Brief Definitive Report

Interleukin 4 Reduces Expression of Inhibitory Receptors on B Cells and Abolishes CD22 and FcγRII–mediated B Cell Suppression

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Abstract

Inhibitory receptors CD22, FcγRII (CD32), CD72, and paired immunoglobulin-like receptor (PIR)–B are critically involved in negatively regulating the B cell immune response and in preventing autoimmunity. Here we show that interleukin 4 (IL-4) reduces expression of all four on activated B cells at the level of messenger RNA and protein. This reduced expression is dependent on continuous exposure to IL-4 and is mediated through Stat6. Coligation of FcγRII to the B cell receptor (BCR) via intact IgG increases the B cell activation threshold and suppresses antigen presentation. IL-4 completely abolishes these negative regulatory effects of FcγRII. CD22 coligation with the BCR also suppresses activation — this suppression too is abolished by IL-4. Thus, IL-4 is likely to enhance the B cell immune response by releasing B cells from inhibitory receptor suppression. By this coordinate reduction in expression of inhibitory receptors, and release from CD22 and FcγRII-mediated inhibition, IL-4 is likely to play a role in T cell help of B cells and the development of T helper cell type 2 responses. Conversely, B cell activation in the absence of IL-4 would be more difficult to achieve, contributing to the maintenance of B cell tolerance in the absence of T cell help.

Key words: interleukin 4 • B cells • CD22 • FcγRII • inhibitory receptors

Introduction

Activation of peripheral B cells is inhibited by a number of cell surface molecules such as CD22, FcγRII (CD32), CD72, and paired immunoglobulin-like receptor (PIR)–B. Little is known about what releases the B cell from this inhibitory thrall, allowing it be appropriately activated in response to pathogens, and inappropriately activated by self-antigen resulting in autoimmunity. These inhibitory receptors contain immune tyrosine inhibitory motifs (ITIMs) in their cytoplasmic tails which, under specific circumstances, such as when FcγRII and CD22 are coligated with the B cell receptor (BCR), are phosphorylated by the tyrosine kinase lyn. ITIM motifs can then dock with the SH2 domains of phosphatases – predominantly SH2-containing protein tyrosine phosphatase Shp-1 (CD22, CD72, and PIR–B), and the inositol phosphatase SH2-containing inositol 5-phosphatase (SHIP; FcγRII). These phosphatases then dephosphorylate incompletely defined downstream targets and reduce cellular activation (1).

Consistent with the similarity in the signaling pathways of FcγRII, CD22, and CD72, mice deficient in them display defects in their antibody responses. These include increased serum IgG and IgM and increased B cell responsiveness to antigen (2–4). Moreover, mice deficient in lyn or Shp-1, molecules responsible for the signaling of the ITIM-containing inhibitory receptors, also exhibit evidence of B cell hyperactivity (5). FcγRII is ligated to the BCR by antigen and IgG in immune complexes, and may therefore provide feedback inhibition. Coligation of CD22 to the BCR reduces B cell activation, while sequestering CD22 away from the BCR results in B cell hyperactivity (for a review, see reference 5). Interaction of CD22 with its sialic acid residue ligand may promote B cell activation in appropriate lymphoid environments (5) and/or reduce the

likelihood of autoreactivity (6). Ligands for CD72 may include CD5 and CD100 (4), and for PIR–B, MHC I (7), though their exact roles in B cell regulation are yet to be determined. An activatory version of PIR–B, PIR–A, is also expressed on B cells. Both these molecules share essentially identical extracellular domains and it is thought that their ratio on the B cell surface may determine their net effect on activation (8).

Not only are the ITIM-containing inhibitory receptors important in controlling the B cell response to exogenous antigen, they also appear to be important in maintaining self-tolerance. Mice deficient in FcγRII, CD22, lyn, or Shp-1 all display a predisposition to autoimmunity (5, 9, 10). Partial defects in these molecules may contribute to the development of spontaneous systemic lupus erythematosus (SLE)–like disease in polygenic models, with FcγRII being implicated in SLE in a number of mouse models (11), CD22 in the NZW mouse (12), and CD72 in the MRL mouse (13).

Relatively small reductions in expression of these molecules can result in increased B cell activation and/or a contribution toward the breakdown of self-tolerance. Thus, a defect in tolerance in the hen egg lysozyme (HEL)/anti-HEL system occurs in mice heterozygous for lyn, CD22, and SHP-1 (14). In addition, mice heterozygous for deletions in FcγRII or CD22 exhibit only modest reductions in protein expression, but nonetheless have a reduced but significant predisposition to autoimmunity (J. Ravetch, personal communication; and reference 12). The normal regulation of surface expression of inhibitory receptors is therefore likely to be important in controlling the outcome of B cell activation in both normal and autoreactive immune responses.

IL-4 is produced predominantly by T cells and has many effects, as the IL-4R is widely expressed (reviewed 15). Most of the IL-4–mediated effects on lymphocytes, including the mitogenic effect, are reduced or abolished in Stat6-deficient mice (15). B cell effects of IL-4 include isotype switching to IgG1 and IgE (in the mouse, IgG4 and IgE in humans), increased expression of MHC II, CD23, and the IL-4R, prevention of apoptosis in culture (for a review, see reference 15), and morphological changes (16). Importantly, IL-4 increases B cell proliferation when acting in concert with other activatory signals, but does not have this effect alone (17). The mechanisms by which IL-4 stimulation promotes B cell proliferation and activation are not well defined.

Here we show that signals through the IL-4R reduce the expression and function of ITIM-containing inhibitory receptors on activated B cells, suggesting the release of the B cell from ITIM-mediated suppression is likely be an important mechanism of action of IL-4.

Materials and Methods

Mice. C57BL/6 FcγRII$^{-/-}$ mice (3) were provided by Jeff Ravetch and Silvia Bolland (The Rockefeller University, New York, NY). C57BL/6 Stat6$^{-/-}$ mice were provided by Eva Sev-

erinson (Karolinska Institute, Stockholm, Sweden). Control mice were from Charles River Laboratories.

Cell Purification and Stimulation. Splenic B cells were selected by magnetic cell purification using anti-CD19 beads (Miltenyi Biotech), to >95% purity. Results were confirmed by negative selection using anti-CD43 beads (Miltenyi Biotech). Cells were cultured (2 × 10^6 cells/ml) in 24-well flat bottomed plates (Corning Inc.) in RPMI-1640 (Sigma-Aldrich) and 10% fetal calf serum (Invitrogen) and stimulated with either goat anti–mouse IgM μ-chain specific F(ab')$_2$ or intact IgG (Jackson ImmunoResearch Laboratories) or LPS (Sigma-Aldrich). Cytokines added to these cultures were IL-4 (usually 10 ng/ml: PeproTech), IL-2, 5, 6, 9, and 13 (all 10 ng/ml), IL-10 (100 ng/ml), IFN-γ (1,000 U/ml; R&D Systems), and TGFβ (PeproTech). CD40L expressing J558L cells were the gift of Prof. Martin Glennie (University of Southampton, Southampton, UK). Rat anti–mouse IL-4 neutralizing antibody (Serotec) or isotype control (Caltag) were used at 30 μg/ml.

Flow Cytometric Analysis and Intracellular Calcium ([Ca^{2+}]i) Measurement. Cells were stained with FITC-conjugated 2.4G2 (anti-FcγRII/III), anti-CD22, anti-CD72, or isotype control (BD PharMingen) and counterstained with RA6–3B2 (anti-B220 PE) and 7-aminoactinomycin D (Molecular Probes) to identify B cells and exclude dead cells respectively. Cells were analyzed using a FACSCalibur™ flow cytometer (Becton Dickinson) and FCS Press software (Ray Hicks, University of Cambridge, Cambridge, UK). To determine [Ca^{2+}]i flux, B cells from three mice were pooled, purified, and stimulated for 48 h with LPS (10 μg/ml) with or without IL-4 (10 ng/ml). They were then prepared and loaded with Indo-1 as described (11) before staining and analysis using a FACS Vantage™ flow cytometer (Becton Dickinson). Thapsigargin (100 ng/ml; Molecular Probes) stimulation was used to confirm equality of maximal responsiveness and Indo-1 loading (not shown).

Real Time Semiquantitative Reverse Transcription-PCR. RNA was extracted from equal numbers of B cells using TRIzol® Reagent (GIBCO BRL), and reverse transcribed using Super RT (HT Biotechnology). Levels of FcγRII, CD22, CD72, PIR-B, PIR-A, IL-2Rγ chain, and CD23 mRNA were assessed relative to GAPDH using real time semiquantitative reverse transcription (RT)-PCR (ABI Prism 7700 Sequence Detection System; Applied Biosystems). The GAPDH control primers and probe were Taqman Rodent control reagents (Applied Biosystems). IL-2Rγ chain primers and probe were supplied by Dr. A. Gonzalea-Munoz (University of Cambridge), and all other primers and probes were designed using Primer Express software (Applied Biosystems) and manufactured by Sigma-Genosys and Biosource International.

Proliferation Assays. Purified B cells (2 × 10^6/ml) were stimulated with anti-IgM μ-chain specific F(ab')$_2$ or intact antibody (Jackson ImmunoResearch Laboratories). Tritiated thymidine (0.5 μCi; Amersham Pharmacia Biotech) was added from 24 to 48 h and incorporation determined using a scintillation counter (Packard Bioscience).

Antigen Presentation Assay. Purified B cells from BALB/C$^{-/-}$ and control mice were cultured (10^6/ml) with the ovalbumin specific T cell hybridoma D011.10 (18) at 2.5 × 10^5/ml. Ovalbumin or bovine serum albumin (negative control; Sigma-Aldrich) was added at either 2 and 10 mg/ml. Cells were stimulated with anti IgM F(ab')$_2$ or intact antibody, either alone or with 2.4G2 (5 μg/ml) or murine IL-4 (10 ng/ml), for 24 h at 37°C. IL-2 was then assayed using a sandwich ELISA (Becton Dickinson).

Results

IL-4 Reduces Expression of FcγRII, CD22, and CD72 on Activated B Cells. Regulation of ITIM-containing inhibitory receptors is likely to be important both in controlling the normal immune response, and in preventing the development of autoimmunity. IL-4 has previously been shown to reduce FcγRII binding on activated B cells (19). We sought to study the influence of IL-4 and other cytokines on B cells, further characterizing the effect on FcγRII expression, and determining if expression of other inhibitory receptors was similarly controlled. Splenic B cells were activated with anti-IgM F(ab')$_2$ fragments in the presence of IL-4, other cytokines, or medium alone. IL-4 had no effect on expression if cultured with unstimulated B cells, but upon B cell activation a progressive relative reduction of FcγRII, CD22, and CD72 could be seen relative to B cells activated in medium alone (Fig. 1 A). This effect was consistently seen (Fig. 1 B), and was also seen if the B cells were activated by LPS in place of anti-IgM (data not shown). Expression of the inhibitory receptors was not influenced when B cells were activated in the presence of IL-2, 3, 5, 6, 9, 10, or 13, TGF-β, TNF-α, IFN-γ, or CD40-ligand expressing J558L (data not shown). The effect of IL-4 could be titrated, and its specificity was confirmed by blocking with neutralizing anti–IL-4 antibodies (data not shown). Reduction in inhibitory receptor expression required constant exposure of the B cell to IL-4, as removal of the latter after 24 h by washing (Fig. 1 C) or addi-

tion of neutralizing anti–IL-4 antibodies (data not shown) resulted in rapid restitution of inhibitory receptor levels to normal. Once the B cell had been activated in the absence of IL-4, subsequent addition of IL-4 could still reduce inhibitory receptor expression (Fig. 1 C). Thus the reduction in inhibitory receptor expression caused by IL-4 requires constant exposure to it, and is not "set" at the time of initial activation of the B cell.

Resting splenic B cells were labeled with 5-(and-6)-carboxyfluorescein (CFSE). IL-4 increased B cell proliferation (indicated by reduced CFSE staining), but when gates were placed upon B cells having undergone similar numbers of divisions, reduction in inhibitory receptor expression in the IL-4–treated population could still be clearly seen relative to control (data not shown). Similar experiments showed that in B cells expressing similar levels of CD69 or CD86, the effect of IL-4 on inhibitory receptor expression was still observed (data not shown). Activated B cells did not express the plasma cell marker CD138 (syndecan). Thus, reduced inhibitory receptor expression is specifically dependent on IL-4, and is not the nonspecific consequence of increased proliferation or differentiation.

IL-4 Reduces Levels of FcγRII, CD22, CD72, and PIR-B mRNA in Activated B Cells, but Increases PIR-A. A marked reduction in CD22, FcγRII, and CD72 mRNA levels was seen as early as 24 h after activation in the presence of IL-4 (Fig. 2), a time at which little difference is seen in surface
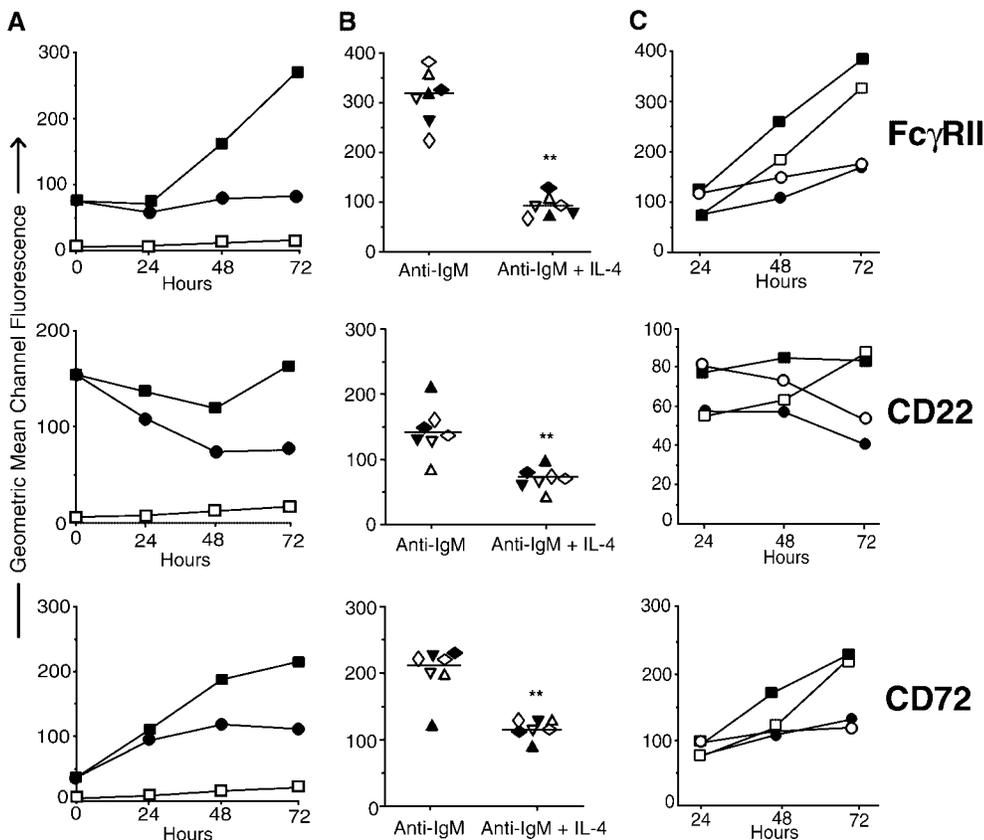
Figure 1. IL-4 reduces surface expression of inhibitory receptors on activated B cells. (A) Purified splenic B cells were activated with anti-IgM F(ab')$_2$ antibodies with (closed circles) or without (closed squares) IL-4. Levels of expression of FcγRII, CD22, and CD72 were determined by flow cytometry at the times shown. Isotype control staining is shown (open squares). Variation in control bead mean channel fluorescence was < ±5% between time points (not shown). (B) Expression levels at 72 h for seven experiments. Individual symbols correspond to single experiments. Mean expression (horizontal bars) of all three molecules was significantly lower when activation occurred in the presence of IL-4 (**P < 0.005, Mann-Whitney U test). (C) B cells were activated with anti-IgM F(ab')$_2$ in the continuous presence (closed circles) or absence (closed squares) of IL-4. In addition, in some cultures IL-4 was only added after 24 h (open circles) or was added at the start but removed at 24 h (open squares).
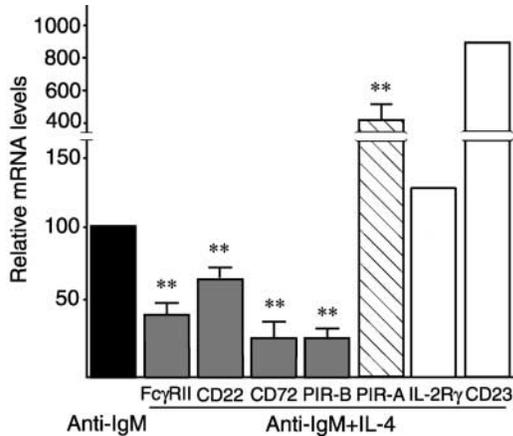
Figure 2. IL-4 reduces mRNA expression of FcγRII, CD22, CD72, and PIR-B, and increases PIR-A. B cells were cultured with anti-IgM F(ab')₂ with or without IL-4 for 24 h, RNA isolated and quantified relative to GAPDH by real time RT-PCR. Shown is the mean (± 1 SD) of a number of independent experiments (FcγRII, five experiments; CD22, four experiments; CD72, six experiments; PIR-B, four experiments; and PIR-A, three experiments). These means were significantly different from levels in B cells cultured with anti-IgM F(ab')₂ alone (**$P < 0.005$, Mann-Whitney U test). CD23 mRNA levels are shown as a positive control and IL-2Rγ chain levels are shown as an example of an mRNA little influenced by IL-4.

expression (Fig. 1 A). In addition, a similar reduction in PIR–B mRNA was observed (Fig. 2). Moreover, PIR–A mRNA levels were increased by IL-4, and the PIR–A/B ratio increased 17-fold. The relative ratio of PIR–A and B in the membrane is thought to determine their net activatory effect (8), as is true for a number of paired inhibitory/activatory receptors, and thus this reciprocal regulation of their expression would shift the B cell from an inhibitory toward an activatory state.

The Effect of IL-4 on Inhibitory Receptor Expression Is Predominantly Mediated through Stat6. Stat6 signaling is known to mediate IL-4's control of the expression of MHC II, CD23, and the IL-4R, but the mechanism by which Stat6 contributes to IL-4–mediated proliferation is unclear. Experiments in Stat-6–deficient mice demonstrated that the reduction in inhibitory receptor expression caused by IL-4 is dependent on signaling through Stat6 (Fig. 3).

Possible mechanisms of IL-4/Stat6 suppression of inhibitory receptors include the production of inhibitory intermediates such as the suppressors of cytokine signaling (SOCS) proteins (20) or competition with nuclear factor (NF)–κB (21). The promoter regions of the inhibitory receptors have not been exhaustively examined (but see FcγRII, [11]; CD22, [22]; CD72, [4]; PIR–B, [23]). Nonetheless, there are a number of putative Stat binding elements upstream of the start sites of all four inhibitory receptors examined in this study, though consensus sequences typical of those thought to preferentially bind Stat6 (24) are only seen in CD22 and PIR–B. This raises the additional possibilities that Stat6 could operate either
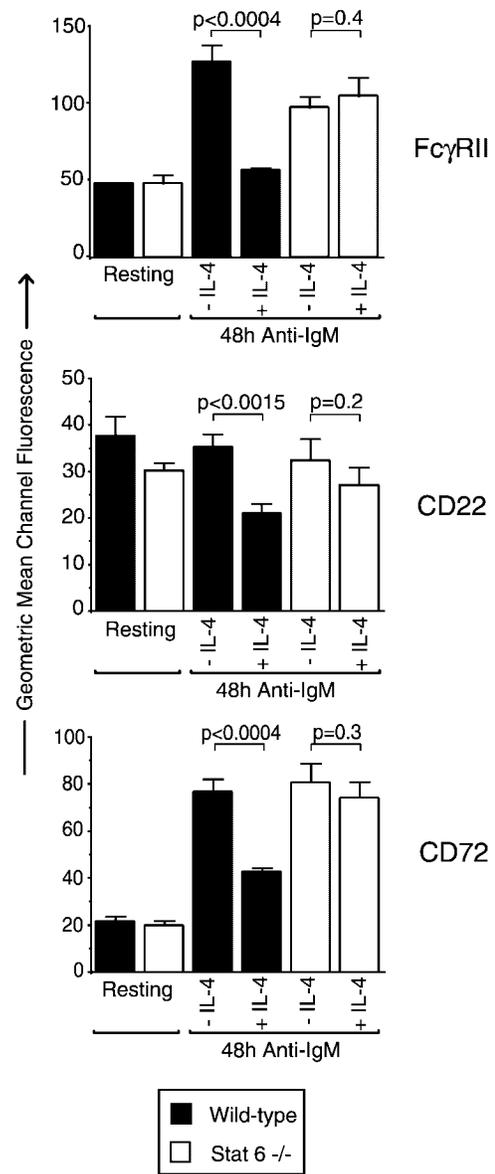
Figure 3. The reduction in inhibitory receptor expression caused by IL-4 is mediated by Stat6. B cells were activated by anti–IgM F(ab')₂ with or without IL-4 in C57BL/6 mice and in Stat6-deficient mice on the C57BL/6 background. Average geometric mean channel fluorescence ± 1 SD of FcγRII, CD22, and CD72 on B cells from three individual mice cultured for 48 h is shown. Representative one of two experiments shown. Significant reductions in inhibitory receptor expression were seen in B cells from control mice, but not in Stat⁻/⁻ mice (Mann-Whitney U test).

by direct binding to, or by competing with, other Stat family members.

IL-4 Abolishes the Inhibitory Effect of FcγRII Ligation on B Cell Activation. When the BCR is cross-linked by anti-IgM F(ab')₂, B cells proliferate. Coligation of FcγRII into this BCR complex, for example by stimulation with intact instead of F(ab')₂ anti-IgM IgG, reduces this proliferation. IL-4 has been reported to overcome this inhibition (25). We confirmed and extended these findings, demonstrating that stimulation by intact IgG increased the threshold for B

A

B
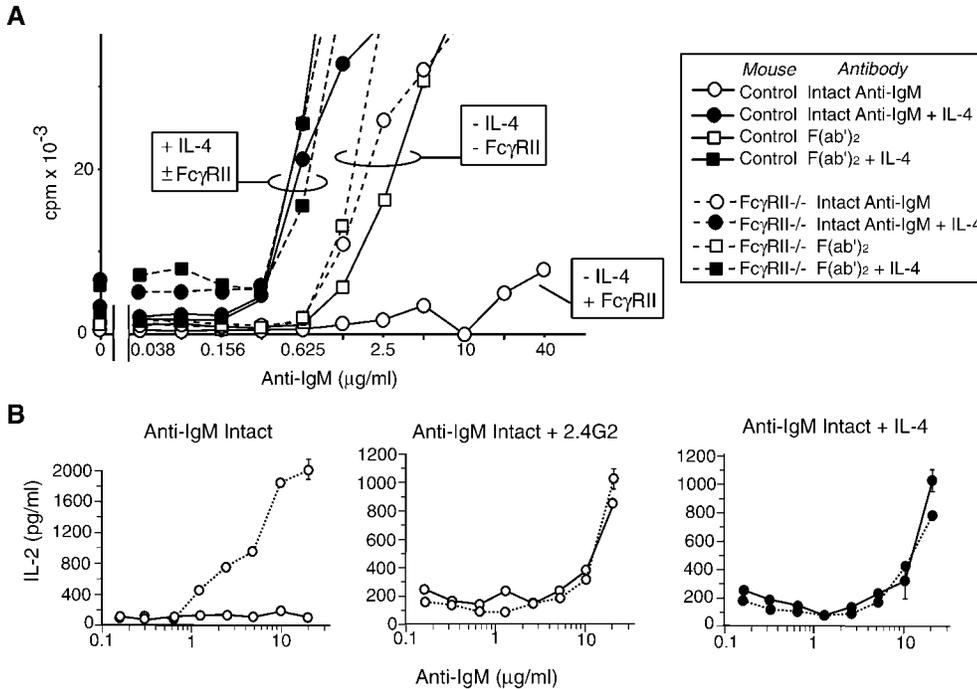
Figure 4. IL-4 abolishes Fc-γRII-mediated suppression of B cell activation and antigen presentation. (A) Activation threshold: B cells from control and FcγRII-deficient mice were stimulated with F(ab′)₂ or intact anti-IgM antibody in the presence or absence of IL-4. In the absence of IL-4, recruitment of FcγRII by intact anti-IgM results in an increased activation threshold. This is not seen if FcγRII is not engaged – when either F(ab′)₂ anti-IgM or FcγRII$^{-/-}$ B cells are used. When B cells are activated in the presence of IL-4, however, no inhibitory effect of FcγRII is seen (one of three experiments shown). (B) Antigen presentation: B cells were stimulated for 24 h with F(ab′)₂ or intact anti-IgM antibody with or without IL-4 or 2.4G2, then incubated with the T cell hybridoma DO11.10 and soluble OVA. Antigen presentation by B cells to the T cell line resulted in IL-2 production, measured by ELISA. Antigen presentation is suppressed in control B cells stimulated with intact anti-IgM when compared with FcγRII$^{-/-}$ B cells (left panel). This suppression is mediated by FcγRII, as it does not occur when FcγRII is blocked by the addition of 2.4G2 (central panel). The addition of IL-4 (right panel) reverses the suppression of antigen presentation by intact anti-IgM as effectively as 2.4G2.

cell activation, and that this increase was dependent on Fc-γRII as it did not occur in FcγRII-deficient mice. In the presence of IL-4 the activation threshold was no longer increased by recruitment of FcγRII (Fig. 4 A). IL-4 also induces a small but consistent reduction in activation threshold that is independent of FcγRII.

IL-4 Abolishes the Inhibitory Effect of FcγRII Ligation on B Cell Antigen Presentation. Activated B cells can present antigen to T cells, and such presentation is likely to be important in maintaining T cell help to promote ongoing B cell responses, and in maintaining an effective T_H2 response. B cell antigen presentation is increased in the presence of costimulation through the BCR and this increase is reduced by cross-linking FcγRII to the BCR (26). We confirmed that, compared with activation by anti IgM F(ab′)₂, FcγRII recruitment by intact anti-IgM reduced MHC II-restricted presentation of fluid-phase OVA to the T cell hybridoma DO11.10. This inhibition was FcγRII dependent, as it was not seen if FcγRII was blocked by the mAb 2.4G2, nor in FcγRII-deficient mice (Fig. 4 B). The addition of IL-4 reversed this inhibition: antigen presentation occurred as effectively in B cells stimulated by intact anti-IgM in the presence of IL-4 as it did in those stimulated by anti-IgM F(ab′)₂ alone (Fig. 4 B).

IL-4 Abolishes the Effect of CD22 Sequestration on B Cell Intracellular Calcium Flux. Coligation of CD22 to the BCR results in suppression of B cell activation (27), and conversely sequestration of CD22 away from the BCR results in a reduced activation threshold (28) and enhanced [Ca²⁺]i flux (29). We examined the effect of CD22 seques-

tration on [Ca²⁺]i concentrations. Upon BCR cross-linking with anti-IgM F(ab′)₂, an exaggerated [Ca²⁺]i response was seen in those cells in which CD22 had been sequestered (Fig. 5). In cells which had been cultured in IL-4 for 48 h an enhanced response was seen whether or not CD22
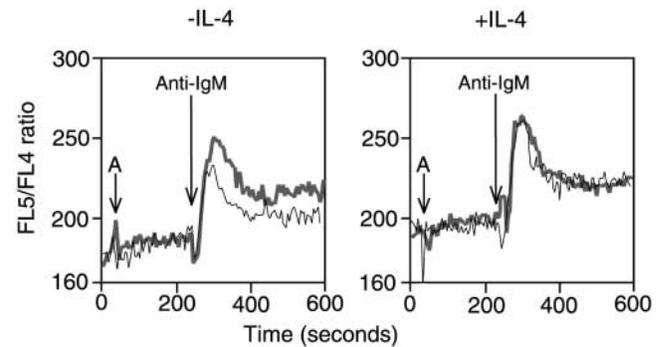
Figure 5. IL-4 abolishes CD22-mediated inhibition of [Ca²⁺]i flux after stimulation through the BCR. B cells were stimulated for 48 h with LPS either with (right panel) or without (left panel) IL-4, then loaded with Indo-1 and stained with biotinylated anti-CD22 antibody. Changes in FL5/FL4 fluorescence ratio are proportional to [Ca²⁺]i and were measured by flow cytometry. After 30 s (A) either Kreb's Ringer phosphate solution/BSA (thin line) or avidin 1 mg/ml (thick line) was added. After a further 210 s anti-IgM F(ab′)₂ was used to cross-link the BCR. Additional sequestration of CD22 with avidin released the B cells from inhibition, as seen by increased [Ca²⁺]i (left panel). In cells activated in the presence of IL-4, sequestration of CD22 had no effect on [Ca²⁺]i, indicating no discernible suppression of [Ca²⁺]i by CD22. Representative of three experiments.

was cross-linked, indicating that the suppressive effect of CD22 was abolished by IL-4.

The IL-4–mediated decrease in inhibitory receptor expression and function provides a novel mechanism by which IL-4 may enhance B cell activation. The reduction would be expected to have a functional effect for at least three reasons. First, the reduction in inhibitory receptors caused by IL-4 is of the same degree as that seen in mice heterozygous for deletions in individual inhibitory receptors, which results in a significant phenotype (FcγRII; J. Ravetch, personal communication, CD22; reference 12). Second, it would be expected that a quantitatively similar reduction in all four inhibitory receptors on activated B cells would have an additive or synergistic effect on B cell function, as has been demonstrated in mice heterozygous for CD22, lyn, and Shp-1 (14). Third, IL-4 completely abolished the inhibitory effects of both CD22 and FcγRII on B cell activation, and of FcγRII on antigen presentation. This implies that continuous T cell production of IL-4 can override inhibitory signals, consistent with, though not directly implicating, the observed reduction in their expression.

Thus, the reduction in inhibitory receptor expression and function brought about by IL-4 could enhance T cell driven B cell activation early in "undifferentiated" immune responses (Th0), and could drive such activation in a more prolonged fashion in Th2 responses. Reduction in negative regulation by IL-4 may also help recruit ongoing T cell help by permitting continued antigen presentation by B cells not subject to FcγRII-mediated feedback. This amplifying effect could account for the IL-4–dependent production of very large amounts of IgG1 and IgE seen in Th2 responses, and in particular in allergy and in chronic parasitic infections (15).

IL-4 could predispose to autoimmune disease, as it abolishes CD22- and FcγRII-mediated inhibition, mice deficient in either of these are prone to autoimmunity (2, 10), and the observed reduction in four different inhibitory receptors are likely to have at least additive effects. Once tolerance had been breached, IL-4 production could override FcγRII-mediated feedback mechanisms allowing continued production of autoantibody and the recruitment of further T cell help. These possibilities are supported by studies demonstrating that transgenic overexpression of IL-4 leads to an SLE-like illness in mice (30).

IL-4 reduces the expression of FcγRII, CD22, CD72, and PIR-B, all ITIM-containing inhibitory receptors, on activated B cells. In addition, IL-4 reverses the inhibitory effect of CD22 on B cell activation, and of FcγRII on activation threshold and antigen presentation. This suggests a new role for IL-4 in the T cell dependent B cell response – in removal of ITIM-mediated suppression rather than solely in direct activation.

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