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Cutting Edge

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 (FCRL1–6) 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, FCRL1–6 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 distinctly expressed by cytotoxic T and NK cells, is upregulated on expanded populations of terminally differentiated CD8+ T cells in patients with HIV and B cell chronic lymphocytic leukemia, and possesses a cytoplasmic ITIM that is capable of 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.

Materials and Methods

Cells

43-1 FCRL6Δx 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 SfiI-flanked primers: forward 5'-TAT AGG CCA TTT AGG CGG CCC TTC and reverse 5'-TAT AGG CCA CCG CGG CGG GTG AAC AAG ACT TG-3', and inserted into the pEF.CD3Δx backbone to generate a lentiviral vector plasmid termed pEF.FCRL6Δx. Vector packaging, transduction, and transductant sorting was performed as previously described (9).

Full-length HLA-DRα and HLA-DRβ1 cDNAs were amplified from human PBL cells and the SUDHL6 cell line, respectively. The DRB3 (DRB3*010101), DRB4 (DRB4*01030101), and DRB5 (DRB5*010101) allele cDNAs were purchased from Open Biosystems (Huntsville, AL). HLA-DRx (DRA*0101), HLA-DRβ1 (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-DRα-β1-β3-5 cells were sorted with a PE-labeled anti–HLA-DR mAb (Sera-Mark, St. Louis, MO); singly transduced HLA-DRx and HLA-DRβ1 lines were sorted for GFP.

Human tonsil and spleen samples were obtained from the University of Alabama at Birmingham (UAB) Tissue Procurement program (Birmingham, AL). Blood specimens were obtained from healthy adult volunteers with Institutional Review Board approval following informed consent according to the Declaration of Helsinki. Mononuclear cells were isolated as detailed previously (8). Dendritic cells were generated from FACS-sorted monocytes as described (11).

43-1 experiments

For Ab stimulation experiments, Immunulon 96-well high-binding plates (Thermo Fisher Scientific, Waltham, MA) were coated with Abs for 1 h at
room temperature and plated with 3 × 10^4 43-1 cells for 18 h. For coculture experiments, primary cells were added at a 5:1 ratio and dendritic cell and cell lines at a 2:1 ratio with 43-1 reporter cell lines. In blocking experiments, 30 μl of hybridoma supernatant or purified Ab diluted in culture media was added to stimulator cells prior to plating the 43-1 cells. After coculture, cells were stained with anti-mouse CD3 PE (Southern Biotechnology Associates, Birmingham, AL) to delineate the 43-1 fraction and then analyzed by flow cytometry. To distinguish between the 43-1 and BWS147 cell lines, BWS147 cells were fluorescently labeled with the PKH26 cell membrane labeling kit (Sigma-Aldrich) prior to coculture.

Abs

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 FCRL6ε cell GFP induction by SUDHL6. Hybridomas were subcloned by limiting dilution and isotype identified using the Southern Biotechnology Associates cloneotyping system. The resulting mAbs are molgG1k (L2-1E2), molgG3k (L2-3F7, L2-3H2), and molgMx (L2-1A8, L2-2A6, L2-2B1, L2-3C5, L2-3C6, L2-4C2) isotypes. The anti-FCRL6-specific mAbs 7B7 and 1D8 (both molgG1k) were generated as previously described (8), and Fab digests were prepared using the ImmunoPure Fab kit (Pierce, Rockford, IL). The following additional Abs were used: control hamster IgG (anti-KLH) (BD Biosciences, San Jose, CA), HLA-DR8 clone DA2 (Santa Cruz Biotechnology, Santa Cruz, CA), control mouse IgG Fab fragments (Rockland Immunocchemicals, Gilbertsville, PA), and the hamster anti-mouse CD3 clone 145-2C11 (a gift from Dr. Chandar Raman, UAB).

Fc chimeric proteins

Fc soluble recombinant proteins were generated by modifying the pRB1-Ecmut plasmid (a gift of Dr. Roberto Biasionti, Gaslini Institute, Genoa, Italy), which contains a cDNA encoding the CH2-3 Fc portion of the human IgG1 Hc (IGHG1) with 3 aa residues mutated (L234A, L235A, and G237A) to abrogate Fc binding. The FCRL6-Fc and control-Fc proteins were generated by inserting the endogenous FCRL6 leader and ectodomain sequence or a murine Igκ leader sequence, respectively, upstream of the CH2 portion of IGHG1.

Final DNA constructs were subcloned into the pShuttle-CMV adenovirus transfer vector (Quantum Biotechnologies, Laval, Quebec, Canada) for transfection and expression in HEK293 cells. Soluble protein containing supernatants were purified over protein A-sepharose columns (GE Healthcare, Piscataway, NJ) before SDS-PAGE and analysis by Coomassie staining (Bio-Rad, Hercules, CA) and Western blot.

Fcε soluble recombinant proteins were incubated with Alexa-Fluor 647-labeled protein A (Invitrogen, Carlsbad, CA) at a 2.5:1 molar ratio for 30 min at room temperature, then diluted and used to stain BWS147 transductants at a 200 nM concentration for 1 h at 37°C.

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 FCRL6ε, 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 CD3ε, was transduced into a murine T cell hybridoma, 43-1, which harbors an inducible 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 CD3ε-driven activation cascade, resulting in NFAT nuclear translocation and GFP expression.

Following transduction of the chimeric construct into 43-1 cells, FCRL6ε surface expression was verified by staining with an FCRL6ε-specific Ab (Fig. 1A). The 43-1 cells have an intact TCR complex (13); therefore, stimulation of parental or FCRL6ε cells with mAbs to mouse CD3 resulted in potent GFP induction by both cell lines, whereas stimulation with an FCRL6ε-specific mAb (7B7) only induced GFP expression in

FIGURE 1. Development of a GFP-inducible system to detect FCRL6 engagement. A, 43-1 cells transduced with the FCRL6ε reporter construct were stained with the anti-FCRL6ε 7B7 mAb (black line) or with an isotype-matched control (gray shade). B, Untransduced 43-1 control (gray columns) or 43-1 FCRL6ε 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, FCRL6ε cells were stimulated as in B with different concentrations of plate-bound anti-FCRL6ε mAbs. The percentage of GFP+ 43-1 cells is indicated in each histogram and demarcated by horizontal bars.

FCRL6ε 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 FCRL6ε 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).

To further define FCRL6 ligand expression and facilitate its eventual cloning, representative human cell lines were analyzed. Coculture with EBV-immortalized B lymphoblastoid (721.221), diffuse large B lymphoma (SUDHL6), and mantle B cell lymphoma-derived (Mino) cell lines activated FCRL6ε 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 was blocked by both intact and monomeric Fab fragments of an FCRL6ε-specific mAb (1D8), as well

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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 FCRL1\(z\) transductant that only differs from human FCRL6\(z\) 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 FCRL6\(z\) 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 FCRL6\(z\) cells utilizing an anti–HLA-DR\(B\) mAb (clone DA2). Addition of this reagent in coculture experiments with SUDHL6 cells

![FIGURE 2. FCRL6 interacts with a molecule expressed by APCs.](image)

**FIGURE 2.** FCRL6 interacts with a molecule expressed by APCs. FCRL6\(z\) (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, FCRL6\(z\) 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. FCRL6 directly binds to HLA-DR.](image)

**FIGURE 3.** FCRL6 directly binds to HLA-DR. A, FCRL6\(z\) cells were cocultured for 18 h with SUDHL6 B cells either alone (No treatment) or in the presence of an HLA-DR\(B\)-specific mAb (clone DA2) and assayed for GFP expression. B, FCRL6\(z\) (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-DR\(A\) or HLA-DR\(B\) (both surface HLA-DR\(^+\)), or doubly transduced with HLA-DR\(A\) and B1 (surface HLA-DR\(^+\)) 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\(^*\)0401, DRB3\(^*\)01010201, DRB4\(^*\)01030101, and DRB5\(^*\)010101). Histograms are representative of three independent experiments.
indeed abrogated FCRL6ζ 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-DR+), but not parental or singly transduced control cells (surface HLA-DR−), triggered FCRL6ζ 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 FCRL6ζ 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 β2-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 ancestor, it is quite remarkable that individual members have evolved to bind structurally and phylogenetically related ligands. Even more noteworthy are the parallels with members of the leukocyte receptor complex, an expanded monophyletic family of Ig-like receptor genes in a region of human chromosome 19q13 (20, 21). This dense locus encompasses the FcR for IgA (FCAR) as well as the multigene killer cell Ig-like receptor (KIR) and leukocyte Ig-like receptor (LILR) families that interact with classical and nonclassical MHC class I molecules (22). Despite their separate genomic locations, both the FcR/FCRL and leukocyte receptor complex Ig-like multigene families encode activating and/or inhibitory receptors that bind either Ig or MHC and have expanded or contracted in a species-specific fashion (21, 23).

In addition to highlighting an interesting evolutionary relationship, the finding that human FCRL6 is a receptor for HLA-DR may have important implications for understanding the interactions between cytotoxic lymphocytes and APCs. Dendritic cells can activate resting NK cells, and conversely, NK cells can induce dendritic cell activation and maturation (24, 25). 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.

References


Supplemental Figure 1. 43-1 GFP induction by human B cell lines is FCRL6-specific. The indicated 43-1 transductants were cultured for 18 h alone (white columns) or with the SUDHL6 (grey columns) and 721.221 (black columns) B cell lines. GFP expression was measured by flow cytometry.

Supplemental Figure 2. Generation of a panel of FCRL6 ligand-reactive monoclonal antibodies. A SUDHL6 cells stained with the indicated monoclonal hybridoma supernatants (black line) or isotype-matched control mAbs (grey shade), were counterstained with a PE-labeled goat anti-mouse Ig secondary reagent and analyzed by flow cytometry. B FCRL6ζ cells were co-cultured with SUDHL6 cells either alone (“no treatment”) or in the presence of the indicated hybridoma supernatants and assayed for GFP expression. Control antibodies were used at a final concentration of 25μg/mL and were diluted in the same culture media used for growing hybridomas. Columns represent the mean ± s.d.; n=3.
Supplemental Figure 3. FCRL6 ligand-reactive mAbs recognize HLA-DR. A Control BW5147 cells singly transduced with the HLA-DRα chain (top row) or co-transduced with the HLA-DRα and HLA-DRβ1 chains (bottom row) were stained with FCRL6 ligand-reactive monoclonal hybridoma supernatants (black line) or isotype-matched control mAbs (grey shade), counterstained with goat anti-mouse Ig PE, and analyzed by flow cytometry. B FCRL6ζ cells were co-cultured with BW5147 HLA-DRα+β1 transductants in the presence of the indicated hybridoma supernatants and assayed for GFP expression as in Figure 2B.

Supplemental Figure 4. FCRL6 binds HLA-DR molecules composed of distinct DRβ subunits. FCRL6ζ (black columns) or untransduced 43-1 control cells (grey columns) were cultured for 18 h either alone (“none”) or in the presence of the indicated BW5147 transductants and analyzed for GFP expression.