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Molecular Characterization of a Novel Immune Receptor Restricted to the Monocytic Lineage

Helena Aguilar,* Damian Álvarez-Errico,* Andrés C. García-Montero,† Alberto Orfao,‡ Joan Sayós,‡ and Miguel López-Botet‡

Homology basic local alignment search tool search was conducted using a sequence encoding for a novel inhibitory receptor (IREM-1) cloned in our laboratory and a previously described homologous sequence termed CMRF-35. On the basis of this information, we cloned a full length cDNA corresponding to a novel member of this family, termed immune receptor expressed by myeloid cells 2 (IREM-2). The gene, located in chromosome 17q25.1, encodes for a protein of 205 aa that contains an extracellular region comprising an Ig-like domain and a transmembrane region with a positively charged amino acid residue (lysine), that predicted its putative association with an adapter molecule. Indeed, the interaction between IREM-2 and DAP-12 was confirmed in transfected COS-7 cells. By generating specific Abs and using bone marrow and PBMCs, we observed that IREM-2 expression appeared to be restricted to mature hemopoietic cells of the monocytic and myeloid dendritic cell lineages. In vitro differentiation to macrophages or immature dendritic cells down-regulated IREM-2 expression. Upon engagement with the specific mAbs, IREM-2 expressed in rat basophilic leukemia cells together with DAP-12, induced NFAT transcriptional activity; moreover, IREM-2 engagement on monocytes induced TNF-α production. Taken together, our results indicate that IREM-2 is a novel activating receptor of the Ig-superfamily in the monocytic lineage. The Journal of Immunology, 2004, 173: 6703–6711.

Immunoreceptors expressed on the surface of hemopoietic cells are involved in activation and inhibition of cell functions. Inhibitory receptors display cytoplasmic domain regulatory components known as ITIMs (1). Upon receptor-ligand interaction, ITIMs become tyrosine phosphorylated, recruiting and activating SH2-containing phosphatases, like Src homology region 2 domain-containing phosphatase 1 and SHIP, which mediate the inhibitory signal. In contrast, immune activating receptors have a short cytoplasmic domain lacking ITIM but contain a positively charged amino acid residue in the transmembrane region. This residue is involved in association with ITAM motif-bearing adapter transmembrane proteins, such as CD3ζ, FceRIγ, and DAP-12. Receptor engagement promotes tyrosine phosphorylation of ITAM-bearing adaptors and the recruitment and activation of the Syk and Zap-70 kinases (2, 3). Some of these receptors belong to the Ig superfamily, whereas others are lectin-like molecules. The first group includes several multigenic families of receptors (killer Ig-related receptor, Ig-like transcript, receptor for IgA Fc and leukocyte-associated Ig-like receptor) that are encoded within the leukocyte receptor complex on chromosome 19 (19q13.4), as well as other gene families (i.e., signal-regulatory protein (SIRP), paired Ig-like receptor, and the family of triggering receptors expressed by myeloid cells (TREM)§ located in different chromosomes (1, 2, 4). Lectin-like receptors comprise, among others, CD94/NKG2s heterodimers and members of the Ly49 family (5, 6).

Myeloid cells are able not only to recognize and eliminate a wide range of pathogens, but through their professional Ag-presenting function they connect the innate and adaptive immune systems. A number of families of activating/inhibitory receptors including Ig-like transcript (7), SIRP (8), and TREM (4, 9, 10) play an important role in the regulation of myeloid cell function. For instance, TREM comprise two activating receptors; TREM-1 controls inflammation, whereas TREM-2 has been known to regulate the development and function of dendritic cells, microglia, and osteoclasts (4).

In the present study, we report the identification of a novel activating Ig-like receptor, expressed on monocytes and a subset of dendritic cells, that has been termed immune receptor expressed by myeloid cells 2 (IREM-2). This molecule pertains to a multigenic family of activating/inhibitory receptors encoded in human chromosome 17 (17q25.1) and includes previously identified molecules termed CMRF-35 and CMRF35-H/IRP60. Herein, we describe the distribution as well as the molecular and functional characteristics of IREM-2.

Materials and Methods

Cells and Abs

The cell lines P815 (murine mastocytoma), 300.19 (mouse pre-B cell line), Jurkat, U937, THP-1, K562, MonoMac 6, HL60, Raji, and NKL were grown at 37°C with 5% CO2 atmosphere in RPMI 1640/Glutamax medium (Invitrogen Life Technologies, Paisley, United Kingdom) supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Rat basophilic leukemia (RBL) and COS-7 cells were grown in

§Abbreviations used in this paper: TREM, triggering receptors expressed by myeloid cells; IREM-2, immune receptor expressed by myeloid cells 2; RBL, rat basophilic leukemia; BM, bone marrow; PB, peripheral blood; SIRP, signal regulatory protein; HA, hemagglutinin; ssc, side scattering.
complete DMEM with 10% heat-inactivated FCS, 2 mM glutamine, and 1 mM sodium pyruvate. PBMCs were purified from human blood by standard gradient density centrifugation (Lymphoprep; Axis-Shield PoC, Oslo, Norway), washed with PBS, and activated with 2 µg/ml PHA. For isolation of monocytes, monocytes were resuspended at a final concentration of 2 x 10^6/ml in RPMI 1640 with 10% FCS and allowed to adhere to plastic for 60 min to 37°C. Nonadherent cells were eliminated, and attached cells were washed twice with PBS. Isolated monocyte-enriched cell populations were maintained for 5 days either in medium alone or in the presence of different stimuli used at the following concentrations: 500 µM GM-CSF (Amgen, Thousand Oaks, CA), 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ), and 1 µg/ml LPS (Sigma-Aldrich, St. Louis, MO). Cultured cells were harvested after incubation with 1 mM EDTA PBS for 15 min to enhance detachment of adherent cells.

Anti-human HA (H1) mAb 12CA5 was previously described in detail (11). Phosphotyrosine-specific mAbs directly conjugated to HRP was from Zymed (San Francisco, CA). Anti-DAP-12 Ab was a generous gift from Dr. L. L. Lanier (University of California, San Francisco, CA).

**DNA reagents**

For expression in COS-7 cells, FcɛRIγ was amplified by PCR and cloned into the HidIII/BamHI sites of pFLAG-CMV-2 (Sigma-Aldrich) with the 5'-sense primer 5'-CgA ATG ATT CCA gCg A Tg Tgc tGC-3' and the 3'-antisense primer 5'-CgA ggg gAg tCC CTA Ctg Tgg Tgg TTT TCC-3'. Mammalian expression vectors encoding for human c-fyn (11), CD34 (12), and DAP-13 (13) have been previously described.

**Cloning of IREM-2**

Nested PCR was used to clone the full length IREM-2 sequence with PBMCs cDNA as a template. A first PCR round was performed with the 5’-CCA gaa CCA CTA gaa ATC ATT Agg ACC-3’ and 5’-gTC gAT gAg gAg gGa Tg Tg TcA Cag Cag-3’, primers that mapped in the 5'-untranslated region and the 3'-untranslated region, respectively. A second PCR round was conducted using the first PCR as a template with the following pair of primers: 5’-CCT Aga TAg gAt cTC AgT gCT AgT gA Cg AgT-3’ and 5’-gCc gCC gTC gAc ACa CCT Agg CcT gCT gAT gGt gCT-3’. In both rounds, PCR conditions were 94°C for 3 min and 30 cycles of 94°C for 1 min, 65°C for 1.5 min and 72°C for 1 min. PCR products were resolved in 1% agarose gels and visualized by ethidium bromide staining. Amplified fragments were cloned into a pcR.2 vector (Invitrogen, San Diego, CA) and sequenced with ABI PRISM Bigs Dyes Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and the universal T7 primer. For expression in COS-7 cells and RBL cells, IREM-2 cDNA (without signal peptide) was amplified by PCR and cloned into the BglII and SalI sites of pcDisplay (Invitrogen) with the 5’-CCT AgA tCT TCA ggC TgT TgT TCT TgC-3’ and 5’-gCC gTC gAC CTA gTC Agg Agg gAg CCA Cag Cag-3’.

**Cell transfection**

300,199 cells and RBL cells were stably transfected with 20 µg of IREM-2/HA pcDisplay and with or without DAP-12 constructs by electroporation at 250 V and 950 µF in a Gene Pulser Electroporator (Bio-Rad Laboratories, Hercules, CA) and further selection with 1 mg/ml G-418 (Invitrogen Life Technologies). Expression of IREM-2/HA on the cell surface was detected by flow cytometry (FACScan; BD Biosciences, San Jose, CA) in the presence or absence of 250 µl of a luciferase reporter plasmid in serum-free RPMI. After 15 days, immunization was repeated; 15 days later, mice were injected with 150 mg/kg IgE and counted up to 12 days. After 12 days, mice were sacrificed by cervical dislocation. The serum IgE levels were determined by a mouse ELISA using recombinant murine IREM-2 as a coating antigen.

**Immunoprecipitation and Western blotting**

Transfected RBL and COS-7 cells were lysed using Triton X-100 lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM HEPES [pH 7.5], 50 mM NaF, 1 mM sodium orthovanadate, 1 mM EGTA, 2% PMSF, and 1% protease inhibitors). Cell lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4°C, and supernatants were used for preparation of protein samples. Lysates were processed with 30 µl of protein G-Sepharose beads (Invitrogen Life Technologies) and 5 µl of mouse serum for 1 h at 4°C. Afterward, immunoprecipitations were performed with the indicated Abs for 14 h. Beads were washed three times with lysis buffer, sample buffer was added, and samples were denatured under reducing and nonreducing conditions by heating for 6 min at 100°C. Precipitates were treated in the presence or absence of N-glycans. F enzyme (New England Biolabs, Beverly, MA) overnight at 37°C. Samples were electrophoresed in 12% polyacrylamide gels, and proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by semidy string blotting. Membranes were blocked in 5% nonfat milk in 20 mM Tris (pH 7.6), 150 mM NaCl with 2% Tween 20 for 1 h and then probed with the indicated Abs followed by HRP secondary Abs (Amersham Pharmacia Biotech, Piscataway, NJ). Bound HRP-conjugated goat-anti-mouse Ab was visualized using enhanced chemiluminescence (Western Pico Supersignal kit; Pierce, Rockford, IL) and Hyperfilms (Amersham, Little Chalfont, United Kingdom). Cells were surface labeled with 125I (Amersham) and lysed with buffer containing 1% Triton X-100; the solubilized proteins were immunoprecipitated using the UP-H1 mAb and protein G-Sepharose beads. Samples were analyzed by SDS-PAGE followed by autoradiography.

**Luciferase assays**

A total of 4 x 10^4 RBL-IREM-2/HA cells were transfectively transfected with 10 µg of a luciferase reporter plasmid in serum-free RPMI. After electroporation, cells were incubated at 37°C for 24 h in RPMI. Cells were stimulated in 6 h with UP-H1 and UP-H2 (or isotypic controls) using the murine mastocytoma P815 cell line as a presenting cell. IgE 10 µg/ml was used as positive control. Following a washing with PBS, the cells were lysed with 5% lysis buffer (Promega) and 100 µl aliquots were measured for luciferase activity with a Dual Luciferase reporter kit (Promega) following the manufacturer’s instructions.

**Measurement of TNF-α release**

Purified monocytes were stimulated for 24 h in 96-well flat-bottom plates (Costar, Corning, NY) coated with F(ab)’2 sheep anti-mouse IgG (10 µg/
**FIGURE 1.** Nucleotide and predicted amino acid sequence of IREM-2. The nucleotide sequence of IREM-2 containing an open reading frame is shown in upper case and the untranslated region is shown in lower case. The predicted amino acid sequence is shown below the nucleotide sequence. The Ig-like domain is marked in bold. The signal peptide and the transmembrane domain are single- and double-underlined, respectively; the transmembrane charged lysine residue is circled. The potential N-glycosylation site is boxed.

**FIGURE 2.** IREM-2 is a single Ig domain receptor encoded in chromosome 17. A. Alignment of IREM-2 Ig-like domain with Ig domains from related proteins (IRP60, CRMF-35, and NKP44). Identical residues are indicated in a black background, and similar residues are in gray. The cysteines marked with an asterisk are highly conserved. B, Schematic organization of the CRMF35/IREM locus on chromosome 17 and genomic organization of IREM-2. Exons are represented by solid boxes; introns are represented by the connecting lines. Exon-intron junctions of the IREM-2 gene are shown in the lower box.
ml) followed by UP-H2 (anti-IREM-2), B73.1 (anti-CD16), or HP-1F7 (anti-MHC class I). Cells were plated at a concentration of $10^4$ cells/well. Supernatants were collected and tested for production of TNF-α by ELISA according to the manufacturer’s instructions (Bender MedSystems, Vienna, Austria).

**Results**

*Cloning and sequence analysis of IREM-2*

Using a three-hybrid strategy in yeast, we have recently identified a novel cell surface-inhibitory receptor termed IREM-1 (accession...
number Ay303545). This molecule shares homology with two previously cloned receptors called CMRF-35 (17) and IRP60/CMRF35-H (18, 19). The genes encoding for these three molecules are located at the same region (17q21.5) on human chromosome 17 close to the genes encoding for some 17 and appear to belong to a multigenic family of activating/inhibitory receptors. To identify new members of this family of receptors, we searched the Ensembl genome database (20) using the sequence of IREM-1 and CMRF-35. We identified a predicted cDNA sequence that shared homology with both IREM-1 and CMRF-35. We designed primers and cloned a cDNA containing an open reading frame of 618 nucleotides, encoding for a polypeptide of 205 aa (Fig. 1). The deduced polypeptide conformed to a type I transmembrane domain. As shown in Fig. 2A, the monocytic cell lineage and CD16+ cells of CD14 expression (Table II).

Amino acid sequence alignment of the extracellular Ig domain of IREM-2 with CMRF-35 and IRP60 sequences (Fig. 2A) showed a high degree of homology between the Ig domain of IREM-2 with IRP60 (71%) and CMRF-35 (73%). As in the case of Nkp44 and TREM-2 (4), it is of note that the presence of two conserved cysteine residues (C20 and C39) besides the two cysteine residues (C50 and C104) that are involved in the secondary structure of the Ig domain. As shown in Fig. 2A, the Nkp44 Ig domain also displays a high degree of homology with IREM-2 (54%); moreover, similar levels of homology were noticed with TREM-1 (49%) and TREM-2 (58%) (data not shown).

The gene encoding for IREM-2 gene is located at the region 17q25.1 on human chromosome 17 close to the genes encoding for CMRF-35, IRP60 and IREM-1 (Fig. 2B). By alignment of IREM-2 cDNA with the genomic sequence, we determined the organization of the IREM-2 gene (Fig. 2B), that spans ~11 kb and consists of four exons. Exon 1 encodes the 5′-untranslated region and the leader sequence. Exon 2 encodes for the single Ig domain, whereas exon 3 encodes the extracellular region proximal to the transmembrane domain. The transmembrane, cytoplasmic and 3′-untranslated region are encoded by exon 4. The sequences of the intron-exon boundaries conform with the GT-AC rule (Fig. 2B) (21).

### Table I. IREM-2 expression on normal populations of PB and BM hemopoietic cells

<table>
<thead>
<tr>
<th>Hematopoietic precursors</th>
<th>Adult Healthy Individuals</th>
<th>Peripheral blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythroid precursors</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Myeloid precursors</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>CD34+ B cell precursors</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>CD34+ B cell precursors</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Mature cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B lymphocytes</td>
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<td>Negative</td>
</tr>
<tr>
<td>T/NK lymphocytes</td>
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<td>Negative</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Basophils</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Lymphoplasmocytoid DCs</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>CD16+ DCs</td>
<td>80 ± 6%</td>
<td>100%</td>
</tr>
<tr>
<td>(74–85%)</td>
<td>82%</td>
<td></td>
</tr>
<tr>
<td>Myeloid DCs</td>
<td>43 ± 12%</td>
<td>100%</td>
</tr>
<tr>
<td>Monocytes</td>
<td>(26–59%)</td>
<td>48%</td>
</tr>
</tbody>
</table>

* Results expressed as mean ± SD (range) and median percentage of IREM-2+ cells.

DCs, Dendritic cells.

IREM-2 is selectively expressed on PBMC and dendritic cells

The expression pattern of IREM-2 determined by RT-PCR showed the presence of mRNA encoding for this receptor in nonactivated PBMCs as well as in PHA-activated PBMCs from different donors, whereas no transcripts were detected from NK cells (data not shown). Moreover, no IREM-2 transcripts were detected in any of the different cell lines assayed of B (721.221 and RPMI-8866), T (Jurkat, Molt-4, Tall, and Skw), NK (NKL), and myeloid (THP-1, U937, HL60, and K562) cell lineages (data not shown).

To examine the cellular expression of IREM-2, two mAbs (UP-H1 and UP-H2) were generated, immunizing mice with the murine pre-B cell line 300.19 transfected with IREM-2 tagged with the HA epitope. Both Abs specifically stained HA-IREM-2-transfected RBL cells, but not wild-type or HA-IREM-1-transfected RBL cells (Fig. 3A). In addition, COS cells transfected with a construct encoding for IREM-2 without HA in the presence or absence of DAP-12 were recognized equally well by UP-H1 (Fig. 3B) or UP-H2 (data not shown). As shown in Fig. 3C, UP-H1 Ab was specifically bound to the surface of monocytes, whereas no staining was detected on T, B, and NK cells. The different cell lines tested of B (721.221 and RPM-8866), T (Jurkat, Molt-4, Tall, and Skw), NK (NKL and NK3.3) and myeloid cell lineage (THP-1, U937, HL60, and K562, and MonoMac6) were also IREM-2 negative (data not shown).

Table I summarizes the expression pattern of IREM-2 (UP-H1) in the different subpopulations of normal bone marrow and peripheral blood nucleated cells from 11 healthy donors. Reactivity for anti-IREM-2 in PB was restricted to mature hemopoietic cells of the monocytic and myeloid dendritic cell lineages, being constantly absent in mature lymphoid, neutrophil, basophil, eosinophil, mast cell, and lymphoplasmocytoid dendritic cells. Analysis of IREM-2 in bone marrow samples confirmed that IREM-2-positive monocyctic cells appeared at a relatively late stage of maturation, after monocytic precursors have already acquired high levels of CD14 expression (Table II).

### Table II. Maturation-associated patterns of expression of IREM-2 in the monocytic cell lineage and CD16+ monocytic-associated dendritic cells from normal BM samples

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>Relative Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD64+/CD14+/CD45+ /IREM-2+</td>
<td>29 ± 6% (26–36%) 26%</td>
</tr>
<tr>
<td>CD64+/CD14+++/CD45+ /IREM-2+</td>
<td>34 ± 7% (27–39%) 38%</td>
</tr>
<tr>
<td>CD64+/CD14+++/CD14+/IREM-2+</td>
<td>33 ± 12% (22–45%) 33%</td>
</tr>
<tr>
<td>CD64+/CD14++++/CD14+/IREM-2+</td>
<td>3 ± 1% (2–5%) 3%</td>
</tr>
</tbody>
</table>

* Results expressed as mean ± SD (range) and median percentage of IREM-2+ cells.
IREM-2 expression is down-regulated upon in vitro differentiation of monocytes

To analyze the pattern of IREM-2 expression on in vitro-derived macrophages and immature dendritic cells, monocytes were cultured in the presence of GM-CSF or GM-CSF plus IL-4 as described above. After 6 days of culture, FACS analysis showed down-regulation of IREM-2 expression on cells cultured in the presence of GM-CSF (CD14<sup>+</sup>/H11001<sup>+</sup>/CD1a<sup>+</sup>/H11002<sup>+</sup>/CD80<sup>+</sup>/H11001<sup>+</sup>) and GM-CSF plus IL-4 (CD14<sup>+</sup>/H11002<sup>+</sup>/CD1a<sup>+</sup>/H11001<sup>+</sup>/CD80<sup>+</sup>/H11001<sup>+</sup>), corresponding to macrophages and immature dendritic cells, respectively (Fig. 4). By contrast, unstimulated and LPS-treated monocytes maintained IREM-2 expression (Fig. 4).

Biochemical characterization of IREM-2

We conducted immunoprecipitations with UP-H1, UP-H2 and anti-HA mAbs from the HA-IREM-2 stably transfected RBL cell line. All three Abs immunoprecipitated a protein with a molecular mass of ~34 kDa when resolved by SDS-PAGE (Fig. 5A). The apparent molecular mass was reduced to 31 kDa after N-deglycosylation. This result was consistent with the expected molecular mass for IREM-2 and the presence of a putative N-glycosylation site in the extracellular domain (Fig. 5A). The mobility of the protein did not change under reducing or nonreducing conditions, indicating that this receptor did not dimerize through disulfide bonds (data not shown). Similar results were obtained from the analysis of IREM-2 immunoprecipitated from surface 125I-labeled monocytes (Fig. 5B) or by Western blotting with the UP-H2 mAb (Fig. 5C).

IREM-2 associates with DAP-12 in transfected COS-7 cells

The presence of a lysine in its transmembrane region suggested that IREM-2 might associate with ITAM-containing adaptor molecules. Three ITAM-bearing adaptors (DAP-12, FcεRIγ, and CD3ζ) have been reported to associate through a negatively charged amino acid present in their transmembrane domains with a number of activating receptors (3). To determine whether IREM-2 was able to bind any of these adaptors, we cotransfected COS-7 cells with the Src kinase c-fyn, IREM-2-HA, and the adaptors DAP-12, FcεRIγ, and CD3ζ. All three adaptors were tyrosine phosphorylated in the presence of the c-fyn kinase (Fig. 6, left). Western blot analysis with anti-phosphotyrosine mAb of anti-HA immunoprecipitates from cotransfected cells showed that IREM-2 was able to bind DAP-12, whereas no interaction was detected with FcεRIγ and CD3ζ (Fig. 6).

**FIGURE 4.** Immunophenotypic analysis of human monocytes after treatment with LPS, GM-CSF or GM-CSF/IL-4. Human monocytes were cultured under standard conditions with LPS and GM-CSF/IL-4 for 6 days; subsequently, IREM-2, CD14, CD1a, and CD80 expression was analyzed by flow cytometry. Stimulation of monocytes with LPS maintains the expression of IREM-2 as compared with unstimulated cells. In vitro differentiation to macrophages or dendritic cells down-regulates IREM-2 expression. The results show a representative experiment of four performed. FL, Fluorescence.
IREM-2 induces NFAT transcriptional activity in RBL-transfected cells and TNF-α release in monocytes

To explore the possible stimulatory function of IREM-2, we co-transfected IREM-2 and DAP-12 in RBL cells, which did not express any of these molecules. Expression of IREM-2 was verified by surface staining and DAP-12 was monitored by Western blot (data not shown). Cells were transiently transfected with a plasmid encoding the firefly luciferase gene as reporter under the control of an NFAT/AP1-dependent promoter (22). Transfected cells were stimulated with UP-H1, UP-H2 or isotypic Ig controls as described in Materials and Methods. As shown in Fig. 7A, significant luciferase activity was induced in RBL/IREM-2/DAP-12 cells engaged by the UP-H1 and UP-H2 mAbs as compared with the isotypic controls. In all four experiments conducted, UP-H2 was consistently more efficient than UP-H1 for inducing activation. As a positive control, we used IgE to trigger the FcεRI receptor-activating pathway (Fig. 7A). No induction of luciferase activity was detected in wild-type RBL cells or in IREM-2−/−DAP-12−/−transfected RBL cells stimulated with UP-H1 and UP-H2 (data not shown). To further confirm that IREM-2 could act as a stimulatory receptor, we engaged IREM-2 present on the surface of monocytes with the UP-H2 mAb, and we measured by ELISA the release of TNF-α. As shown in Fig. 7B, cross-linking of IREM-2 with UP-H2 induced significant TNF-α production as compared with an isotypic control; despite this, the levels of TNF-α detected were reproducibly lower than those produced upon engagement of CD16 (23). Taken together, our data support that IREM-2 functions as an activating receptor.

FIGURE 6. IREM-2 associates with the adaptor protein DAP-12 in COS-7 cells. COS-7 cells were transiently cotransfected with IREM-2 and c-fyn together with DAP-12, CD3ε, or FcεRIγ. After 48 h, cells were lysed and immunoprecipitated with anti-HA mAb. Immunoprecipitates were analyzed in 15% SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane. The membrane was probed with either anti-HA mAb or anti-phosphotyrosine (anti-pY) mAb and then visualized by Supersignal.

FIGURE 5. IREM-2 is a monomeric 32-kDa glycoprotein. A, Transfected RBLs were subjected to immunoprecipitation (IP) with anti-CD94 mAb as a negative control, anti-HA, UP-H1, and UP-H2 mAbs. The precipitates were analyzed by anti-HA mAb blot under reducing conditions. After N-glycosidase treatment, the molecular mass is reduced to 30 kDa. B, Immunoprecipitation from 125I-labeled monocytes with UP-H1 and UP-H2 mAbs yields a protein that runs as a 30-kDa band in SDS-PAGE under reducing conditions. Anti-CD94 mAb was used as a negative control. C, Comparable electrophoretic patterns were obtained by immunoprecipitation from monocytes and Western blot with the anti-IREM-2 mAb UP-H1.
IREM-2 surface expression did not depend on its association with any adaptor molecules. The ability of IREM-2 to associate with DAP-12 in transfected cells, as well as the fact that it induces NFAT transcripational activity in RBL-transfected cells, indicates that this molecule could act as an activating receptor. Moreover, engagement of IREM-2 induced the production of TNF-α in monocytes. These data confirm IREM-2 as the first identified activating receptor of the CRMF-35 family. Despite the evidence for the interaction between IREM-2 and DAP-12 in COS-7 cells, we could not coprecipitate both molecules from human monocytes. Although this fact could be due to technical limitations, the involvement of another adaptor molecule binding preferentially IREM-2 in monocytes cannot be excluded. In fact, some activating receptors, like NKG2D and SIRPβ1, have been shown to recruit DAP-10 as well as DAP-12 (26–28). Expression of IREM-2 in the surface of RBL cells in the absence of DAP-12 (Fig. 3A) is consistent with results obtained in COS cells where it was detected in the absence of any adaptor; yet the possibility that IREM-2 could associate with endogenous DAP-10 in RBL (28) cannot be excluded. Additional experiments are required to precisely establish the signaling pathways triggered by IREM-2 in monocytes.

The expression pattern of the CRMF-35 molecules appears to be restricted to hemopoietic cells. IRP60 was detected on the surface of NK cells, monocytes, granulocytes, and some subsets of T cells (18). The CRMF-35 distribution is unclear, because the mAb used to study this molecule turned out to cross-react with IRP-60 (29). Nevertheless, by using polyclonal antisera, it has been shown that CRMF-35 was expressed, at least, on the surface of monocytes and T cells (29). These data indirectly exclude the possibility that UP-H1 and UP-H2 mAbs could cross-react with these members of the CRMF-35 family, because the expression of IREM-2 is restricted to myeloid dendritic cells and monocytes. Moreover, because UP-H1 and UP-H2 mAbs did not recognize IREM-1, we can conclude that these Abs do not cross-react with any known member of the CRMF-35 family of receptors.

The presence of IREM-2 on the surface of myeloid dendritic cells (Table I) is in contrast with its down-regulation on monocytes differentiated in vitro to immature dendritic cells (Fig. 4). It is conceivable that down-regulation of IREM-2 in immature dendritic cells may be a transient process and that IREM-2 could be re-expressed on mature dendritic cells (Table I). Despite the fact that in vitro generated dendritic cells can function as professional APCs, the use of cells produced in cytokine-dependent cultures as a model for the study of dendritic cell differentiation is limited, because dendritic cell development in vivo differs in cytokine dependency (30).

CRMF-35 receptors contain a single extracellular Ig domain, with a pair of extra cysteine residues similar to those found in TREM-2 and NKP44 receptors. The crystal structure of NKP44 (31) revealed the existence of a disulfide bridge within the Ig domain that defines a subfamily of Ig-like molecules which is probably important for the interaction with the natural ligands. Triggering of IREM-2 by its ligand would modify the cytokine network and could modulate the process of mononcytic differentiation. Human monocytes differentiate into macrophages, dendritic cells, and osteoclasts under the influence of different microenviromental signals, whereas the lack of appropriate exogenous stimuli leads monocytes to undergo apoptosis (32, 33). It has been reported that CML-1, a member of the murine CRMF-35 orthologous family (25), regulates osteoclastogenesis; thus, IREM-2 might play a similar role. In contrast, putative viral or bacterial ligands could regulate monocyte activation and differentiation.
In conclusion, we have cloned and characterized a novel activating receptor termed IREM-2 that is selectively expressed by monocytes and myeloid dendritic cells; the identification of its ligand is required to determine the physiological role of this receptor.

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References