Cutting Edge: FcR-Like 5 on Innate B Cells Is Targeted by a Poxvirus MHC Class I-Like Immunoevasin

Jessica A. Campbell, Randall S. Davis, Lauren M. Lilly, Daved H. Fremont, Anthony R. French and Leonidas N. Carayannopoulos

*J Immunol* 2010; 185:28-32; Prepublished online 2 June 2010; doi: 10.4049/jimmunol.1000240
http://www.jimmunol.org/content/185/1/28

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2010/06/01/jimmunol.1000240.DC1

**References**
This article cites 25 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/185/1/28.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: FcR-Like 5 on Innate B Cells Is Targeted by a Poxvirus MHC Class I-Like Immunoevasin

Jessica A. Campbell,* Randall S. Davis,†‡§ Lauren M. Lilly,‡ David H. Fremont,¶ Anthony R. French,*† and Leonidas N. Carayannopoulos†‖

Under selective pressure from host immunity, viruses have retained genes encoding immunoevasins, molecules interfering with host viral recognition and clearance. Due to their binding specificities, immunoevasins can be exploited as affinity labels to identify host-encoded molecules of previously unsuspected importance in defense against the relevant class of virus. We previously described an orthopoxvirus MHC class I-like protein (OMCP) that binds with high affinity to the activating receptor NKG2D on NK and T cell subsets, implicating NKG2D in antiorthopoxvirus immunity. In this study, we report that OMCP also binds in an NKG2D-independent manner to B cells and monocytes/macrophages. We identify murine FcR-like 5 (FCRL5), an orphan immunoregulatory protein highly expressed by innate B lymphocytes, as a specific receptor for OMCP. The three N-terminal Ig domains of FCRL5 are required for OMCP binding. The targeting of FCRL5 by an orthopoxvirus immunoevasin strongly implicates it in Contributing to host defense against zoonotic orthopoxviruses. The Journal of Immunology, 2010, 185: 28–32.

Effective immune responses depend on functional communication within and between host cells. At the molecular level, communication is controlled by receptor–ligand interactions. Every stage of an immune response to foreign Ag ultimately relies on such interactions, be they host–host or host–microbe in nature. Following receptor ligation, downstream signaling events regulate cellular activation and effector functions. The latter impose great selective pressure on invading microbes. Therefore, microbial countermeasures aimed at preventing or avoiding host immunity often disrupt receptor–ligand interactions. To accomplish this, microbial immunomodulatory proteins (immuno-evasins) must specifically bind one or more host-encoded molecules. In this regard, immunoevasins can be viewed as probes with which the complex network of host-encoded proteins can be searched to identify those that are important to defense against the respective class of viruses.

We previously described an orthopoxvirus MHC class I-like protein (OMCP) encoded by monkeypoxvirus and cowpoxvirus (CPXV) (1). OMCP is secreted from infected cells and binds with high affinity to human and mouse NKG2D, an activating receptor expressed by NK and T cell subsets. As a soluble NKG2D antagonist, OMCP potently inhibits NKG2D-dependent killing of target cells. These findings strongly implicated NKG2D in host defense against zoonotic orthopoxviruses, and this conclusion was recently verified by Sigal and colleagues (2) using experimental mousepox.

Interestingly, OMCP also binds to B cells and monocytes/macrophages. The lack of NKG2D on these cell types (3–5) suggested the existence of a separate, non-NKG2D receptor targeted by OMCP. In this study, we report that OMCP binds to the protein FcR-like 5 (FCRL5), an immunoregulatory receptor of unknown specificity that is highly expressed by murine marginal zone (MZ) and B1 B cells. In addition to describing the first known ligand for FCRL5, these data implicate FCRL5 and innate B cells in defense against poxviruses.

Materials and Methods

Retroviral library

WEHI231 cell-derived mRNA was reverse-transcribed with Superscript II (Invitrogen, Carlsbad, CA) and amplified and digested with the SMART cDNA Library Construction Kit (BD Clontech, Palo Alto, CA). SfiI-digested cDNA was ligated into pMXs-ires-EGFP (courtesy of Dr. T. Kitamura, University of Tokyo, Tokyo, Japan) in which SfiIA and SfiIB sites had been incorporated, then transformed into XL10 Gold Ultracompetent cells (Stratagene, La Jolla, CA) for a final library complexity of $1.4 \times 10^6$. Retrovirus were produced and used to infect C1498 cells as described previously (6). C1498 transductants were stained with OMCP tetramers and sorted for the most brightly staining enhanced GFP (EGFP)* cells. After four successive sorts, retroviral inserts from individual clones were amplified with primers

---

*Division of Pediatric Rheumatology, Department of Pediatrics, †Department of Pathology and Immunology, and ‡Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110; †Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110; and ‡Department of Medicine, †Department of Microbiology, and ‡Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294

A.R.F. and L.N.C. contributed equally to this work.

1A.R.F. and L.N.C. contributed equally to this work.

2Current address: Merck Research Laboratories, Rahway, NJ.

Received for publication January 28, 2010. Accepted for publication May 4, 2010.

This work was supported by National Institute of Allergy and Infectious Diseases Grants R01 AI075552 and R01 AI067467 and National Heart, Lung, and Blood Institute Grant T32 HL073171.

Address correspondence and reprint requests to Dr. Anthony R. French, Department of Pediatrics, Washington University, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail address: french_a@kids.wustl.edu

The online version of this article contains supplemental material.

Abbreviations used in this paper: CPXV, cowpoxvirus; DIII, domain III; EGFP, enhanced GFP; FCRL5, FcR-like 5; FO, follicular; IRES, internal ribosome entry site; MZ, marginal zone; OMCP, orthopoxvirus MHC class I-like protein.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1000240
subjected to basic local alignment search tool analysis.

Plasmid constructs, transductants, and transformants

Full-length FCRL5 was ligated into pMX-ires-EGFP. FCRL5-expressing Ba/F3 transductants were generated as described previously (6). To express truncated versions of FCRL5, specified BALB/c FCRL5 Ig domains were PCR-spliced to the mature N terminus of Thy1.1 as previously described (1). FCRL5 D1-5 was amplified from MSGSF…to…ISIFD; D1 from MSGSF…to…EPESD; D2 from FLVLQ…to…VQQQE; D3 from LFPFR…to…IPVQR; D1-3 from MSGSF…to…VQQQE and D2-3 from FLVLQ…to…IPVQR. The FCRL5 leader peptide from MSGSF…to…VNGQH was spliced to the N terminus of D2 and D3. Amplicons were spliced to Thy1.1, cloned into pcDNA3 (Invitrogen), and transfected into 293T cells. Soluble FCRL-Fc fusion constructs were produced by amplifying FCRL1 from MLPW1…to…SLGLT and FCRL5 from MSGSF…to…ISIFD, then cloned into pFUSE-hlgG1-Fc1 (Inviogen, San Diego, CA). A 6-His tag was cloned onto the 3’ end of the Fc to generate pFUSE-FCRL-Fc-His plasmids.

Recombinant protein production

rOMCP, MULT1, RAE1, and West Nile virus glycoprotein domain III (DIII) were produced and biotinylated when indicated as previously reported (1, 7, 8). Soluble FCRL1-Fc and FCRL5-Fc fusion proteins were produced by 293T cells transfected with pFUSE-FCRL-Fc-His plasmids and purified from spent supernatant with Ni-NTA beads (Qiagen, Valencia, CA). Abs and flow cytometry

Mouse IgG2a-PE, streptavidin-PE, streptavidin-APC, anti-mouse CD11b-PE, B220-PE, NK2G2D (CX5), CD5-FITC, Thy1.1-PE, anti-human CD14-FITC, and CD19-PE were purchased from eBioscience (San Diego, CA). Rat IgG1k, rat IgG2a, anti-human NK2G2D (1D11), anti-mouse CD21-FITC, CD23-PE, and NK1.1-PE were from BD Biosciences (San Jose, CA). Anti-mouse CD5-PE and CD19-FITC were from Biolegend (San Diego, CA). Anti-FCRL5 clone 9D10 has been described (9). For blocking experiments, cells were incubated with Abs or recombinant soluble proteins for 30 min prior to OMCP tetramer addition. Data were collected on an FACS Calibur (BD Biosciences) and analyzed with Flowjo (Tree Star, Ashland, OR).

ELISA

OMCP or RAE1b was plated at 0.2 μg/ml in ELISA Coating Buffer and blocked with Assay Diluent (eBioscience), followed by incubation with the indicated FCRL-Fc concentration, then with 2 μg/ml anti-human IgG Fc-HRP (Southern Biotechnology Associates, Birmingham, AL). Tetramethylbenzidine (eBioscience) was added to visualize bound proteins, and the reaction was stopped with sulfuric acid.

Results

OMCP binds to B cells and monocytes/macrophages

To determine whether OMCP displays NKG2D-independent binding activity, primary mouse and human cell preparations were cotained with rOMCP tetramers and leukocyte subset markers. OMCP tetramer staining was observed on C57BL/6 splenic CD11b+ macrophages and B220+ B cells (Fig. 1A, top panel). To examine murine splenic B cell subsets more closely, follicular (FO) and MZ B cells were identified based on CD21 and CD23 expression. This revealed a much higher level of OMCP tetramer binding to CD21hiCD23hi MZ B cells than to CD21intCD23hi FO B cells (Fig. 1A, middle panel). Analysis of murine peritoneal B cells demonstrated that CD5+ B1a and CD5− B1b B cells also express relatively high levels of a receptor for OMCP (Fig. 1B). Although B cells do not express NKG2D, macrophages have been reported to express this receptor in certain circumstances (10, 11). To exclude NK2G2D as the receptor responsible for OMCP binding on B cells and macrophages, cells were incubated with either soluble MULT1, a high-affinity host-encoded NK2G2D ligand (7), or anti-NKG2D mAb prior to OMCP tetramer staining. Binding was not due to NKG2D expression because neither anti-NKG2D mAb nor soluble MULT1 (lines 4 and 6, respectively, in Fig. 1A, top and middle panels, and 1B) inhibited OMCP tetramer binding in contrast to effective blocking on NK, NKT, and CD8+ T cells (Fig. 1A, bottom panels) (1). Similar results were obtained with splenic and peritoneal cells from BALB/c, 129, and SJL mouse strains (data not shown). Examination of human PBMCs showed that OMCP tetramers bind to CD14+ monocytes and CD19+ B cells (Fig. 1C). As was observed with murine B cells and macrophages, preincubation with anti-NKG2D mAb did not prevent OMCP tetramers from binding to human B cells and monocytes (Fig. 1C, line 4). Together, these data demonstrate that B cells and monocytes/macrophages express one or more distinct non-NKG2D receptors for OMCP.

FCRL5 is a receptor for OMCP on innate B cells

To identify the receptor on B cells recognized by OMCP, we transduced the OMCP tetramer-negative cell line C1498 with a retroviral cDNA library derived from WEHI231 cells, a mouse B cell line with high cell-surface OMCP-binding activity. After four successive rounds of enrichment for OMCP
tetramer-positive cells by flow cytometric sorting (Fig. 2A), retroviral inserts were obtained from individual clones by PCR. A predominant amplicon (15 of 17 amplifiable clones) of ∼1.8 kbp was identified by basic local alignment search tool analysis as FCRL5, a protein expressed by WEHI231 cells, MZ, B1a, and B1b B cells (Supplemental Fig. 1) (9, 12).

To confirm the interaction between OMCP and FCRL5, Ba/F3 cells were transduced with FCRL5 amplified from murine spleens. FCRL5 is polymorphic, with C57BL/6 and BALB/c strains representing the two distinct alleles (9). Expression of either FCRL5 allele endowed Ba/F3 transductants, but not untransduced cells, with the ability to bind OMCP tetramers, but not control tetramers (Fig. 2B). The specificity of the OMCP–FCRL5 interaction was verified by preincubating the cells with anti-FCRL5 mAb prior to OMCP tetramer staining. Although preincubation with isotype control mAb did not affect OMCP tetramer binding to either Ba/F3-FCRL5 transductants or WEHI231 cells, OMCP tetramers were unable to bind either cell type after anti-FCRL5 mAb blockade, establishing the specificity of the interaction between OMCP and FCRL5 (Fig. 2C). The OMCP–FCRL5 interaction was further validated with an ELISA, which showed that rFCRL5-Fc, but not FCRL1-Fc, bound to plate-immobilized OMCP (Fig. 2D). FCRL5-Fc did not bind to plate-immobilized RAE1α, a host-encoded NKG2D ligand. Together, these results demonstrate that FCRL5 is specifically targeted by OMCP.

Having established a specific interaction between OMCP and FCRL5, we proceeded to test whether the binding of OMCP tetramers to primary B cells and macrophages could be blocked by anti-FCRL5 mAb (Fig. 2E, 2F). OMCP tetramers were completely inhibited from recognizing MZ, B1a, and B1b B cells after preincubation with anti-FCRL5 mAb. As expected, anti-FCRL5 mAb did not inhibit OMCP tetramer binding to NK cells, which express the OMCP receptor NKG2D. Consistent with the lack of FCRL5 expression by FO B cells and macrophages (Supplemental Fig. 1) (9, 12), pretreatment with anti-FCRL5 mAb also did not inhibit OMCP tetramer binding to these subsets. Thus, OMCP binds specifically to FCRL5 expressed by murine innate B cells.

**OMCP binds to the three N-terminal Ig domains of FCRL5**

FCRL5 is composed of five Ig domains (9, 12), termed D1–D5, with D1 being the most N-terminal domain. To identify the domains that bind OMCP, truncated versions of FCRL5 were cloned and expressed in 293T cells. To ensure surface expression of the shortened constructs, different combinations of FCRL5 Ig domains were fused to the mature N terminus of the GPI-linked protein Thy1.1 to generate a series of FCRL5 ectodomain-Thy1.1 transfectants. Neither FCRL5 D1, D2, or D3 nor Thy1.1 alone bound OMCP tetramers (Fig. 3). 293T cells expressing FCRL5 D1–3 retained OMCP tetramer-binding ability, indicating that D4 and D5 are dispensable.
for this activity. Cells expressing the D1-2 and D2-3 constructs were incapable of recognizing OMCP. Together, these experiments demonstrate that the three N-terminal Ig domains of FCRL5 are necessary and sufficient for binding to OMCP.

Discussion

This study reveals a novel interaction between an FCRL family member and a virally encoded, MHC-like immunomodulator. The EBV nuclear Ag 2 transcription factor has been shown to induce expression of human FCRL5 (13), but OMCP is the first known virally encoded protein to recognize an FCRL member directly. Furthermore, it is the first identified ligand of either microbial or host origin for FCRL5. The function of OMCP with regard to FCRL5, however, remains to be identified.

One possibility involves OMCP acting as a cross-linker between cells expressing different OMCP receptors. For example, OMCP could link an NKG2D+ NK cell to an FCRL5+ B cell to induce cytolysis against the B cell, formally possible because FCRL5 and NKG2D recognize independent binding sites (not shown). However, given the rapid dissociation kinetics of OMCP binding sites (not shown), yet OMCP targets multiple receptors, the in vivo effect of OMCP on FCRL5+ cells will best be directly evaluated by infection with vaccinia virus (23), implying that MZ and B1 B cells may contribute to control of orthopoxvirus infection. If OMCP indeed prevents FCRL5 from interacting with its activating natural ligand(s), this may inhibit or delay MZ and B1 B cells from mounting antiviral responses. Infection of mice with an rOMCP-null CPXV may be used to test the cumulative impact of OMCP on its target cells. However, because OMCP targets multiple receptors, the in vivo effect of OMCP on FCRL5+ cells will best be directly evaluated by infection with rCPXV expressing mutant OMCP unable to bind FCRL5, but still capable of interacting with its other targets. Generation and use of these recombinant viruses is in progress.

Murine macrophages and FO B cells bind OMCP tetramers in an FCRL5- and NKG2D-independent manner (Figs. 1, 2). A likely explanation is that these cell types express yet another receptor(s) targeted by OMCP. We hypothesize that the human ortholog of this putative third receptor may mediate binding of OMCP to human B cells and monocytes. Candidate human receptors include the six transmembrane FCRL family members (FCRL1–6) expressed on human leukocytes (25). Indeed, three of these members (FCRL3–5) contain domains with homology to the three N-terminal Ig domains of mouse FCRL5 necessary for recognition of OMCP (Fig. 3). However, when we tested the ability OMCP tetramers to recognize human FCRL molecules ectopically expressed on 293T cells, none of these six individual human FCRL molecules bound OMCP tetramers (data not shown). Additional studies to resolve this issue are currently underway.

Complete understanding of the contributions of OMCP and FCRL5 to poxvirus pathogenesis and host immunity awaits identification of OMCP’s remaining target receptor(s) and the natural ligand(s) of FCRL5. However, the targeting of intracellular domain of human FCRL5 (another FCRL with dual ITIM and ITAM-like sequences) inhibits BCR-induced calcium mobilization in a B cell line (15). A similar phenomenon has been observed with murine FCRL5 (9). However, we have found no functional impact of soluble, monomeric OMCP on FCRL5+ B cell proliferation, BCR-induced calcium flux, or FCRL5 cell-surface levels (data not shown). Interestingly, despite 64% identity and 75% similarity over the majority of the mouse and human FCRL5 intracellular domains, an abrupt sequence divergence and truncation occurs just prior to the mouse C terminus, causing loss of a tyrosine moiety critical to inhibitory signaling by human FCRL5 (15). Thus, we hypothesize that FCRL5 may use its ITAM-like sequence to function as an activating or costimulatory receptor. In this scenario, OMCP would serve as a competitive antagonist of FCRL5 in a manner analogous to that for NKG2D (1).
FCRL5 by a microbial immunoevasin implies that FCRL5 plays an important role in antiviral immunity and establishes CPXV infection as in vivo model with which to probe FCRL5 biology.

Disclosures

R.S.D. holds a patent on the mouse FCLR5 sequence.

References


**Supplemental Figure 1.** FCRL5 is expressed by marginal zone and B1 B cells. B6 splenocytes (A) or peritoneal cells (B) were gated on the indicated populations and additionally stained with the secondary antibody alone (lines 1), isotype control mAb (lines 2), or anti-FCRL5 mAb (lines 3).
Supplemental Figure 1.