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'-CCGTCAGCTGTTTCAAAGTCATTGTTGGTTT-3, Y288F using forward primer 5'-CAGCTGACGGCGGCTTCATGACTCTGAACC-3 and reverse 5'-GGTTCAGAGTCATGAAGCCGCCGTCAGCTG-3 and Y304F using forward primer 5'-GATGATAAAAACATCTTCCTGACTCTTCCTCCCAAC-3' and reverse 5'-GTTGGGAGGAAGAGTCAGGAAGATGTTTTTATCATC-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 fromTHP 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' CAGGAAAAAGCGGATTTCAG-3') and 10 pmol of reverse primer for *FCGIIA*(5'-GATTGGCTGGGGTTGTCTTA-3') or 10 pmol of forward primer for *FCGRIIB* (5' AAGGACAAGCCTCTGGTCAA-3') and 10 pmol of reverse primer for *FCGRIIB* (5' TCAAATCCCAATGCAAGACA-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′-CACTGTTATTAACAGATAATA-3′; sense, CUGUUAUUAACAGAUAAUATT; antisense, UAUUAUCUGUUAAUAACAGTG), the hFcγRIIA-encoding gene *FCGR2A* (5′-CTCAGAATGTATGTCCCAGAA-3′; sense, CAGAAUGUAUGUCCCAGAATT; antisense, UUCUGGGACAUACAUUCUGAG), the human Syk-encoding gene *SYK* (5′-CCCGCTCTTAAAGATGAGTTA-3′; sense, CGCUCUUAAAGAUGAGUUATT; antisense, UAACUCAUCUUUAAGAGCGGG), the human SHP-1-encoding gene *PTPN6* (5′-CCGGAACAAATGCGTCCCATA-3′; sense, GGAACAAAUGCGUCCCAUATT; antisense, UAUGGGACGCAUUUGUUCCGG). A universal negative control siRNA (target DNA sequence, AATTCTCCGAACGTGTCACGT; sense, UUCUCCGAACGUGUCACGUdTdT; 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

(moderate), or 3 (severe). 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-hFcγRII (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-hFcγRI clone 10.1, anti-hFcγRIIA clone IV.3 (Stem Cell Technologies), anti-hFcγRIIB (home made), anti-hFcγRIII 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-mIgG1 and FITC-mIgG2b 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

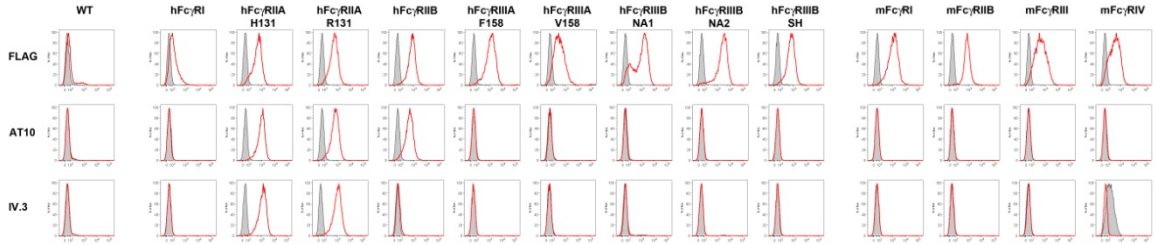
in the figure legends. Degranulation was induced by 0.1 µg/ml DNP-HSA antigen (Sigma-Aldrich) at 37°C for 45 minutes.

Chemotaxis assay. BMM chemotaxis was measured in 24-well Micro Chemotaxis Transwell plates (Corning; Costar). Cells (10^5/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

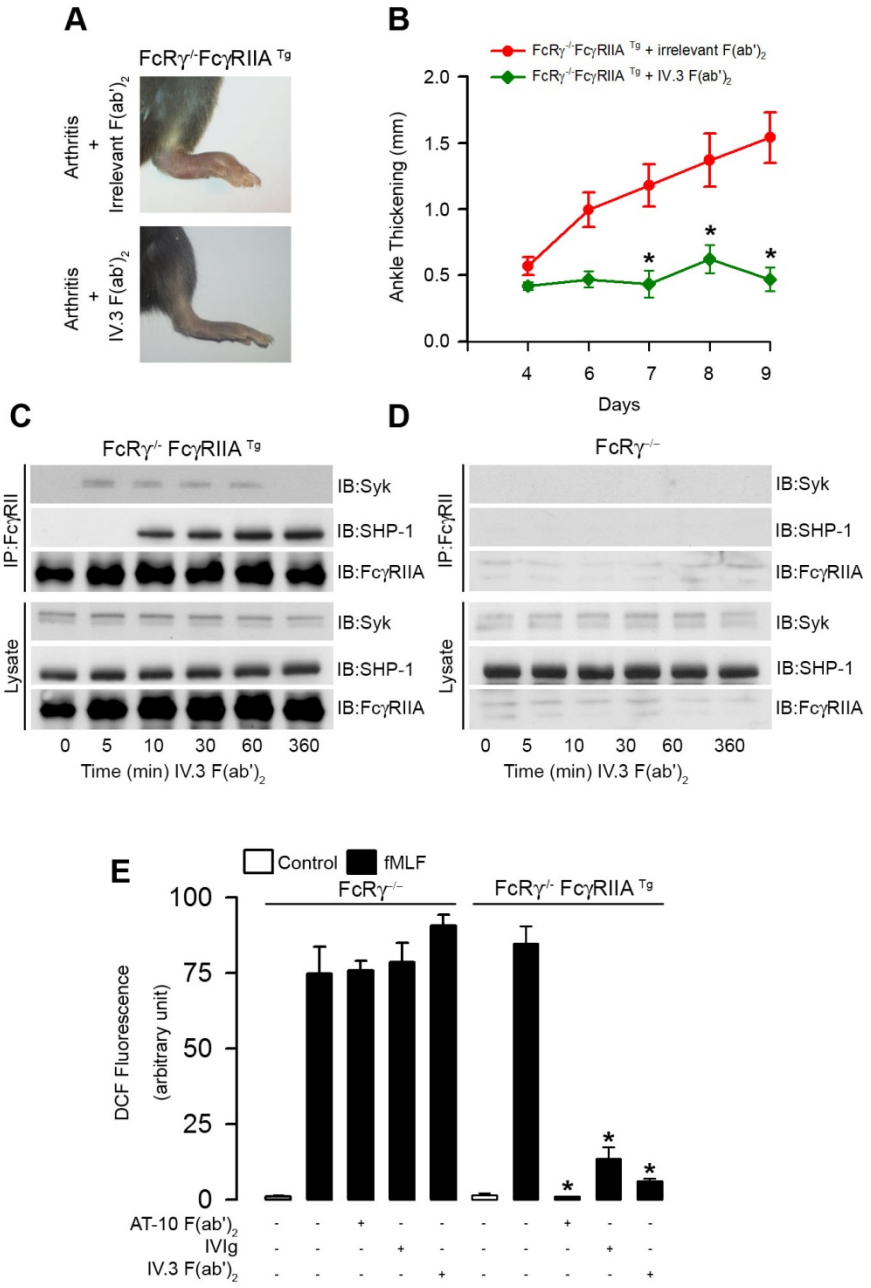
1. Bruhns, P., Iannascoli, B., England, P., Mancardi, D.A., Fernandez, N., Jorieux, S., and Daeron, M. 2009. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. *Blood* 113:3716-3725.
2. Mancardi, D.A., Iannascoli, B., Hoos, S., England, P., Daeron, M., and Bruhns, P. 2008. FcgammaRIV is a mouse IgE receptor that resembles macrophage FcepsilonRI in humans and promotes IgE-induced lung inflammation. *J Clin Invest* 118:3738-3750.
3. Roa, M., Paumet, F., Le Mao, J., David, B., and Blank, U. 1997. Involvement of the ras-like GTPase rab3d in RBL-2H3 mast cell exocytosis following stimulation via high affinity IgE receptors (Fc epsilonRI). *J Immunol* 159:2815-2823.

Supplementary Figure 1



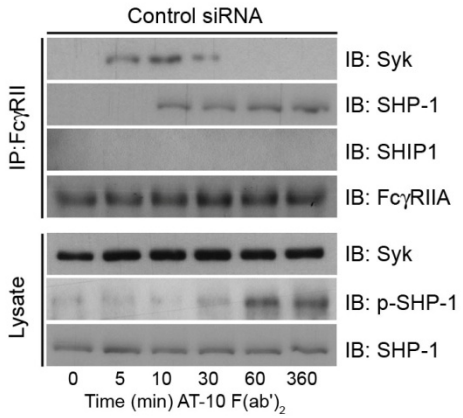
Supplementary Figure 1: mAb AT10 (Santa Cruz) is specific for human FcγRII and mAb IV.3 (Stem cell Technologies) for human FcγRIIA. Representative histogram plots of anti-FLAG, anti-hFcγRII mAb (clone AT10) and anti-hFcγRIIA mAb (clone IV.3) staining (red line) of CHO transfectants expressing the indicated FLAG-tagged human and mouse FcγRs. Shaded histograms indicate staining with the respective isotype controls.

Supplementary Figure 2



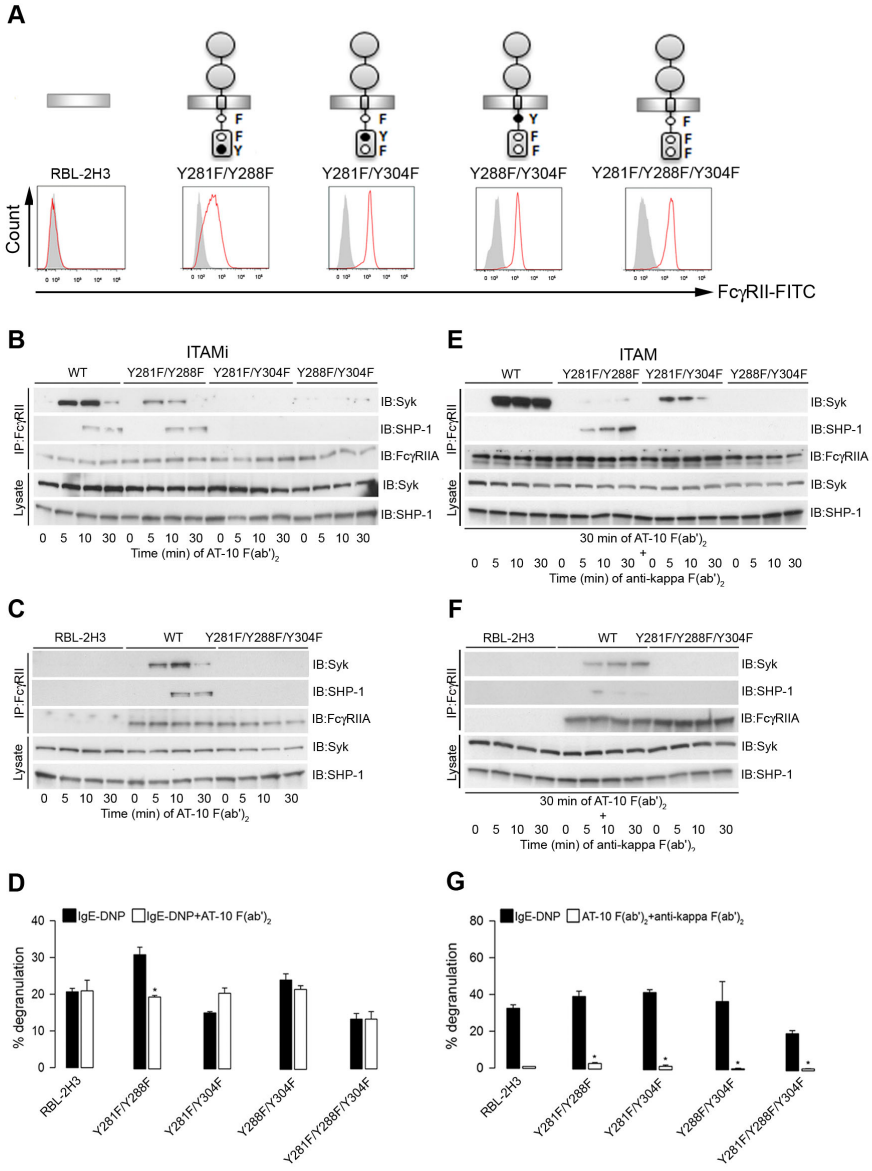
Supplementary Figure 2: Bivalent targeting of hFcγRIIA by the specific hFcγRIIA antibody IV.3 induces SHP-1-dependent inhibitory signaling and inhibits ROS production induced by fMLF. (A) Representative images of hind paws from FcRγ$^{-/-}$hFcγRIIA^{Tg} mice treated with irrelevant F(ab')$_2$ (top) or 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 IVIg (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



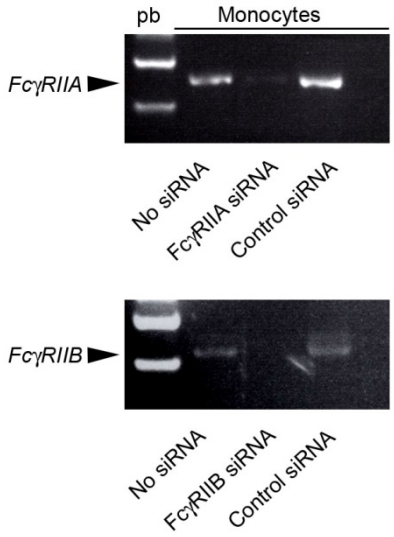
Supplementary Figure 3: Control siRNA had no effect on hFcγRIIA inhibitory signaling. THP-1-hFcγRIIA-R131^{+}-CD14^{+}cells transfected with the control siRNA were incubated for the indicated times with AT-10 F(ab')$_2$ (10 µg/ml) at 37°C. Cells were solubilized in 1% digitonin lysis buffer. Lysates 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



Supplementary Figure 4: hFcγRIIA ITAMi 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



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.