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Cutting Edge: FcR-Like 6 Is an MHC Class II Receptor

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Receptors for the Fc portion of Ig have been extensively characterized and are known to regulate humoral responses, but members of the closely related FcR-like (FCRL) family have not been found to bind Ig, and to date, no ligand has been identified for any FcRL. Using a cell-based GFP reporter system and a recombinant Fc chimeric protein, we show that human FCRL6, a receptor selectively expressed by cytotoxic T and NK cells, directly binds HLA-DR, an MHC class II molecule. Given the similarity among constant regions of Ig and MHC molecules, these findings suggest that representatives of the FcR and FCRL multigene families may have independently evolved to engage two ancestral elements fundamental to adaptive immunity. This discovery may offer new insight into the interaction between cytotoxic lymphocytes and APCs and may have important implications for better understanding HLA disease susceptibility and pathogenesis.

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The characterization in recent years of multiple receptor-gene families with activating or inhibitory potential has disclosed that the immune system is integrated with a remarkable number of regulatory mechanisms to balance effector responses. The classical FcRs for IgG and IgE are located on human chromosome 1q21–23 and play fundamental roles in both positive and negative immune regulation (1–3). The discovery of an extended family of FcR-like (FCRL) genes (FCR1–6) positioned in the same human chromosomal region has uncovered unexpected diversity at this locus (4–6). In addition to their genomic linkage and similar genetic organization, FCR1–6 share many features with the FcRs, including related extracellular Ig-like domains and cytoplasmic tyrosine-based signaling capability of their encoded type I transmembrane protein products (6). Of these, human FCRL6 is distinctively expressed by cytotoxic T and NK cells, is upregulated previously (8). Dendritic cells were generated from FACS-sorted monocytes in the presence of IL-4 and TNF-α; singly transduced the SUDHL6 cell line, respectively. The DRB3 (DRB3*01010201), DRB4 (DRB4*01030101), and DRB5 (DRB5*010101) allele cDNAs were purchased from Open Biosystems (Huntsville, AL). HLA-DRA (DRA*0101), HLA-DRB1 (DRB1*040101), and DRB3-5 cDNAs were subcloned into the pMX-PIE retroviral vector, which contains the GFP gene, and used to transduce BW5147 mouse T cells as described previously (10). Doubly transduced HLA-DRαβ1 and β2-3 cells were sorted with a PE-labeled anti-HLA-DR mAb (Sigm-Aldrich, St. Louis, MO); singly transduced HLA-DRα and HLA-DRβ1 lines were sorted for GFP.

Materials and Methods

Cells

43-1 FCRL6 cells were generated as previously described (9). Briefly, the FCRL6 extracellular region was PCR amplified from full-length cDNA with the KOD polymerase (Novagen/EMD Chemicals, Gibbstown, NJ) using the following Styl-flanked primers: forward 5'TAT AGG CCA TTA TTG GG CGG CCA CCG CGG CCA CGT AAC AAG ACT TG-3 and reverse 5'TAT AGG CCA CCG CGG CCA CCG CGG CCA CGT AAC AAG ACT TG-3', and inserted into the pEF.CD3 backbone to generate a lentiviral vector plasmid termed pEF.CD63. Vector packaging, transduction, and transductant sorting was performed as previously described (9).

Full-length HLA-DRA and HLA-DRB1 cDNAs were amplified from human PBL cells and the SUDHL6 cell line, respectively. The DRB3 (DRB3*01010201), DRB4 (DRB4*01030101), and DRB5 (DRB5*010101) allele cDNAs were purchased from Open Biosystems (Huntsville, AL). HLA-DRA (DRA*0101), HLA-DRB1 (DRB1*040101), and DRB3-5 cDNAs were subcloned into the pMX-PIE retroviral vector, which contains the GFP gene, and used to transduce BW5147 mouse T cells as described previously (10).

Dendritic cells were generated from FACS-sorted monocytes as described (11).

43-1 experiments

For Ab stimulation experiments, Immulon 96-well high-binding plates (Thermo Fisher Scientific, Waltham, MA) were coated with Abs for 1 h at being phosphorylated and recruiting the Src homology 2-domain containing phosphatase 2 (7, 8).

Despite the many similarities between the FcR and FCRL families, no FCRL has been shown to bind Ig, and thus ligands for these receptors remain unknown. In this study, we report that the MHC class II molecule HLA-DR is a ligand for human FCRL6. Using a cell-based reporter system, FCRL6 ligand reactivity was found to be restricted to APCs, and the development of a panel of blocking Abs facilitated the identification of HLA-DR as the interacting partner. This association was further confirmed using HLA-DR transductants for FCRL6-specific induction assays and selective binding of a soluble FCRL6-Fc chimeric molecule.
FCRL6 ligand mAbs were generated by immunizing BALB/c mice with the SUDHL6 and Mino cell lines according to established techniques (12). All procedures were approved by the UAB Institutional Animal Care and Use Committee. Hybridoma supernatants were screened for reactivity with SUDHL6 and their ability to block 43-1 FCRL6 cell GFP induction by SUDHL6. Hybridomas were subcloned by limiting dilution and isotype tested using the Southern Biotechnology Associates clonotyping system. The resulting mAbs are moIgG1 (L2-1E2), moIgG3 (L2-3F7, L2-3H2), and moIgM (L2-1A8, L2-2A6, L2-2B1, L2-3C5, L2-3C6, L2-4C2) isotypes. The anti-FCRL6-specific mAbs 7B7 and 1D8 (both moIgG1) were generated as previously described (8), and Fab digests were prepared using the ImmunoPure A or FCRL6 intact TCR complex (13); therefore, stimulation of parental FCRL6-specific mAb (7B7) only induced GFP expression in GFP induction by both cell lines, whereas stimulation with an isotype-matched control (gray shade). B. Untransduced 43-1 control (gray columns) or 43-1 FCRL6 cells (black columns) were stimulated at 5 μg/ml with the indicated plate-bound Abs for 18 h, and GFP induction was assayed by flow cytometry. Columns represent the mean ± SD; n = 3. C. FCRL6 cells were stimulated as in B with different concentrations of plate-bound anti-FCRL6 mAb. The percentage of GFP+ 43-1 cells is indicated in each histogram and demarcated by horizontal bars.

**Results and Discussion**

**Development of a GFP-inducible system to detect FCRL6 engagement.** To assay functional engagement between FCRL6 and potential ligand sources, a reporter cell line was engineered. A chimeric molecule termed FCRL6C, which possesses the three FCRL6 Ig-like extracellular domains in frame with an uncharged hydrophobic transmembrane region and the ITAM-containing cytoplasmic portion of mouse CD3ζ, was transduced into a murine T cell hybridoma, 43-1, which harbors an inducible GFP gene downstream of three tandem NFAT transcription factor binding sites (9, 13). The rationale behind this approach was that an encounter between FCRL6 and its ligand should initiate a T cell, CD8+ T cells, or granulocytes (Fig. 2A). Monocyte-derived dendritic cells were also found to evoke a robust response (Fig. 2C).

To further define FCRL6 ligand expression and facilitate its eventual cloning, representative human cell lines were analyzed. Co-culture with EBV-immortalized B lymphoblastoid (721.221), diffuse large B lymphoma (SUDHL6), and mantle cell lymphoma-derived (Mino) cell lines activated FCRL6 cells, whereas no detectable response was observed following coculture with Jurkat T cells or the U937 and K562 myeloid cell lines (Fig. 2D). Conditioned media from SUDHL6-cultured cells failed to stimulate GFP, suggesting that the interacting counterpart is a cell surface-associated molecule (data not shown). The FCRL6 specificity in this reporter system was supported by several lines of evidence. Induction by SUDHL6 in coculture assays cells was blocked by both intact and monomeric Fab fragments of an FCRL6-specific mAb (1D8), as well

**FIGURE 1.** Development of a GFP-inducible system to detect FCRL6 engagement. A, 43-1 cells transduced with the FCRL6C reporter construct were stained with the anti-FCRL6 7B7 mAb (black line) or with an isotype-matched control (gray shade). B, Untransduced 43-1 control (gray columns) or 43-1 FCRL6 cells (black columns) were stimulated at 5 μg/ml with the indicated plate-bound Abs for 18 h, and GFP induction was assayed by flow cytometry. Columns represent the mean ± SD; n = 3. C, FCRL6 cells were stimulated as in B with different concentrations of plate-bound anti-FCRL6 mAb. The percentage of GFP+ 43-1 cells is indicated in each histogram and demarcated by horizontal bars.

FCRL6C cells (Fig. 1B). The response to mAb stimulation was dose dependent, such that a larger percentage of 43-1 cells expressed GFP when exposed to higher concentrations of stimulating Ab (Fig. 1C).

**FCRL6 interacts with a surface molecule on APCs**

Having established a functional readout for FCRL6 engagement, we began investigating different lymphoid tissues to explore the cellular distribution of the FCRL6 ligand(s). In coculture experiments, mononuclear cells isolated from blood, tonsil, and spleen were found to induce GFP expression in FCRL6C cells but not in control 43-1 cells (Fig. 2A). To ascertain which cell type(s) was responsible for this reactivity, distinct leukocyte populations were sorted from blood samples and assayed for FCRL6-dependent GFP induction. The putative FCRL6 ligand was found only on cells with Ag-presenting capability, namely, B cells and monocytes, but not on resting CD4+ T cells, CD8+ T cells, or granulocytes (Fig. 2B). Monocyte-derived dendritic cells were also found to evoke a robust response (Fig. 2C).
as by the addition of a soluble chimeric FCRL6-Fc fusion protein but not a control Fc-only recombinant molecule consisting of the CH2–CH3 domains of human IgG1 (Fig. 2E). Furthermore, a mouse FCRL1z43-1 transductant that only differs from human FCRL6z cells in its extracellular region failed to respond following coculture with 721.221 or SUDHL6 B cell lines (Supplemental Fig. 1).

A panel of mAbs obstructs binding between FCRL6 and its ligand

As a tool to molecularly characterize the unknown FCRL6 ligand, we developed a panel of ligand-reactive mAbs. Hybridoma supernatants from mice immunized with the SUDHL6 and Mino B cell lines were tested for mAbs that would both react with SUDHL6 cells by flow cytometry and suppress SUDHL6-induced GFP expression by FCRL6z cells. This strategy led to the isolation of nine mAb-secreting clones that fulfilled both criteria (Supplemental Fig. 2A, 2B). However, prior to utilizing these mAbs in approaches to identify the FCRL6 ligand biochemically, we were struck by several compelling evolutionary observations that provided useful clues for predicting its possible nature.

**HLA-DR is an FCRL6 ligand**

Striking phylogenetic relationships are evident between Ig and MHC C regions (14–19). Thus, the finding that an FcR/FCRL family member recognized a surface determinant common to APCs prompted us to consider that the ligand for FCRL6 could be MHC class II. To test this hypothesis, we first performed competition assays with 43-1 FCRL6z cells utilizing an anti–HLA-DRβ mAb (clone DA2). Addition of this reagent in coculture experiments with SUDHL6 cells

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** FCRL6 interacts with a molecule expressed by APCs. FCRL6z (black columns) or untransduced 43-1 control cells (gray columns) were cocultured with mononuclear cells isolated from the indicated tissues (A); FACS-sorted blood CD4+ T cells (CD3+CD4+), CD8+ T cells (CD3+CD8+), B cells (CD19+), monocytes, or granulocytes (both defined by light scatter) (B); monocyte-derived dendritic cells (C); or the indicated human cell lines (D). After 18 h, 43-1 cell GFP expression was analyzed by flow cytometry. 43-1 control cells cultured alone (None) were employed to determine background GFP levels. E, FCRL6z cells were cocultured with the SUDHL6 B cell line either alone (No treatment) or in the presence of the indicated blocking reagents and assayed for GFP expression. Columns represent the mean ± SD; n ≥ 3.

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** FCRL6 directly binds to HLA-DR. A, FCRL6z cells were cocultured for 18 h with SUDHL6 B cells either alone (No treatment) or in the presence of an HLA-DRβ-specific mAb (clone DA2) and assayed for GFP expression. B, FCRL6z (black columns) or untransduced 43-1 control cells (gray columns) were cultured for 18 h either alone (None) or in the presence of parental BW5147 cells, BW cells singly transduced with HLA-DRα or HLA-DRβ1 (both surface HLA-DRz), or doubly transduced with HLA-DRα and B7 (surface HLA-DRz) and analyzed for GFP expression. Columns represent the mean ± SD; n = 3. C, rFCRL6-Fc (black line) or a control Fc-only protein (gray shaded) was complexed with Alexa Fluor 647-labeled protein A and used to stain the indicated BW5147 cells transduced with the following HLA-DR alleles (DRA*0101, DRB1*040101, DRB3*01010201, DRB4*01030101, and DRB5*010101). Histograms are representative of three independent experiments.
Indeed abrogated FCRL6 γ GFP induction (Fig. 3A). A series of BW5147 murine thymoma cell transductants expressing HLA-DRα and/or HLA-DRβ1 was then generated and employed in coculture reporter assays. Surprisingly, HLA-DRα+ β1 BW cells (surface HLA-DR4), but not parental or singly transduced control cells (surface HLA-DR4), triggered FCRL6 γ GFP expression (Fig. 3B). Importantly, the panel of FCRL6 ligand-reactive mAbs all selectively stained the HLA-DRα+ β1 transductants, and all but the L2-1E2 clone were capable of blocking FCRL6 γ activation when added to coculture experiments with the HLA-DRα+ β1 BW cells (Supplemental Fig. 3A, 3B).

MHC class II is both extremely polymorphic and polygenic. Therefore, we explored the potential of FCRL6 to bind HLA-DR heterodimers composed of different DRβ-chains. HLA-DRβ3, β4, and β5 were each stably transduced into BW HLA-DRα cells and used for coculture experiments with 43-1 cells. Interestingly, although HLA-DRα+ β1, HLA-DRα+ β4, and HLA-DRα+ β5 cells invoked moderate to high levels of activation in the 43-1 system, HLA-DRα+ β3 cells induced much lower levels of GFP expression (Supplemental Fig. 4). To independently verify the FCRL6–HLA-DR interaction, we then tested the ability of a recombinant soluble FCRL6-Fc fusion protein to stain the panel of HLA-DR transductants. Multimeric FCRL6-Fc selectively bound to HLA-DRα+ β1, HLA-DRα+ β4, and HLA-DRα+ β5 BW cells and to a lesser extent HLA-DRα+ β3 transductants, but not to control cells (Fig. 3C). These results demonstrate that FCRL6 is capable of binding MHC class II molecules and suggest that it may have differential affinity for HLA-DR that varies according to its HLA-DR β-chain composition.

Concluding remarks

In summary, these findings clearly indicate that MHC class II is an FCRL6 ligand. Phylogenetic analyses suggest that the nonpolymorphic C region domains of IgH isotypes and the membrane-proximal MHC class I α3 and MHC class II α2 and β2 domains, as well as β3-microglobulin, arose from a common ancestral C1-set Ig-like domain (14–19). Given the strong likelihood that the FcR and FCRL families arose from a common ancestral C1-set Ig-like domain (14–19). Additionally, NK cells and CD8+ T cells can kill autologous dendritic cells, a mechanism proposed to regulate adaptive immune responses (24–27). What influence, if any, the FCRL6–MHC class II interaction may have in this cross talk is subject to further investigation. FCRL6 contains a cytoplasmic domain with a consensus ITIM motif that is capable of phosphorylation and Src homology 2-domain containing phosphatase 2 recruitment (7, 8). It is therefore tempting to speculate that FCRL6 may function as an inhibitory receptor for MHC class II. However, studies using mAbs to ligate FCRL6 have failed to demonstrate convincing inhibitory function (7, 8), and definitive functional studies for this receptor await further exploration.

The discovery of this interaction raises many questions. For example, do other FCRL family members bind MHC molecules, and is FCRL6 capable of recognizing other MHC class II isotypes? The data shown in this study demonstrate that FCRL6 is capable of binding HLA-DR molecules composed of distinct DRβ subunits; future studies addressing whether FCRL6 differentially binds MHC class II allelic variants may provide insight into the molecular mechanisms underlying HLA-based disease associations.

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Disclosures

R.S.D. is a coinventor on U.S. Patent 7,317,087, which covers the FCRL6 molecule. In addition, R.S.D., D.M.S., and J.P.C. are coinventors on pending patent applications.

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