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CD84 Negatively Regulates IgE High-Affinity Receptor Signaling in Human Mast Cells

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CD84 is a self-binding receptor from the CD150 (or signaling lymphocyte activation molecule [SLAM]) family that is broadly expressed in hematopoietic cells. It has been described that the adaptors SLAM-associated protein (SAP) and EWS-FLI1-activated transcript 2 (EAT-2) are critical for CD150 family members' signaling and function. We observed that human mast cells express CD84 but lack SAP or EAT-2, that CD84 is tyrosine phosphorylated upon FcεRI engagement, and that the release of granule contents is reduced when FcεRI is coengaged with CD84 in LAD2 and human CD34⁺-derived mast cells. In addition, we observed that the release of IL-8 and GM-CSF was also reduced in FcεRI/CD84-costimulated cells as compared with FcεRI/Ig control. To understand how CD84 downregulates FcεRI-mediated function, we analyzed signaling pathways affected by CD84 in human mast cells. Our results showed that CD84 dampens FcεRI-mediated calcium mobilization after its co-cross-linking with the receptor. Furthermore, FcεRI-mediated Syk-linker for activation of T cells-phospholipase C-γ1 axis activity is downregulated after CD84 stimulation, compared with FcεRI/Ig control. The inhibitory kinase Fes phosphorylates mainly the inhibitory motif for CD84. Moreover, Fes, which has been described to become phosphorylated after substrate binding, also gets phosphorylated when coexpressed with CD84. Consistently, Fes was observed to be more phosphorylated after CD84 and FcεRI co-cross-linking. The phosphorylation of the protein phosphatase Src homology region 2 domain-containing phosphatase-1 also increases after CD84 and FcεRI coengagement. Taken together, our results show that CD84 is highly expressed in mast cells and that it contributes to the regulation of FcεRI signaling in SAP- and EAT-2-independent and Fes- and Src homology region 2 domain-containing phosphatase-1-dependent mechanisms. *The Journal of Immunology*, 2011, 187: 000–000.

Mast cells (MCs) have been long recognized as the key effector cells of allergic inflammation and disease (1). More recently, MCs have been described to play a diverse role within the immune system, including the regulation of innate immune responses, autoimmunity, tolerance, and transplant, among others (2). To manifest these biological functions,

MCs are equipped with a myriad of surface receptors that allow them to respond to environmental signals. The most widely studied receptor in MCs is the high affinity receptor for IgE (FcεRI) that belongs to the Fc receptor family. In human MCs (huMCs), the FcεRI is a tetrameric receptor that comprises an IgE-binding α-chain, a signal amplification β-chain, and a γ-chain homodimer that regulates signal transduction. After FcεRI engagement by cognate Ag, two independent but complementary pathways are triggered by the Src family kinases Lyn and Fyn, leading to optimal MC responses (3). The consequential phosphorylation of the β- and γ-chain ITAMs results in the recruitment and activation of Syk kinase, a key step in FcεRI signal transduction. The activation of Syk is essential for all known FcεRI-mediated responses, including degranulation, secretion of allergic mediators, and induction of gene transcription (4). These early phosphorylation events lead to the recruitment of other molecules, for example, linker for activation of T cells (LAT), Src homology 2 (SH2)-containing leukocyte protein of 76 kDa, and the linker for activation of B cells (also called non-T cell activation linker) (5), and to the activation of enzymes such as phospholipase C-γ (PLC-γ), which regulates intracellular calcium release and protein kinase C activation. Downstream events regulate the activation of MAPK family members, ERK, c-Jun N-terminal kinase, and p38, which control the activity of numerous transcription factors important for the synthesis and secretion of lipid mediators and cytokines (6).

MC responses are tightly regulated, and a number of receptors that dampen FcεRI signals have been described. These include CD300a (7), CD300f (8), MAFA (9), gp49B1 (10), and inhibitory Fc receptors such as FcγRIIb (11), among others. Most of these receptors act by recruiting either protein tyrosine phosphatases such as Src homology region 2 domain-containing phosphatase

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The online version of this article contains supplemental material.

Abbreviations used in this article: EAT-2, EWS-FLI1-activated transcript 2; huMC, human mast cell; ILT2, Ig-like transcript 2; LAT, linker for activation of T cells; MC, mast cell; p, phospho; PLC-γ, phospholipase C-γ; SAP, SLAM-associated protein; SCF, stem cell factor; SH2, Src homology 2; SHP, Src homology region 2 domain-containing phosphatase; shRNA, short hairpin RNA; SLAM, signaling lymphocyte activation molecule; WT, wild-type.

(SHP)-1 or SHP-2, or inositol phosphatases such as SHIP-1 upon receptor coligation and phosphorylation of their ITIMs in their cytosolic domains.

The CD150 (signaling lymphocyte activation molecule [SLAM]) family of immune receptors is a subgroup of the CD2 family that is characterized by their capacity to recruit the cytoplasmic adaptor SLAM-associated protein (SAP) by means of at least one SAP-binding motif known as immunoreceptor tyrosine-based switch motifs (consensus sequence is TV/IYxxV/I, where X denotes any amino acid) (12). SAP/SH2D1A consists almost entirely of a single SH2 domain protein, and it was first identified as the product of the gene mutated in X-linked lymphoproliferative disease, a rare immune disorder commonly triggered by EBV (13).

CD84 is a self-binding receptor (14) from the CD150 family that has two Ig domains in the extracellular portion and four tyrosine residues in the cytoplasmic tail (15). CD84 is broadly expressed in the immune system and is present in T cells, B cells, as well as several myeloid cells, including macrophages and MCs (16). Our group has shown that CD84 expression in transfected RBL 2H3 cells can downregulate MC activation through homophilic interaction (17). However, these experiments were conducted in a heterologous system that required transfection of human CD84 into rat cells. Thus, the role of endogenous CD84 in the regulation of primary nontransfected MCs remained unclear. In the present work, we therefore analyzed the contribution of CD84 to FcεRI-mediated signaling in huMCs, showing that CD84 can inhibit FcεRI signaling through SAP–EWS–FLI1–activated transcript 2 (EAT-2)–independent and Fes, SHP-1–dependent mechanisms.

Materials and Methods

Cells and Abs

The LAD2 huMC line, provided by A. Kirshenbaum and D. Metcalfe (National Institutes of Health, Bethesda, MD), was grown in StemPro-34 serum-free medium (Invitrogen Life Technologies, Carlsbad, CA), supplemented with StemPro-34 nutrient, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 100 ng/ml recombinant stem cell factor (SCF; Amgen). Primary huMCs derived from CD34-positive peripheral blood cells were obtained from healthy donors following informed consent on a protocol (98-1-0027; Principal Investigator, A. Kirshenbaum) approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board, and differentiated *in vitro* for 8 wk in the presence of 100 ng/ml IL-6 and 100 ng/ml SCF, as described (18). After 8 wk, the purity of the cultures was assessed by surface FcεRI and c-Kit expression. COS-7 (10⁷) cells were transiently transfected using Nucleofector (Amaxa, Gaithersburg, MD) following the manufacturer's instructions, and then lysed for immunoprecipitation assays, as previously described (19).

Anti-CD84 (clone CD84 1.7; IgG1, k) and anti-human CD229 mAb (clone Ly9 1.25; IgG1, k) used as isotopic control and SAP mAb were described elsewhere (14, 19). Polyclonal rabbit anti-CD84 and anti-SHP-1 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine monoclonal mixture and anti-total ERK Abs were from Zymed Laboratories (Invitrogen). Anti-phospho (p)-LAT^{Y191}, anti-p-Syk^{Y352}, anti-p-PLC-γ1^{Y783}, anti-Fes, anti-p-ERK, and anti-p-Akt were purchased from Cell Signaling Technology (Danvers, MA). Biotinylated human IgE was from Abbiotec (San Diego, CA). PE-conjugated FcεRI Ab was purchased from eBioscience (San Diego, CA), and PE-conjugated c-Kit Ab and PE-conjugated streptavidin were from BD Biosciences (San Jose, CA).

DNA reagents and transfections

Full-length human CD84 cloned in pCINeo, SAP cloned in pCMV2-Flag (20), and Fyn cloned in pSRα (21) were used. The murine lyn construct pcDNA1-mLyn and Lyn K275M plasmid coding for kinase-dead mutant were provided by M. Hibbs (Ludwig Institute for Cancer Research, Melbourne, VIC, Australia). Human Fes (Fes-pCMV6-XL4) was purchased from Trueclone (Origene, Rockville, MD). Csk, SHP-1, and Ig-like transcript 2 (ILT2)/CD85j cytoplasmic tail constructs for three-hybrid system were previously described (22).

To analyze the contribution of CD84 cytoplasmic tail tyrosine residues, single-mutant Y279F and deletion-mutant ΔY324 were employed, as described (17). Single Y324F mutant was generated using CD84 wild-type (WT) pCINeo as a template, and primers for annealing were (forward) 5'-CCCAAGCTTTTCCACAGAAGGTTAGAC-3', (reverse) 5'-GGATCCCT-AGATCACAATTTCAGAGCT-3'. The double mutant Y279F/Y324F was generated using CD84 Y279F in pCDNA3 TOPO as a template and the primers mentioned above. PCR products from both mutants were cloned in pCDNA3 TOPO vector (Invitrogen). The integrity of all constructs was confirmed by sequencing with an ABI PRISM Big Dyes Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and universal T7 primer.

RNA extraction and RT-PCR

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany) from 2 × 10⁶ MCs sensitized with biotinylated IgE, incubated with CD84 or control Abs, and challenged with streptavidin. cDNA was generated from mRNA High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions.

To amplify CD84 from LAD2 or primary human CD34⁺-derived MCs (huMCs), the following primer pairs were used: forward, 5'-CCCGAAT-TCCGTTTGTTCAGAG-3'; reverse, 5'-AAAGGATCCGCCAGCAGC-CTAG-3'. The primers used to analyze SAP expression were as follows: forward, 5'-AGTGGCTGTGTATCATGGCAA-3'; reverse, 5'-CTTGATC-TGGCTTCTGAAATG-3'.

Real-time PCR

Real-time PCR for SAP, EAT-2, and CD84 was performed using TaqMan Gene Expression Assay (Applied Biosystems) on an ABI-Prism 7300 Sequence Detector (Applied Biosystems). The 18S RNA amplification control was used for cycle normalization. Data were analyzed with 7500 SDS Software (Applied Biosystems). All PCRs were set up in triplicates.

Yeast three-hybrid screen

The yeast three-hybrid system was performed, as described elsewhere (20). The cytoplasmic tail of human CD84, cloned in pBridge, was cotransformed with SHP-1 or Csk (cloned in pGAD 424 and pACT2, respectively) in the yeast strain CG1945. ILT2 cloned in pBridge was used as a positive control. Yeast clones were then tested by the β-galactosidase assay. The β-galactosidase liquid culture assay using *o*-nitrophenyl β-D-galactopyranoside as a substrate was carried out as described in the Clontech (Palo Alto, CA) yeast protocols handbook.

FACS staining

CD84 expression was analyzed using biotinylated anti-CD84 for 30 min at 4°C, and the staining was developed by streptavidin-PE. FcεRI and c-Kit expression were analyzed by direct staining with the indicated Abs for 30 min at 4°C. Cells were then analyzed using a FACSCalibur flow cytometer (FACScan; BD Biosciences, Mountain View, CA).

Lentiviral short hairpin RNA gene silencing

Lentiviral particles carrying human Fes short hairpin RNA (shRNA) or nontarget control shRNA sequences were purchased from Santa Cruz Biotechnology. Infection of LAD2 cells was done according to manufacturer's instructions. Targeted cells were selected with 0.4 µg/ml puromycin for 1 wk. Fes expression was analyzed by Western blot, and silencing percentage was related to actin.

β-hexosaminidase release assay

Cells were sensitized overnight with 100 ng/ml biotinylated IgE in StemPro-34 media with SCF and IL-6 (only SCF for LAD2 cells). Cells were then incubated with biotinylated anti-CD84 or anti-CD229 mAbs as isotype control in Tyrode's buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1.8 mM CaCl₂, 1.3 mM MgSO₄, 5.6 mM glucose, and 0.025% BSA) for 30 min at 37°C. After washing, cells were stimulated with 100 ng/ml streptavidin (Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min. Several streptavidin concentrations were tested to verify excess of streptavidin in the assay and lack of competition between biotinylated IgE and either biotinylated anti-CD84 or isotype control. No significant differences were observed at all streptavidin concentrations studied (Supplemental Fig. 2). IgE-dependent MC degranulation was monitored by β-hexosaminidase release, as described (23). The resulting β-hexosaminidase activity was expressed as the percentage of maximum response (samples treated with Triton X-100). Percentage of β-hexosaminidase

release = (sample release – spontaneous release/maximum release – spontaneous release) × 100.

Calcium mobilization

Calcium mobilization in LAD2 cells was followed by fluorimetric analysis of cytoplasmic-free calcium with Fluo-4 AM fluorescent dye (Molecular Probes, Invitrogen). A total of 0.2×10^6 cells/point was loaded with 5 μ M Fluo-4 AM for 30 min at 4°C in the dark, washed twice with Tyrode's buffer, and resuspended. Fluorimetric measurements were done in a Modulus II Microplate Multimode Reader (Turner Biosystems, Promega, CA), according to the manufacturer's instructions.

Cell activation, immunoprecipitation, and immunoblotting

MCs were sensitized overnight with biotinylated IgE (100 ng/ml) in culture medium. The following day, cells were incubated for 30 min at 37°C with biotinylated anti-CD84 or anti-CD229 mAbs, washed twice with Tyrode's buffer, and then stimulated with 100 ng/ml streptavidin or 250 ng/ml as indicated in the same buffer for the indicated times at 37°C. For immunoprecipitation studies, cells were washed twice with ice-cold PBS and solubilized in lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 50 mM Tris [pH 7.4], 150 mM NaCl, 100 mM NaF, 1 mM Na_3VO_4 , 1 mM PMSF). Cell lysates were precleared by centrifugation; the whole-cell lysate fraction was collected and incubated first with the indicated primary Abs and subsequently with protein A-Sepharose beads (Amersham Pharmacia Biotech). After rotation for 2 h at 4°C, the beads were washed three times with lysis buffer. Immunoprecipitated proteins were eluted by heat treatment at 100°C for 5 min with 3× sampling buffer. Immunoprecipitates and total cell lysates were separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were probed with the indicated Abs. In all blots, proteins were visualized by ECL (Pierce, Rockford, IL).

GM-CSF and IL-8 production

IgE-dependent GM-CSF and IL-8 production and release were measured by ELISA with the DuoSet ELISA Development System, as described by the manufacturer (R&D Systems). Briefly, after overnight IgE sensitization, cells were incubated with either biotinylated anti-CD84 or anti-CD229 as an isotype control for 30 min at 37°C, and then stimulated with streptavidin (1 μ g/ml) for 6 h. Supernatants were then collected and analyzed by ELISA.

Biotinylation titration

Capture ELISA was performed by plating 1 μ g/ml goat anti-mouse Ig (Sigma-Aldrich) in PBS at 4°C overnight. Blocking was done for 1 h at room temperature with 1% BSA in PBS. Biotinylated anti-CD84 and anti-CD229 were incubated for 2 h at room temperature at various concentrations. Streptavidin HRP (R&D Systems) was used to detect biotinylation and was incubated for 30 min. After several washes with PBS plus 0.01% Tween-20, the assay was developed with 3,3',5,5'-tetramethylbenzidine HRP substrate solution (R&D Systems). The enzyme reaction was stopped after 10 min by adding diluted sulfuric acid. Quantitative measurement was carried out using microplate reader. The absorbance was measured at 450 nm.

Protein concentration was previously determined by bicinchoninic acid protein assay (Pierce), according to manufacturer's instructions. Biotinylation titration showed that anti-CD84 and isotype control (anti-CD229) Abs have comparable levels of biotinylation. At equal protein concentration, no differences in streptavidin binding were observed for both Ab preparations (Supplemental Fig. 1).

Statistical data analysis

All results are expressed as mean $\pm$ SD. Student *t* test was used to determine significant differences (*p* value) between two experimental groups after determination of normal distribution of the sample and variance analysis.

Results

CD84, but not SAP or EAT-2, is expressed in huMCs

To explore the role of the CD150 family member CD84 in huMCs in the context of Fc ϵ RI signaling, we initially examined the presence of CD84, both at the mRNA and protein level, in LAD2 cells and primary huMCs and indeed confirmed that CD84 was expressed in both cell types (Fig. 1A–C). It has been reported that

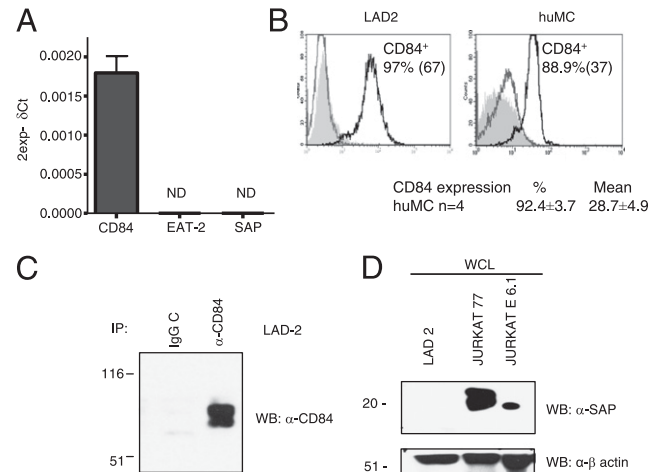


FIGURE 1. CD84 and SAP expression in MCs. CD84, SAP, and EAT-2 expression was analyzed in CD34⁺ peripheral blood-derived huMCs by real-time PCR. Data are representative of three independent experiments (A). CD84 expression on LAD2 cell (left panel) and huMC cell (right panel) surface was analyzed by FACS. Isotype CD229 Ab control (gray line), streptavidin-PE (gray filled), and CD84 expression (black line) is represented on the histograms. Percentage of positive cells and mean of fluorescence (in brackets) are indicated in the figure. Number of donors analyzed is also stated below right panel (B). Western blot against CD84 from anti-CD84 immunoprecipitates from LAD2 cell lysates was performed (C), and Western blot against SAP was done in whole-cell lysates from indicated cells (D). ND, not detectable.

the effects of CD150 family members depend on the presence or absence of the adaptors SAP and/or EAT-2 (24). Despite observing the expression of CD84 in the huMCs, we did not detect the expression of SAP and EAT-2 mRNA (Fig. 1A) or SAP protein levels in these cells (Fig. 1D). The lack of efficient anti-EAT-2 Abs available precluded us from examining the levels of this adaptor at the protein level. SAP and EAT-2 mRNA levels were also undetectable following Fc ϵ RI activation (data not shown). These observations led us to suggest that, in huMCs, CD84 plays a role independently of SAP or EAT-2 signaling.

IgE-mediated huMC degranulation is inhibited by CD84

Following Ag-mediated Fc ϵ RI aggregation, MCs rapidly release preformed proinflammatory molecules stored in cytoplasmic granules. We thus examined whether CD84 could enhance degranulation or modify Fc ϵ RI-mediated degranulation in LAD2 cells and huMCs after ligation or coligation of these two receptors. In both cell types, we observed no effect after CD84 triggering, but a consistent reduction in β -hexosaminidase release following streptavidin-mediated co-cross-linking of biotinylated IgE and anti-CD84, but not with an irrelevant isotype control mAb (Fig. 2A, 2B, respectively). We analyzed whether CD84 ligation had any effect on Fc ϵ RI expression on LAD2 cell surface. No changes on Fc ϵ RI were observed after 10- and 30-min CD84 stimulation either at 37°C or 4°C. Thus, we can rule out that CD84-inhibitory role is due to effects on Fc ϵ RI expression (Supplemental Fig. 3). Thus, CD84 when coligated with Fc ϵ RI inhibits Ag/IgE-mediated MC degranulation.

Fc ϵ RI-mediated cytokine production is negatively regulated by CD84 in huMCs

Later events triggered after Fc ϵ RI aggregation include synthesis and secretion of cytokines such as IL-8 and GM-CSF. These mediators are the most abundant cytokines produced by huMCs; thus, their release upon coengagement of CD84 and Fc ϵ RI was explored. We observed that CD84 reduces Fc ϵ RI-produced IL-8

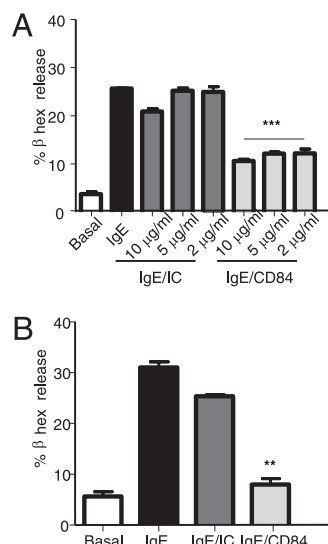


FIGURE 2. Inhibition of FcεRI-mediated degranulation by CD84. β-hexosaminidase contents were measured in the supernatants from activated LAD2 cells and huMCs. Biotinylated IgE-sensitized LAD2 MCs were incubated with the indicated concentrations of either biotinylated anti-CD84 mAb (IgE/CD84), isotype control (IgE/IC), or media (IgE). Coengagement with 100 ng/ml streptavidin was allowed for 30 min (A). Degranulation of huMCs was performed, as described above, in the presence of 5 μg/ml either anti-CD84 or isotype control (IC) (B). Results, expressed as mean ± SD, are representative of three independent experiments; *p* value (***p* < 0.001, ****p* < 0.0001) refers to IgE/CD84 compared against IgE/IC condition. Basal condition represents media with no stimuli.

and GM-CSF detected in cultured supernatants of primary huMCs, when compared with cross-linking of FcεRI and an isotypic control (Fig. 3).

IgE-mediated calcium flux is reduced after CD84 engagement

Calcium mobilization is a key step required for MC degranulation and cytokine production (25). FcεRI aggregation leads to an increase in inositol 1,4,5-triphosphate production, as a consequence of the activation of PLC-γ, and the subsequent release of calcium from intracellular stores. This in turn promotes extracellular cal-

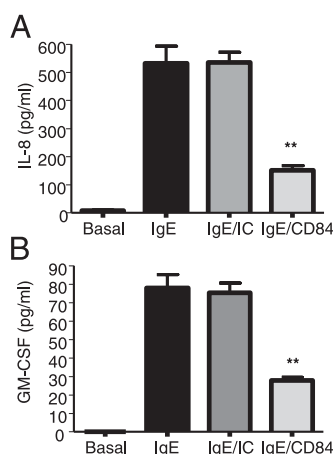


FIGURE 3. CD84 inhibits FcεRI-mediated cytokine secretion. CD34⁺ peripheral blood-derived huMCs were sensitized overnight with biotinylated IgE, incubated with 5 μg/ml biotinylated anti-CD84 (IgE/CD84) or isotype control (IgE/IC) or media (IgE), and then stimulated for 6 h with 1 μg/ml streptavidin. IL-8 (A) and GM-CSF (B) production is represented. Results are representative of three independent experiments and are expressed as the mean ± SD. Statistical significance (***p* < 0.001) is relative to IgE/IC.

cium influx to the cell. We thus evaluated whether CD84 downregulates FcεRI-mediated degranulation and cytokine production by attenuating the ability of aggregated FcεRI to generate the necessary calcium signal. As shown in Fig. 4, CD84 was observed to dampen the FcεRI-dependent calcium response; however, CD84-treated and control cells were able to respond equally to ionomycin, suggesting that cells are viable and CD84 inhibits FcεRI-dependent calcium influx, thus providing support for our conclusion that an attenuated calcium signal accounts for the downregulated degranulation and cytokine production observed after CD84 engagement in huMCs (Fig. 4).

The Syk-LAT-PLC-γ1 axis is downregulated by CD84

The calcium signal elicited by FcεRI aggregation responsible for mediator release requires Syk kinase activation, leading to the phosphorylation of the transmembrane adaptor LAT and subsequently PLC-γ1 activation. We thus analyzed whether the primary signaling pathway leading to calcium flux was altered after CD84 cross-linking. We performed a time course of costimulation with Ag/IgE together with anti-CD84 or isotype control mAb, and evaluated total phosphorylation status of whole-cell lysates. As shown in Fig. 5A, the level of tyrosine phosphorylation of several proteins induced by Ag was reduced after IgE/CD84 cross-linking. The molecular mass of proteins as indicated by the arrows, 160 kDa, 70–72 kDa, and 36–38 kDa, which appeared less phosphorylated, were compatible with those of PLC-γ1, Syk, and LAT. Accordingly, when compared with the co-cross-linking with IgE and an isotype Ig control, coengagement of CD84 and FcεRI showed a marked reduction in PLC-γ1_{Y783}, Syk_{Y352}, and LAT_{Y191} phosphorylation as early as 10 s after stimulation (Fig. 5B). These

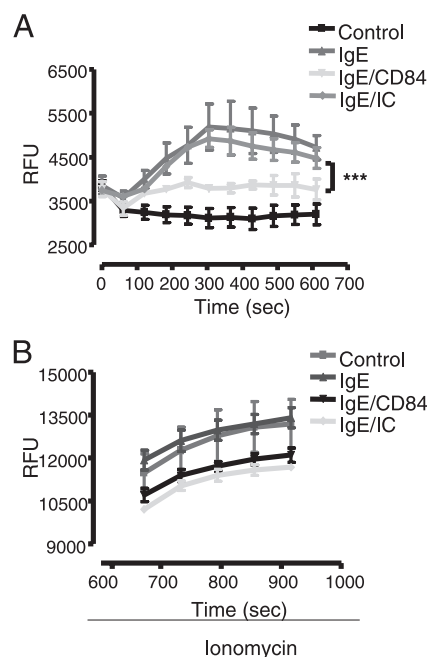
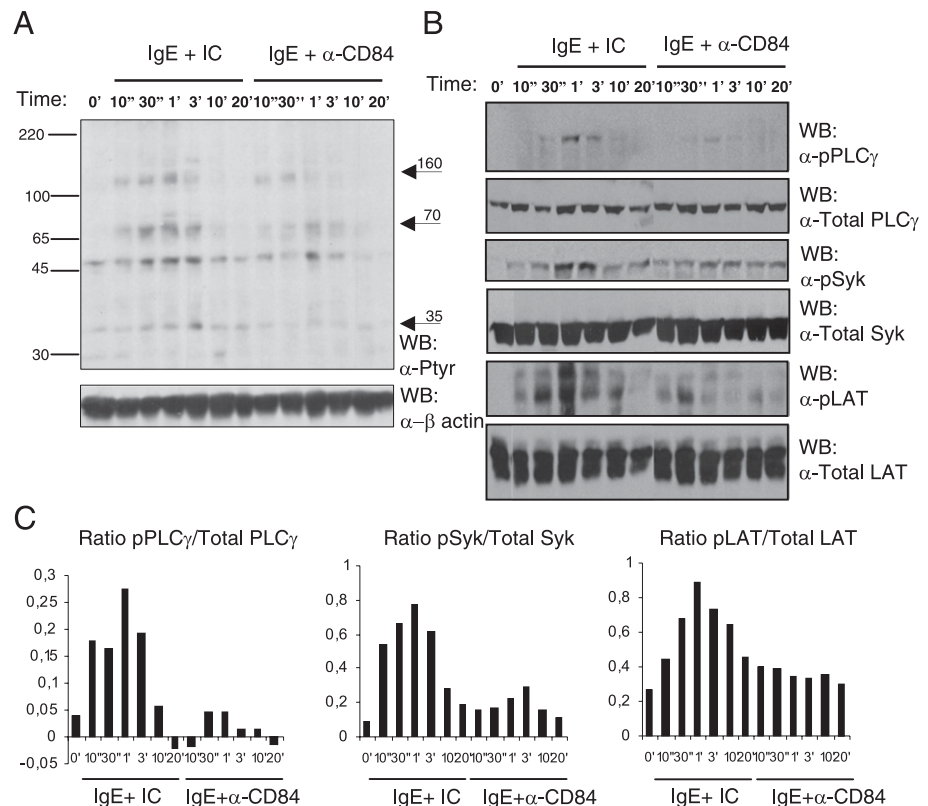


FIGURE 4. Calcium mobilization is downregulated by FcεRI and CD84 coligation. Determination of calcium flux following FcεRI stimulation was performed with biotinylated IgE-sensitized LAD2 cells loaded with Fluo4 dye after incubation with 5 μg/ml biotinylated anti-CD84 mAb or isotype control (IC). Cells were stimulated with 100 ng/ml streptavidin and analyzed by fluorimetric measure of free calcium. Results are expressed as mean ± SD and are representative of three independent experiments. Significance (****p* < 0.0001) represents IgE/CD84 compared with IgE/IC conditions (A). Cells subsequently treated with different stimuli respond to a similar extent to ionomycin (B). RFU, relative fluorescence units.

FIGURE 5. A, FcεRI and CD84 co-engagement reduce the phosphorylation of specific proteins. Phosphotyrosine Western blot of LAD2 cell lysates upon biotinylated IgE and anti-CD84 or isotype control cross-linking with 250 ng/ml streptavidin was performed. The time of stimulation is indicated in the figure. Approximate molecular mass of differentially phosphorylated proteins are indicated by arrows. B, Syk_{Y352}, LAT_{Y191}, and PLC-γ_{1Y783} are less phosphorylated upon receptor coligation. Specific anti-phosphoresidue Abs for pSyk_{Y352}, pLAT_{Y191}, and pPLC-γ_{1Y783} were used to blot membrane with LAD2 cell lysates upon stimulation as described above. The time of stimulation is indicated in the figure. Membranes were reprobbed with anti-total PLC-γ1, anti-total Syk, and anti-total LAT Abs to calculate phosphorylated/total protein ratios and to check loading levels per lane. Densitometric analysis from three independent experiments are represented in the bar charts on the bottom (C).



differences were maintained up to 3 min after stimulation, and signals disappeared after 10 min.

PI3K and ERK pathways are negatively regulated by CD84

The role of PI3K-mediated pathways in the control of cytokine production (26) has been well documented. To investigate whether the inhibitory action of CD84 on cytokine production could be due to the downregulation of these signals, we examined the phosphorylation status of Akt, as a surrogate marker for PI3K activation, at 5, 10, and 20 min following coligation of FcεRI and CD84 or FcεRI aggregation in the presence of an isotypic control. As shown in Fig. 6A, FcεRI-dependent Akt phosphorylation was reduced when coligated with CD84 when compared with the isotype control (Fig. 6A).

Early events including LAT phosphorylation can activate the MAPK ERK1/2 through its interaction with guanine nucleotide exchange factors like Vav and son of sevenless, allowing transcriptional regulation. As MAPK signaling is involved in the phosphorylation of transcription factors that control cytokine expression (27), defective ERK1/2 activation may also account for the reduced cytokine production observed in response to coligation with CD84. Therefore, we additionally investigated the effect of CD84 cross-linking on the ERK cascade, and observed a weaker activation of ERK1/2 following FcεRI and CD84 co-engagement than the one observed when FcεRI was cross-linked with an isotypic control (Fig. 6B). These data support the conclusion that CD84 may downregulate FcεRI-mediated cytokine production through the reversal of PI3K and ERK1/2 activation.

FcεRI aggregation induces CD84 phosphorylation

The ability of inhibitory receptors to downregulate downstream signals vital for cell function requires the recruitment of inhibitory signaling molecules following phosphorylation of specific tyrosine residues contained within the ITIM, or in the case of CD150 family

members, immunoreceptor tyrosine-based switch motifs, consensus sequences. We thus examined the phosphorylation status of CD84 following FcεRI aggregation in LAD2 cells. We observed a very rapid and weak, but consistent increase in CD84 tyrosine phosphorylation after FcεRI stimulation that peaks at 30 s after IgE cross-linking (Fig. 7), which decreased by 3 min.

Lyn and Fes kinases phosphorylate CD84 in COS-7 cells

Most inhibitory receptors on immune cells contain a consensus amino acid sequence, termed the ITIM cytoplasmic domain (28). The prototype ITIM consists of the sequence (I/V/L/S)-x-Y-x-x-(L/V), in which x denotes any amino acid. The two tyrosines in the CD84 cytoplasmic tail, Y²⁷⁹ and Y³²⁴, have proven equally crucial for CD84-mediated degranulation inhibition, but none of them are a SAP-EAT-2 consensus motif (17). Tyrosine 324 (S-S-Y-E-I-V-I) on CD84 features the ITIM consensus, whereas tyrosine 279 (R-I-Y-D-E-I) does not. Ligand-induced clustering of these ITIM-containing receptors results in tyrosine phosphorylation, often by Src-family kinases, which provides a docking site for the recruitment of the tyrosine phosphatases SHP-1 and SHP-2; the inhibitory kinase, Csk; or the inositol phosphatase, SHIP. Nevertheless, studies involving yeast in a phospho-dependent condition failed to show any direct interaction between CD84 and SHP-1 or Csk (Table I). We used as a positive control for these experiments the previously described interaction between ILT2 receptor and SHP-1 and Csk (22). We also were unable to detect any interaction between CD84 and SHP-1, Csk, or SHIP (data not shown) in COS-7, transfected RBL-2H3 cells or LAD2 cells. As shown previously, the CD84 Y²⁷⁹ or Y³²⁴ is not responsible for SHP-2 binding (17). Thus, the role of SHP-2 does not account for CD84-mediated inhibition.

Lyn and Fes kinases have also been implicated in the negative regulation of FcεRI-mediated signaling in MCs (29). Therefore, we analyzed the ability of both Lyn and Fes kinases to phos-

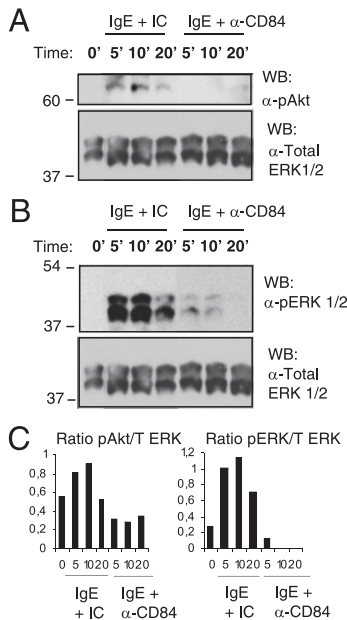


FIGURE 6. Akt and ERK phosphorylation are reduced by CD84 engagement. Biotinylated IgE-sensitized LAD2 cells were incubated with anti-CD84 mAb (CD84) or Ig isotype control (IC) and coengaged with 100 ng/ml streptavidin for 5, 10, and 20 min. Cells were lysed, and total cell lysates were analyzed for Akt phosphorylation as a surrogate marker for PI3K activation (A) and ERK1/2 phosphorylation status (B). Membranes were re-probed with anti-total ERK1/2 Abs to calculate phosphorylated/total protein ratios and to check loading levels per lane. Densitometric analysis and phosphorylated/total ratio for each molecule from three independent experiments are represented in the bar charts on the bottom (C).

phorylate the CD84 residues Y²⁷⁹ and Y³²⁴. To do this, we cotransfected COS-7 cells with WT and mutant CD84 constructs with deletions of these residues together with either Lyn or Fes, and then analyzed phosphorylation status. As shown in Fig. 8A, the major phosphorylation targets for Lyn in CD84 cytoplasmic tail are the residues corresponding to Y²⁷⁹ and Y³²⁴, and disruption of these residues completely abolishes CD84 phosphorylation (Fig. 8A). Lyn specificity was demonstrated by the lack of CD84 phosphorylation when cotransfected together with Lyn K275M mutant that has no kinase activity (Fig. 8A, lane 1).

In a similar set of experiments, we found that CD84 Y³²⁴ fits with the consensus sequence for binding to Fes kinase SH2 domain. Thus, this residue that is part of a putative Fes-binding motif (30) is the major residue within CD84 cytoplasmic tail that gets phosphorylated by Fes (Fig. 8B). Interestingly, we detected a

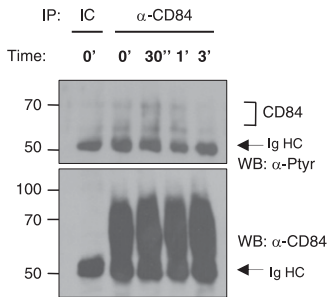


FIGURE 7. CD84 gets tyrosine phosphorylated upon FcεRI engagement. Biotinylated IgE-sensitized LAD2 cells were stimulated with 100ng/ml of streptavidin for 0, 30 s, 1 min, and 3 min, lysed, and immunoprecipitated with anti-CD84 mAb. Membranes were blotted with anti-phosphotyrosine Ab, and total CD84 levels were analyzed by Western blot. Immunoprecipitation with an isotype control (IC) was also loaded.

Table I. Interaction measured by three-hybrid system between negative regulators SHP-1 or Csk and CD84 cytoplasmic tail

	ILT2 (Positive Control)	CD84
SHP-1	++	–
Csk	+	–

ILT2 receptor was used as a positive control. The activity range goes from –, undetectable; +, from 1 to 2 β-galactosidase units; ++, >2 units.

greater increase in Fes phosphorylation when cotransfected with CD84 (Fig. 8C). Mutation of residue Y³²⁴ in CD84 almost completely abolished Fes tyrosine phosphorylation. As it has been described that Fes is activated following binding to its substrates (31), these data strongly suggested that Fes could bind to CD84. However, we have been unable to consistently demonstrate this interaction.

Negative regulators Fes kinase and SHP-1 phosphatase are phosphorylated after FcεRI and CD84 cross-linking in huMCs

To further delineate CD84-inhibitory mechanism in MCs, particularly the contribution of the inhibitory kinase Fes, we examined the phosphorylation of Fes after FcεRI and CD84 cross-linking and compared it with that obtained upon FcεRI aggregation in the presence of an isotypic control. By blotting immunoprecipitated Fes for tyrosine phosphorylation, we observed a rapid phosphorylation of Fes after coengagement of FcεRI and CD84, but not when FcεRI in the presence of an isotypic control was activated. As early as 30 s after FcεRI and CD84 coligation, phosphorylated Fes was already detected and the kinase stayed phosphorylated 1 min after stimulation, concomitantly with CD84 phosphorylation after IgE stimulation. These data further support our conclusion that Fes participates in the inhibitory mechanism exerted by FcεRI in huMCs (Fig. 9A).

After CD84–FcεRI coligation, there is a reduction in the phosphorylation of several proteins, as indicated above. SHP-1 tyrosine phosphatase is a well-known negative regulator of immune receptor signaling, as it catalyzes dephosphorylation of tyrosine residues within signaling mediators (32). SHP-1 gets activated upon recruitment to phosphorylated ITIMs, and in several cases correlation between phosphorylation and increase in phosphatase activity has been described (33). Although we could not observe direct interaction between SHP-1 and CD84, we analyzed SHP-1 phosphorylation status after FcεRI and CD84 cross-linking to verify whether SHP-1 is activated under these conditions. We found an increase in SHP-1 phosphorylation after FcεRI and CD84 coligation as compared with FcεRI stimulation in the presence of an isotypic control (Fig. 9D).

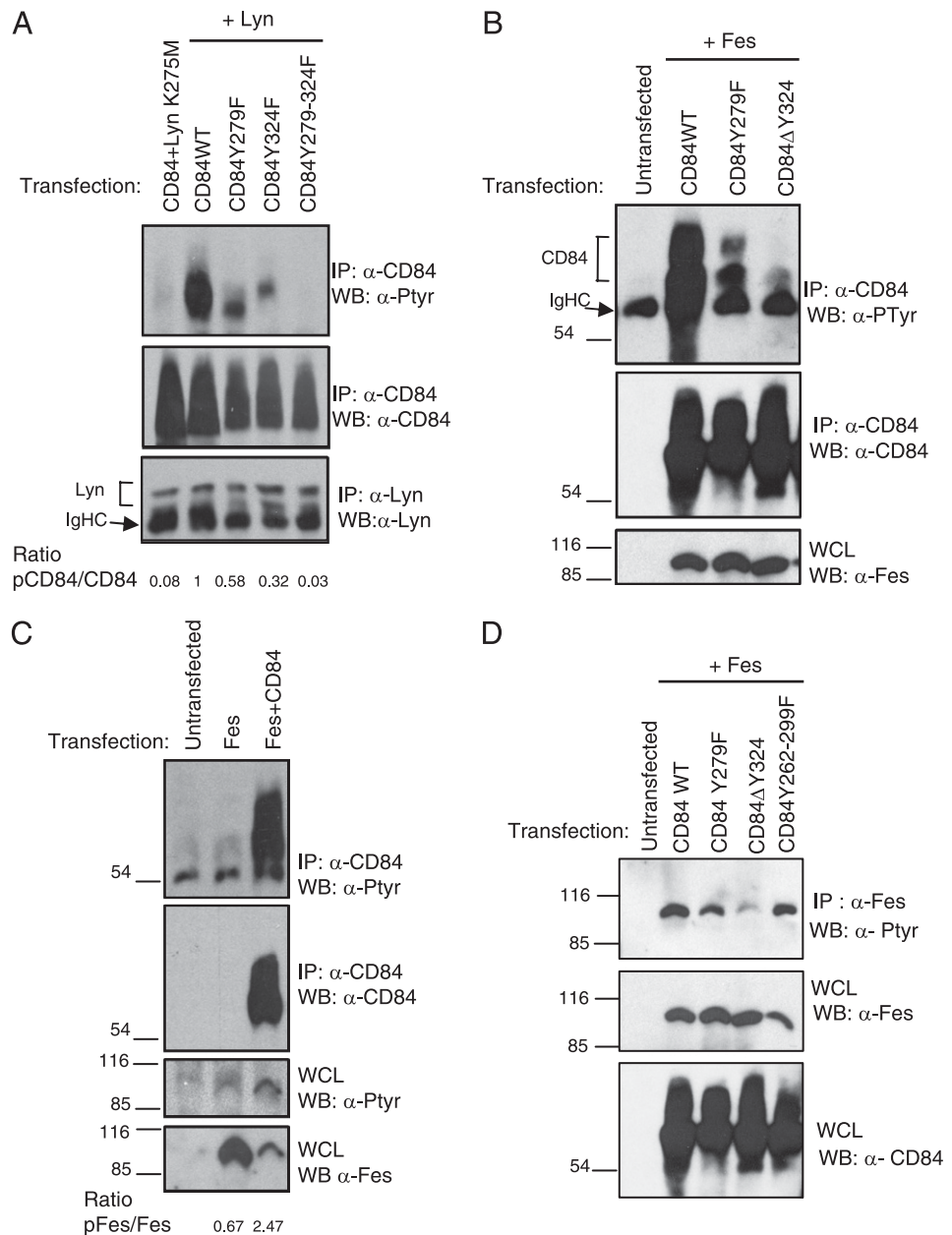
Fes knockdown reverses CD84-mediated inhibition

To further evaluate Fes contribution to CD84-negative regulatory function, we specifically targeted Fes with shRNA (Fig. 9B). We obtained ~50% reduction of normal Fes expression in LAD2, and analyzed these cells for degranulation after FcεRI/IC or FcεRI/CD84 stimulation. We observed that CD84 inhibition on IgE-mediated granule release observed in control shRNA cells was reverted in Fes-targeted cells (Fig. 9C). These results suggest that at least part of CD84-negative signals is mediated by the inhibitory kinase Fes.

Discussion

Inhibitory receptors that reverse signals mediated by activating molecules have been described to play a crucial role in regulating the immune system (28). In the case of MCs, these receptors may

FIGURE 8. A, CD84 Y²⁷⁹ is preferentially phosphorylated by Lyn. Anti-CD84 immunoprecipitation was performed from COS-7 cells cotransfected with WT and CD84 mutants and Lyn kinase. Lyn K275M represents Lyn mutant with ablated kinase activity. Membranes were blotted with anti-phosphotyrosine, anti-CD84, and Lyn Abs. Densitometric analysis for phospho-CD84 versus total CD84 was done. B, CD84 Y³²⁴ is preferentially phosphorylated by Fes. Anti-CD84 immunoprecipitation was performed from COS-7 cells cotransfected with WT and CD84 mutants and Fes kinase. Membranes were blotted with anti-phosphotyrosine, anti-CD84, and anti-Fes Abs. C, Fes phosphorylation is enhanced upon coexpression with CD84. Fes transfection in the presence or absence of CD84 was performed. Blots against phosphotyrosine, CD84, and Fes were carried out. Densitometric analysis of phospho Fes versus total Fes was done. Anti-Fes immunoprecipitation from COS-7 cells cotransfected with WT and CD84 mutants and Fes kinase was done (D). Membranes were blotted with anti-phosphotyrosine, anti-CD84, and anti-Fes Abs.



be considered as potential targets for future therapeutics in allergic inflammation and MC-dependent disease. In this study, we describe that CD84, a member of the CD150 family of receptors, is a novel negative regulator of Fc ϵ RI-initiated signals in huMCs. The CD150 family of receptors are key regulators that can mediate opposite functions in immune cells such as NK, NK-T, macrophages, T, and B cells (34). A “switch of function” dual role model for CD150 receptor family members has been proposed by Veillette et al. (35), whereby alternative activating/costimulatory and inhibitory functions are dictated by the presence or absence of the adaptors SAP and/or EAT-2. It has been observed in NK cells that under conditions in which SAP and EAT-2 are absent, CD150 family members can interact with inhibitory molecules, including phosphatases or inhibitory kinases such as Csk, thereby dampening activating receptor responses (36). The role of the CD150 family of receptors and SAP adaptors has not been elucidated in huMCs to date.

CD84 is highly expressed in MCs (16) and is a self-ligand receptor acting as a homophilic adhesion molecule (14). CD84

interacts with itself with very high affinity through its Ig-like domain 1 in a “head to head” interaction, thus suggesting that CD84 interacts with the CD84 from another cell rather than in the same cell (14, 37). Human CD84 function has been described in T and B lymphocytes. In peripheral blood, CD84–CD84 interactions lead to IFN- γ production in response to suboptimal doses of anti-CD3 (14). In T cells, CD84 appears to also signal in a SAP-independent manner, at least for certain responses. In this respect, cells from X-linked lymphoproliferative disease patients are able to proliferate in a comparable way to WT cells after CD3–CD84 coengagement, suggesting an additional level of complexity to the “switch of function” paradigm (38). In the case of B cells, CD84 is expressed and interacts both with SAP and EAT-2 (20), (39). The relevance of CD84 as a player in adaptive immunity and humoral responses has become evident in CD84-deficient mice, which have a defect in the normal formation of germinal centers in the lymph nodes. CD84 mediates the adhesion necessary for a correct T cell–B cell interaction and for optimal orchestration of humoral responses (40). There are no

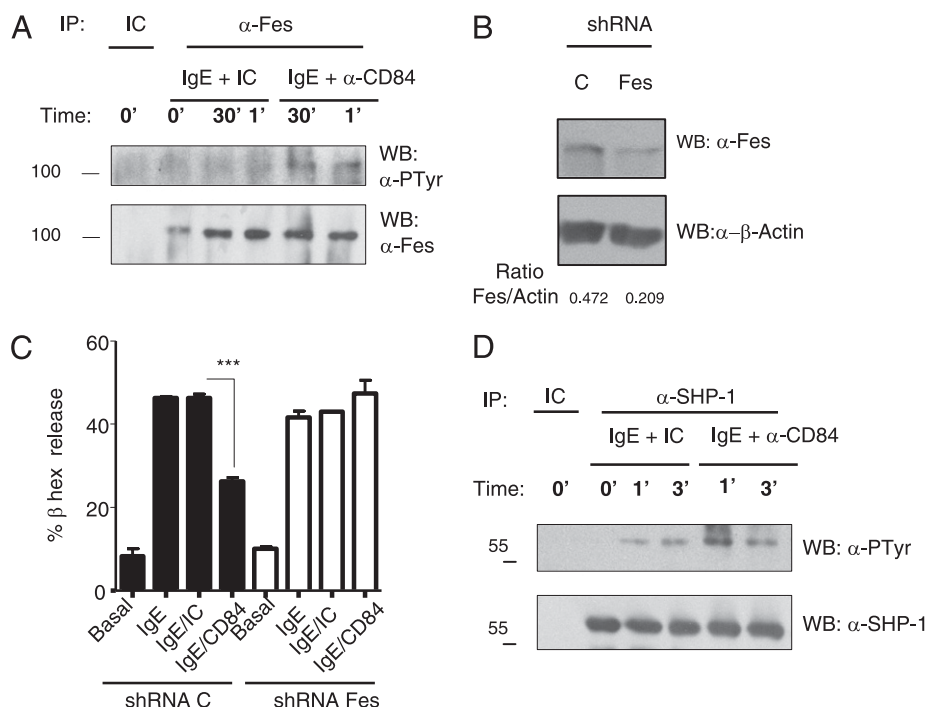


FIGURE 9. A, Fes kinase is phosphorylated upon FcεRI and CD84 coligation. Biotinylated IgE-sensitized LAD2 cells were further incubated with biotinylated anti-CD84 or isotype control for 30 min. Cells were stimulated at 0, 30 s, or 1 min with 100 ng/ml streptavidin, washed, and lysed. Anti-Fes immunoprecipitation was performed, and precipitates were analyzed by Western blot against phosphotyrosine. Total Fes was evaluated by anti-Fes Western blot. B, shRNA Fes kinase silencing. Fes in LAD2 cells was targeted with Fes shRNA or an unspecific control shRNA by lentiviral infection approach. Fes reduction was analyzed by anti-Fes Western blot. Fes to actin ratio was measured to calculate Fes silencing percentage. C, Fes knockdown reverses CD84-mediated β-hexosaminidase release inhibition. Biotinylated IgE-sensitized LAD2 cells with normal (black bars) versus reduced (white bars) levels of Fes kinase were incubated with 5 μg/ml biotinylated anti-CD84 mAb (IgE/CD84), 5 μg/ml biotinylated isotype control (IgE/IC), or media (IgE). Coengagement with 100 ng/ml streptavidin was allowed for 30 min, and β-hexosaminidase release was measured. Significance (***) represents IgE/CD84 compared with IgE/IC conditions. D, SHP-1 phosphorylation increases after FcεRI and CD84 coligation. Sensitized IgE LAD2 cells, incubated with anti-CD84 Ab or isotype Ab control, were stimulated, as described above, for 0, 1, or 3 min. Cells were lysed, and SHP-1 was immunoprecipitated. SHP-1 phosphorylation was evaluated by anti-phosphotyrosine Western blot of immunoprecipitates. Total SHP-1 was assessed by anti-SHP-1 Western blot.

data about MC function in CD84 knockout mice to date. It is of note that murine CD84 cytoplasmic tail does not possess the inhibitory Y³²⁴ motif.

By transfecting CD84 into RBL 2H3 cells, our group previously determined that CD84 can dampen FcεRI-initiated signals in this cell line and that residues Y²⁷⁹ and Y³²⁴ (not involved in SAP and EAT-2 recruitment) in CD84 cytoplasmic tail are critical for such inhibition (17). Given the limitations of previous study due to the necessary heterologous transfection of human CD84 into a rat cell line, it was, however, unclear whether these findings were indicative of a potential biological role of endogenous CD84 in huMC function. In the present work, we provide evidence using the more physiologically appropriate primary cultured CD34⁺ peripheral blood-derived huMCs, as well as LAD2 cell line to further demonstrate a negative role for CD84 in FcεRI-mediated responses in the absence of SAP and EAT-2 expression. Our current data suggest that CD84 signals independently of SAP or EAT-2, and our previous results (17) pointed at the tyrosine motifs, which are not involved in SAP and EAT-2 direct binding, as crucial for CD84-inhibitory role, giving altogether the information that this receptor can play an important role as a modulator beyond SAP and EAT-2 recruitment or dependent signaling of these adaptors. Additionally, as reported (41), we also found that CD84 is expressed in MCs isolated from human lung tissue, and have evidence that it also reduces degranulation in such cells (data not shown).

Consistent with these findings is that CD84 gets rapidly phosphorylated upon FcεRI engagement, which most likely allows it to regulate its inhibitory function, as previous results with RBL cells

suggested. Although the Src family tyrosine kinase Lck phosphorylates CD84 in T cells (38), and Fyn phosphorylates all tyrosine residues in CD84-transfected COS-7 cells (17, 20), selective CD84 phosphorylation in its inhibitory residues has not been investigated to date. We found that in cotransfected COS-7 cells, Lyn is able to phosphorylate CD84 primarily at Y²⁷⁹ and also Y³²⁴. Indeed, double deletion of these residues completely abolishes Lyn-mediated phosphorylation of CD84. These data indicate that Lyn is not responsible for phosphorylation of SAP-EAT-2-dependent motifs present in CD84 cytoplasmic tail.

The Lyn-regulated negative pathway of MC modulation involves Csk-dependent activation of Fyn, mediated by Lyn-dependent Cbp phosphorylation (42). However, we were unable to detect differences in Cbp phosphorylation upon FcεRI-CD84 versus FcεRI cross-linking. Given that signals downstream of Fyn activation lead to PI3K activation via Gab2 phosphorylation (43) and that we show that Akt phosphorylation (a surrogate marker for PI3K activation) is reduced upon FcεRI/CD84 coligation, a direct activation of Csk leading to a Fyn activity reduction could not be ruled out. Csk has been shown to interact with specific members of CD150 family such as CD244 (44); however, we were unable to detect such an interaction with CD84 in a three-hybrid system assay using 2B4 as a positive control.

PI3K signaling can also be downregulated by SHIP-1 in MCs (45). SHIP-1 antagonizes the action of the PI3K pathway by converting the PI3K-produced signaling intermediate PI (3–5)P3 to PI (3,4)P2, thereby preventing recruitment of PH domain-containing proteins such as Akt to signaling complexes at the membrane.

Thus, we analyzed SHIP-1 phosphorylation as indicative of SHIP-1 activity. However, again we observed no differences between CD84 and control coligation in LAD2 cells (data not shown).

The more distal tyrosine Y³²⁴ conforms to a Fes kinase-binding docking site (YE_XV/I) (30), and we show that Fes is able to strongly phosphorylate CD84 at this residue. Although we have consistently observed Fes activation in the presence of WT CD84, we have failed to detect a direct interaction between these two molecules. The possibility that CD84 could also indirectly affect Fes activity cannot be ruled out. Given that CD84 is a self-binding adhesion molecule that could modify cell-adhesive properties, one could speculate that cytoskeleton modification under such conditions could in turn affect Fes activation status. Fps/Fes and Fer are a group of nonreceptor cytoplasmic protein-tyrosine kinases that have been implicated in the regulation of innate immunity through generating signals downstream of a number of cytokines, growth factors, and Ig receptors (46). In MCs, Fes negatively regulates FcεRI responses; thus, Fes-deficient bone marrow-derived MCs are hyperresponsive at low doses of Ag (29). In addition, Fes kinase is tyrosine phosphorylated in a Lyn-dependent manner upon Ag engagement of FcεRI; therefore, a negative regulatory axis involving Lyn–Fes kinases has been proposed. We observed that Fes phosphorylation in LAD2 cells is higher after co-cross-linking of FcεRI with CD84, than with a negative control. Furthermore, Fes expression reduction leads to abrogation of CD84-inhibitory effect on IgE-mediated responses such as degranulation. We failed to observe IgE-mediated hyperdegranulation in LAD2 cells with low levels of Fes. One possibility is that the remaining levels of Fes in these cells still can control granule release in the context of FcεRI signaling alone. Another possibility could be that Fes-negative action on FcεRI has been described for low Ag doses, which would not necessarily be comparable to our streptavidin cross-linking system. Considering that Fes has been shown to phosphorylate inhibitory receptors such as PECAM in bone marrow-derived MCs (29), a scenario in which Fes gets activated via CD84 and phosphorylates another inhibitory receptor that then recruits phosphatases is conceivable. Low but detectable phosphorylation of CD84 could reflect its role in the initiation of the negative signal that would then be amplified by other inhibitory receptors able to recruit SHP-1. This would suggest that CD84 could be a player in the negative regulatory axis upstream of Lyn and Fes. Higher concentration of Lyn kinase brought by activated FcεRI could increase Fes activation and trigger CD84 phosphorylation at inhibitory tyrosines.

It is recognized that phosphorylation of Syk at Y³⁵² residue is associated with the conformational change in Syk required for activation. Accordingly, we observed that Syk is less phosphorylated at this residue upon FcεRI–CD84 coligation when compared with that induced by FcεRI aggregation in the absence of CD84. Consistent with a lower activation of Syk, we also observed that the FcεRI-dependent phosphorylation of LAT (Y¹⁹¹) is reduced when CD84 is engaged. LAT is critical for recruitment of PLC-γ1 and calcium signaling (6). Calcium signals are essential for degranulation and optimal production of cytokines in MCs in response to Ag and other stimulants (47). Consistent with these reports is our observation that CD84 triggering decreases calcium signals. In addition to inhibition of the calcium signal, we found that CD84 coengagement with the FcεRI strongly dampens both Akt and ERK phosphorylation. Consistent with weaker upstream signals observed upon costimulation, we observed lower production of the IL-8/CXCL8 and GM-CSF cytokines.

In conclusion, in the present work, we demonstrate that CD84, a member of the CD150 family of receptors, can negatively regulate FcεRI-mediated signals, degranulation, and cytokine secretion in

huMCs in SAP–EAT-2-independent and Fes- and SHP-1-dependent mechanisms. Considering that CD84 is a self-ligand, we hypothesize that this receptor could set a threshold for FcεRI activation that would be overcome when IgE receptor is aggregated by an encounter with Ag. Defining the precise pathway that regulates CD84 signaling in MCs as well as the mechanisms that modulate its expression would be crucial to establish the potential therapeutic value of this dual receptor.

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Disclosures

The authors have no financial conflicts of interest.

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