Supplemental Information

**Shifting FcγRIIA-ITAM from activation to inhibitory configuration reverses arthritis**

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Supplemental Methods

cDNA constructs and transfections. The hFcγRIIA-encoding DNA sequence was amplified from plasmid pEAK8 puro. Point mutations (Y281F using forward primer 5'-AAACCAACAATGACTTTGAAACAGCTGACGG-3 and reverse 5'-CCGTCAGCTTTCAAAAGTCATTGTTGTTT-3, Y288F using forward primer 5'-CAGCTGACGGCGGCTTCATGACTCTGAACC-3 and reverse 5'-GGTTTCAGAGTCATGAAGGCCGTCAGCTG-3 and Y304F using forward primer 5'-GATGATAAAAACATCTTCTGCAGGTCTCTCCTCCCAAC-3' and reverse 5'-GTTGGGAGGAGAGTGAGTGTGTTTATCATC-3') were introduced in the intracytoplasmic domain of hFcγRIIA using GenArt Site-Directed mutagenesis System kit (Life Technology, France). All constructs were verified by sequencing before transfection into RBL-2H3 cells by Amaxa kit V (Lonza, France). Stable puromycin resistant clones were selected, and hFcγRIIA expression levels and degranulation capacity were determined.

RT-PCR. Total RNA was extracted from monocytes or from THP 1-FcγRIIA-131R+CD14+ using the RNeasy mini kit according to the manufacturer's instructions, and reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Cergy-Pontoise, France). cDNA and non reverse transcribed RNA (500 ng) from cells were amplified for 35 cycles in 40 µl total PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4) containing 100 µM dNTP, 1 or 1.5 mM MgCl2, 1 U Taq polymerase, 10 pmol of forward primer for FCGRIIA (5'-CAGGAAAAAGCGGATTTTACG-3') and 10 pmol of reverse primer for FCGIIA(5'-GATTGGCTGGGTTGTCTTA-3') or 10 pmol of forward primer for FCGRIIB (5'-AAGGACAAGCCGCTGCTGAAAAG-3') and 10 pmol of reverse primer for FCGRIIB (5'-TCAATCCCAATGCAAGACA-3'). The thermal cycling program was 94°C for 30 s,
56°C for 30 s, and 72°C for 1 min. Amplification products were run on a 1.5 % agarose gel stained with ethidium bromide and visualized under UV light.

**siRNA.** Experiments were performed using predesigned HP GenomeWide (Qiagen, Courtaboeuf, France) siRNAs for the hFcγRIIB-encoding gene *FCGR2B* (5′-CAGTGTATTAACAGATAATA-3′; sense, CUGUUAUUAACAGAUAAUATT; antisense, UAUAUCUGUUAUACAGTG), the hFcγRIIA-encoding gene *FCGR2A* (5′-CTCAGAATGTATGTCCAGAA-3′; sense, CAGAAUGUAUGUCCAGAAATT; antisense, UUCUGGGACAUACAUUCUGAG), the human Syk-encoding gene *SYK* (5′-CCGCTCTTAAAGATGAGTTA-3′; sense, CGCUUAUAAAGAUGAGUUATT; antisense, UAACUCAUCUUUAAGAGCGG), the human SHP-1-encoding gene *PTPN6* (5′-CCGGAACAAATGCGTCCCATA-3′; sense, GGAACAAAUGCGUCCCAUATT; antisense, UAUGGGACGCAUUUGUUCGG). A universal negative control siRNA (target DNA sequence, AATTCTCCGAACGTGTCACGT; sense, UUCUCCGAACGUGUCAGUdTdT; antisense, ACGUGACACGUUCGGAGAAdTdT) was purchased from Qiagen. Single strand sense and antisense RNA nucleotides were annealed to generate an RNA duplex according to the manufacturer’s instructions. Monocytes or THP-1 cell line were incubated with 5-10nM of each siRNA tested and 2 µl of Lipofectamine® RNAiMAX prepared according to the manufacturer’s instructions (Invitrogen, Saint Aubin, France) for 48 h or 72h at 37°C before use.

**Histopathology and immunohistochemistry.** For histopathological analysis, the hind leg bones were fixed in phosphate-buffered 10% formaldehyde, decalcified with 10% EDTA, and embedded in paraffin. Horizontal sections of hind paws were stained with H&E. Histological features of peri-articular inflammation (extent of inflammatory cell infiltration), synovial thickening (pannus formation with mesenchymal cell proliferation), and score of inflammation were graded as 0 (normal), 1 (mild), 2
Infiltrating neutrophils and monocytes were detected by immunohistochemical staining using respectively an anti–Ly6C/G (GR-1) and anti-CD11b antibodies (1:1,000 dilutions; BD Biosciences). We used avidin-biotin blocking, alkaline phosphatase, and peroxidase substrate kits (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The number of Gr-1/CD11b–labeled neutrophils and monocytes per surface area \( (10^4 \mu m^2) \) was counted on five different hind paw sections for each of the experimental conditions tested.

**Flow cytometry.** Cells were incubated with anti-hFcyRII (AT-10 at 2µg/10^6 cell) or isotype mAb for 30 min at 4°C before incubation with an Alexa Fluor-488 conjugated goat anti-mouse IgG (Life Technology), or with FITC-conjugated antibodies: anti-hFcyRI clone 10.1, anti-hFcyRIIA clone IV.3 (Stem Cell Technologies), anti-hFcyRIIB (home made), anti-hFcyRIII clone 3G8 (BD Biosciences) or the isotype controls IgG1, (clone: MOPC-21) and (IgG2b, clone 27-35) (BD Biosciences). After washing, cells were analyzed with the BD LSRFortessa flow cytometer (Becton Dickinson) and data were analyzed with FlowJo software (Treestar). For CHO cells, FITC-mlgG1 and FITC-mlgG2b control antibodies were purchased from BD Biosciences; FITC-anti-hCD32 (clone AT10) was from Santa Cruz, FITC-anti-hCD32A (clone IV.3) from Stemcell Technologies, and FITC-labeled anti-FLAG (M2) from Sigma-Aldrich. 2x10^5 CHO-K1 stable FLAG-tagged FcR transfectants (1, 2) were incubated on ice for 30 min with the indicated mAb, washed with PBS, 0.5% BSA, 2 mM EDTA and immediately analysed using a MacsQuant flow cytometer (Miltenyi).

**Degranulation assay.** Degranulation was determined by measuring release of the granule enzyme β-hexosaminidase as described (3). Briefly, cells were sensitized overnight at 37°C with IgE anti-DNP (5 µg/ml) and reagents were added as indicated.
in the figure legends. Degranulation was induced by 0.1 μg/ml DNP-HSA antigen (Sigma-Aldrich) at 37°C for 45 minutes.

**Chemotaxis assay.** BMM chemotaxis was measured in 24-well Micro Chemotaxis Transwell plates (Corning; Costar). Cells (10⁵/ml) were placed in the upper chamber, separated from the lower chamber by a polycarbonate membrane (5 μm pore size). MCP-1 (10 ng/ml; R&D Systems) was added to the lower chamber, and cells were allowed to transmigrate for 2 h at 37°C in humidified atmosphere with 5% CO2. Migrated cells in the lower chamber were counted directly by light microscopy.

**References**


Supplementary Figure 1: mAb AT10 (Santa Cruz) is specific for human FcγRII and mAb IV.3 (Stem cell Technologies) for human FcγRIIA. Representative histogram plots of anti-FLAG, anti-hFcγRII mAb (clone AT10) and anti-hFcγRIIA mAb (clone IV.3) staining (red line) of CHO transfectants expressing the indicated FLAG-tagged human and mouse FcγRs. Shaded histograms indicate staining with the respective isotype controls.
Supplementary Figure 2: Bivalent targeting of hFcγRIIA by the specific hFcγRIIA antibody IV.3 induces SHP-1-dependent inhibitory signaling and inhibits ROS production induced by fMLF. (A) Representative images of hind paws from FcRγ−/−hFcγRIIA Tg mice treated with irrelevant F(ab′)2 (top) or with IV.3 F(ab′)2 (bottom) at day 10. (B) Arthritis evaluation by measuring the increase in ankle thickness (mm) for FcRγ−/−hFcγRIIA Tg mice treated as indicated. Bars show the mean ± S.E.M.. (C,D) BMM from hFcγRIIA Tg FcRγ−/− (C), or FcRγ−/− (D) mice were incubated with IV.3 F(ab′)2 (10 μg/ml) for the indicated time-lengths at 37°C. Cells lysates were immunoprecipitated with AT-10 (IP:CD32). Eluted material was analyzed by immunoblotting (IB) for the presence of SHP-1 and Syk. The amounts of SHP-1 and Syk in lysates were analyzed in parallel by immunoblotting. (E) DCF fluorescence recordings of ROS production by BMM isolated from hFcγRIIA Tg FcRγ−/−, or FcRγ−/− mice and treated or not with AT-10 F(ab′)2 (10 μg/ml) or IgG (10 mg/ml) or IV.3 F(ab′)2 (10 μg/ml) for 30 min at 37°C. Data represent one of three independent experiments. *P<0.05
Supplementary Figure 3: Control siRNA had no effect on hFcγRIIA inhibitory signaling. THP-1-hFcγRIIA-R131ϕCD14ϕcells transfected with the control siRNA were incubated for the indicated times with AT-10 F(ab')₂ (10 µg/ml) at 37°C. Cells were solubilized in 1% digitonin lysis buffer. Lysates were immunoprecipitated with AT-10 antibody (IP: CD32). Eluted proteins were analyzed by immunoblotting (IB) with antibodies of the indicated specificities. Total lysates were analyzed likewise by immunoblotting.
Supplementary Figure 4: hFcγRIIA ITAMIi signaling requires the ITAM distal tyrosine and can inhibit cell activation induced by the FcRγ subunit-ITAM associated to the IgE receptor FcεRI.

(A) Y-to-F mutants of hFcγRIIA are schematically presented and their surface expression levels after transfection into RBL-2H3 cells are shown compared with non-transfected cells (gray vs red histograms). (B, C) RBL-2H3 transfectants or parental cell line were incubated with 10 µg/ml of AT-10 F(ab’)2 for the indicated time at 37°C. Cells lysates were immunoprecipitated with AT-10 (IP: CD32). Eluted material was analyzed by immunoblotting (IB) for the presence of Syk, SHP-1 and hFcγRIIA. The amounts of SHP-1 and Syk in lysates were analyzed in parallel by immunoblotting. (D) IgE sensitized RBL-2H3 transfectants or parental cell line were incubated or not with 10 µg/ml of AT-10 F(ab’)2 overnight at 37°C. Degranulation was triggered with DNP-HSA (0.1 µg/ml) for 45 minutes. Net β-hexosaminidase release was determined. (E, F) RBL-2H3 transfectants or parental cell line were incubated with 10 µg/ml of AT-10 F(ab’)2 for 30 minutes at 4°C and incubated with anti-kappa F(ab’)2 for the indicated time at 37°C. Cells lysates were immunoprecipitated with AT-10 (IP: CD32). Eluted material was analyzed by immunoblotting for the presence of Syk, SHP-1 and hFcγRIIA. The amounts of SHP-1 and Syk in lysates were analyzed in parallel by immunoblotting. (G) RBL-2H3 transfectants or parental cell line were incubated or not with AT-10 F(ab’)2 at 10 µg/ml for 30 minutes at 4°C and degranulation was triggered with the anti-kappa for 45 minutes at 37°C. Net β-hexosaminidase release was determined. *P<0.05; n=12.
Supplementary Figure 5: Expression and silencing of hFcγRIIA and hFcγRIIB in blood monocytes. RT-PCR for the mRNA encoding hFcγRIIA (high panel) and hFcγRIIB (low panel) in non transfected and specific-siRNA transfected monocytes.