-E,** Each panel shows the MFI for the panel of SABs.

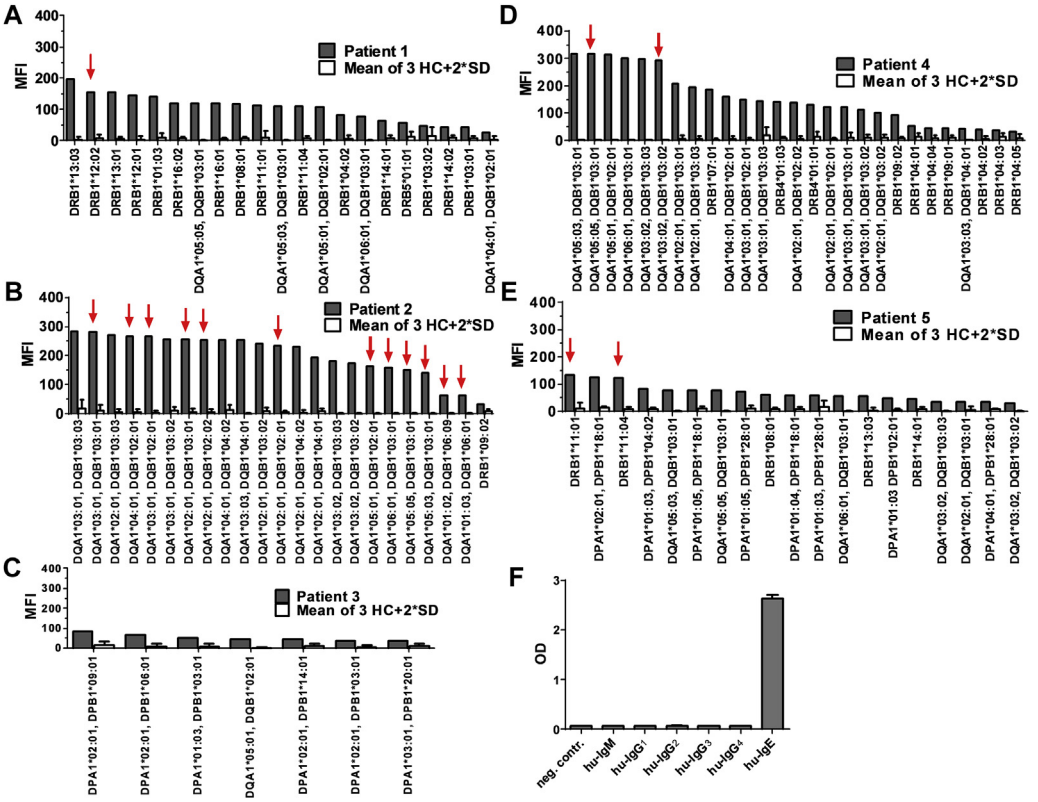


FIG E6. Anti−HLA class II IgE in kidney transplant recipients **(A-E)**. Antigen-specific IgE levels were measured in sera of kidney transplant recipients with high levels of IgG DSAs waiting for retransplantation with One Lambda LABScreen Single Antigen HLA Class II kits. IgE binding to several HLA class II antigens was detectable in 5 of 5 analyzed patients. **A-E,** MFIs representing a single patient were compared with the mean of 3 healthy control subjects *(HC)* plus 2*SDs. *Red arrows* indicate kidney donor−specific bead reactivity. Only positive beads are shown. **F,** Anti-IgE clone MHE-18 was used to detect different human *(hu)* antibody isotypes by using ELISA. Each *column* represents the mean OD of triplicates ± SDs.

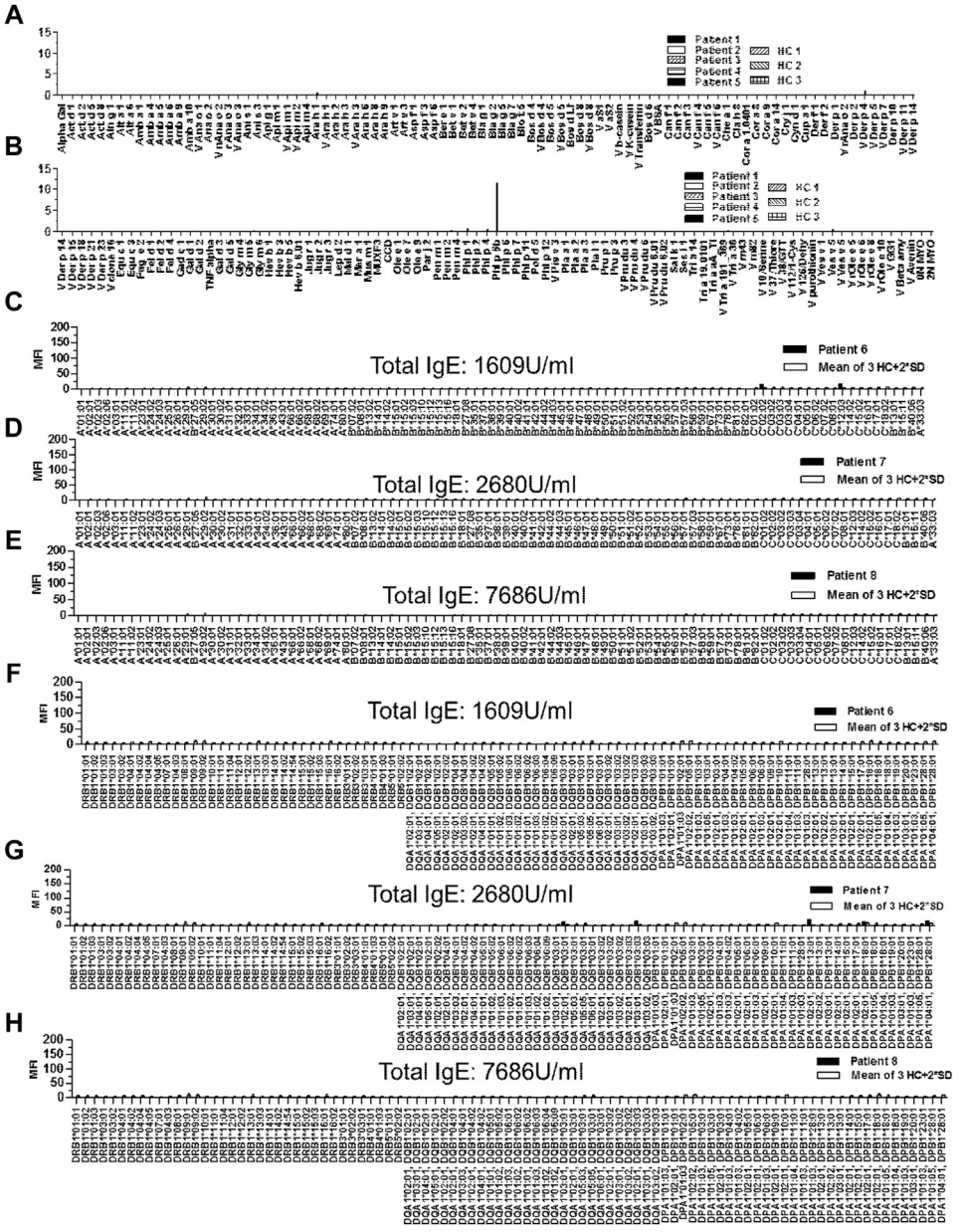


FIG E7. HLA-specific IgE is not linked to atopy. **A** and **B,** Allergen-specific IgE levels were measured in sera of kidney transplant recipients with detectable levels of HLA-specific IgE and 3 healthy control subjects. Allergen-specific IgE levels against 176 relevant allergens *(x-axis)* were measured by using the MeDALL array. IgE levels *(y-axis)* are displayed in ISAC standardized units, as described by Lupinek et al.[E11] **C-H,** HLA class I−specific (Fig E7, *C-E*) and HLA class II−specific (Fig E7, *F-H*) IgE levels were measured in sera of 3 allergic patients with high levels of total IgE (assessed by using ImmunoCAP) with One Lambda LABScreen Single Antigen HLA Class I or II kits. The MFI of each HLA bead was compared with the mean of 3 healthy control subjects *(HC)* plus 2*SDs.

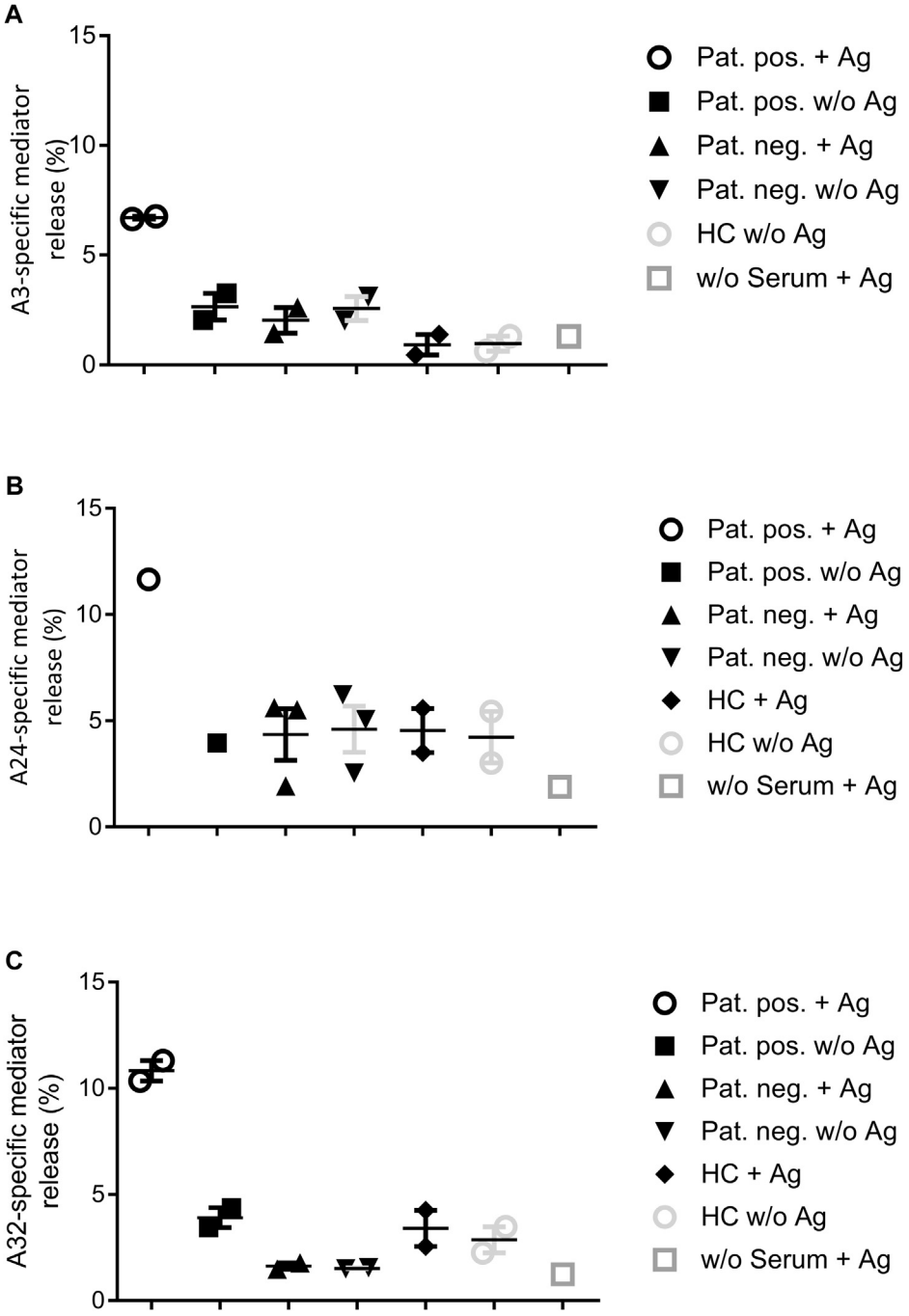


FIG E8. Functional activity of donor HLA-specific IgE in sera of kidney transplant patients. IgE-containing sera from kidney transplant patients were used to load a rat basophil cell line transfected to express human FcεRI. We tested patients who were either IgE positive *(Pat. pos.)* or IgE negative *(Pat. neg.)* for the respective HLA antigen. Cells were then stimulated with beads bearing a single HLA antigen (**A**: A3-positive patients 1 and 4 and A3-negative patients 3 and 5; **B**: A24-positive patient 1 and A24-negative patients 3-5; **C**: A32-positive patients 4 and 5 and A32-negative patients 2 and 3). Mediator release (ie, β-hexosaminidase) was measured and compared with healthy volunteer control sera *(HC)*, sera and cells without *(w/o)* antigen beads, or only beads and cells without sera.

FIG E9. Heat inactivation abrogates MHC-specific basophil degranulation. IgE-containing sera from kidney transplant patients were used to load a rat basophil cell line transfected to express human FcεRI. Cells were then stimulated with SABs bearing 1 donor-specific HLA-antigen (A32) and mediator release (ie, β-hexosaminidase) was measured. Where indicated, sera were heat inactivated *(HI)* to denature IgE.

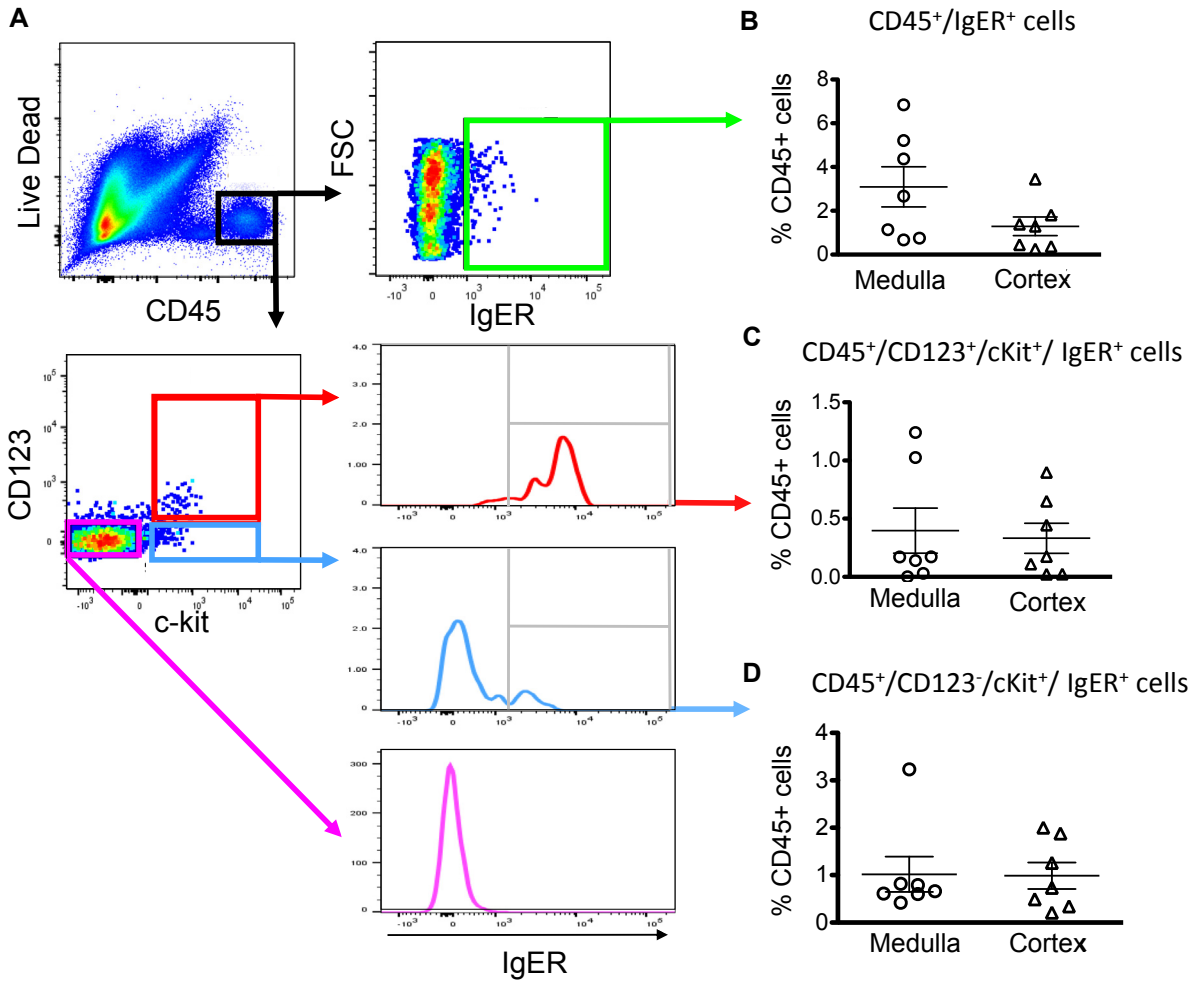


FIG E10. Human kidneys contain FcεRI$^+$ cells. **A,** Representative fluorescence-activated cell-sorting plots obtained from single-cell suspensions of human kidney cells demonstrating live CD45$^+$ cells that are IgER$^+$ (ie, FcεRI$^+$; *upper row*). Live CD45$^+$CD123$^+$cKit$^+$ cells were identified, the majority of which were IgER$^+$ (*middle* and *lower rows*). **B-D,** Quantification of the proportion of IgER$^+$ cells (Fig E10, *B*), CD123$^+$c-Kit$^+$IgER$^+$ basophils (Fig E10, *C*), and CD123$^-$c-Kit$^+$IgER$^+$ mast cells (Fig E10, *D*) within the CD45$^+$ gate in human cortex and medulla kidney samples. Each data point represents a kidney sample from 1 subject.