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J Immunol 2006; 176:819-826; doi: 10.4049/jimmunol.176.2.819
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Progesterone-Induced Blocking Factor Activates STAT6 via Binding to a Novel IL-4 Receptor

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Progesterone-induced blocking factor (PIBF) induces Th2-dominant cytokine production. Western blotting and EMSA revealed phosphorylation as well as nuclear translocation of STAT6 and inhibition of STAT4 phosphorylation in PIBF-treated cells. The silencing of STAT6 by small interfering RNA reduced the cytokine effects. Because the activation of the STAT6 pathway depends on the ligation of IL-4R, we tested the involvement of IL-4R in PIBF-induced STAT6 activation. Although PIBF does not bind to IL-4R, the blocking of the latter with an Ab abolished PIBF-induced STAT6 activation, whereas the blocking of the IL-13R had no effect. PIBF activated suppressor of cytokine signaling-3 and inhibited IL-12-induced suppressor of cytokine signaling-1 activation. The blocking of IL-4R counteracted all the described effects, suggesting that the PIBF receptor interacts with IL-4R α-chain, allowing PIBF to activate the STAT6 pathway. PIBF did not phosphorylate Jak3, suggesting that the γ-chain is not needed for PIBF signaling. Confocal microscopic analysis revealed a colocalization and at 37°C a cocapping of the FITC PIBF-activated PIBF receptor and PE anti-IL-4R-labeled IL-4R. After the digestion of the cells with phosphatidylinositol-specific phospholipase C, the STAT6-activating effect of PIBF was lost, whereas that of IL-4 remained unaltered. These data suggest the existence of a novel type of IL-4R composed of the IL-4R α-chain and the GPI-anchored PIBF receptor. The Journal of Immunology, 2006, 176: 819–826.

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1 This work was supported by Grant OTKA T031737 from the Hungarian National Research Fund, Grant ETT 045/2003 from the Hungarian Ministry of Health, Grant NKFP 1A-057/2004 from the National Research and Development Program, Grant GVOP-3.1.1.-2004-05-0329/3.0 from the Economic Competitiveness Operative Program, and by the Hungarian Academy of Sciences. Most authors are members of a European Network of Excellence on Embryo Implantation Control supported by the European Commission. This paper is Contract No. 512040.

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3 Abbreviations used in this paper: PIBF, progesterone-induced blocking factor; SOCS, suppressor of cytokine signaling; PI-PLC, phosphatidylinositol-specific phospholipase C; siRNA, small interfering RNA; SCR, scrambled.

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0022-1767/06/$02.00
Materials and Methods

Abs and PIBF

The 48-kDa N-terminal recombinant human PIBF was prepared as described earlier (3) and is referred to as PIBF throughout our report. Polyclonal anti-PIBF Abs were generated by immunizing rabbits with the 48-kDa N-terminal recombinant human PIBF. IgG was affinity purified on protein A columns.

Monoclonal anti-human IL-4Rα as well as goat anti-human IL-13Rα1 Abs (all from R&D Systems) were used for treating lymphocytes. Mouse IgG2A-specific Ig (Sigma-Aldrich), and rabbit IgG-specific and goat IgG-specific Abs (both from DakoCytomation) were used as isotype controls. Rabbit polyclonal phospho-specific (Tyrr611) anti-human STAT6, phospho-specific (Tyrr612/Tyr613) anti-human Jak1, phospho-specific (Tyrr612) anti-human Jak3, anti-human SOCS-1, anti-human SOCS-3 Abs (all from Santa Cruz Biotechnology), rabbit polyclonal phospho-specific (Tyrr612) anti-human STAT4 Ab (Zymed Laboratories), and PE-labeled anti-CD45RA (BD Biosciences), and FITC-conjugated PIBF (prepared in our laboratory), PE-labeled mouse, anti-human CD45RA (BD Biosciences), and PE-labeled anti-tubulin were used for Western blotting. Polyclonal rabbit anti-human STAT6, anti-human Jak1, anti-human STAT4 Abs (all from Santa Cruz Biotechnology), and rabbit anti-human β-actin (Sigma-Aldrich) were used for controlling the loading on Western blots. Polyclonal rabbit anti-human STAT6 Ab recommended for gel supershift studies (Santa Cruz Biotechnology) was used for EMSA supershift. Goat polyclonal anti-human IL-4 (R&D Systems), mouse monoclonal anti-human IL-4Rα, HRP-labeled anti-goat IgG (both from DakoCytomation), streptavidin-biotin-otin-HRP (Amersham Biosciences), and polyclonal biotin-conjugated anti-PIBF Abs (prepared in our laboratory) were used for ELISA. Mouse monoclonal anti-human IL-4Rα, PE-labeled rat anti-mouse IgG2A and IgG2B (BD Biosciences), FITC-conjugated PIBF (prepared in our laboratory), PE-labeled mouse, anti-human CD45RA (BD Biosciences), and PE-labeled rat anti-mouse IgG1 (BD Biosciences) were used for immunofluorescence confocal microscopy. PIBF-FITC and mouse monoclonal anti-human IL-4Rα Abs were used for determining the receptor binding of PIBF by flow cytometry.

Treatment of lymphocytes

Ficoll-Paque (Pharmacia) isolated PBL from healthy volunteers were washed in RPMI 1640 medium (Invitrogen Life Technologies), treated with 1 μg/ml PHA (Sigma-Aldrich) for 24 h, and then incubated at 37°C in 5% CO2 with the following: RPMI 1640 medium; 10, 20, and 200 ng/ml and 0.5, 1, and 10 μg/ml PIBF for 5 × 103 cells; PIBF, progestrone (Sigma-Aldrich) or recombinant human IL-4 (R&D Systems) for 1, 10, 20, and 30 min and 24 h; recombinant human IL-4 plus recombinant human IL-12 (both from R&D Systems), or IL-4 plus rIL-12 plus monoclonal anti-IL-4Rα Ab; PIBF plus polyclonal anti-PIBF Ab, or PIBF plus monoclonal anti-IL-4Rα Ab, or PIBF plus rIL-12 plus monoclonal anti-IL-4Rα Ab, or PIBF plus rIL-12 plus monoclonal anti-IL-13Rα1 Ab; rIL-12 plus PIBF or PIBF plus rIL-12 plus recombinant human IL-4; lysate of Escherichia coli that had undergone the same purification procedure as the recombinant human PIBF in a concentration of 200 ng/ml, and isotype controls used with each treatment. 5×106 cells were end-labeled with [32P]ATP using T4 polynucleotide kinase (Promega) according to the manufacturer’s protocol. Binding was performed at room temperature for 30 min in 20 μl volume, containing 300 ng of poly(deoxyinosinic-deoxyctydilic acid), 100 mM NaCl, 0.25 mM EDTA, 1 mM HEPES (pH 7.9), and 20 fmol 32P-labeled oligonucleotide probe. For supershift assays the reaction mixtures were preincubated with 1 μl of rabbit anti-human STAT6 IgG (2 μg/ml) for 1 h at 4°C before adding the 32P-labeled oligonucleotide. Samples were separated on 3.5% acrylamide gel with 0.5 × Tris-borate-EDTA buffer. The gel was dried and exposed to x-ray film.

Phospho-tyrosinol-specific phospholipase C (PI-PLC) treatment

A total of 5 × 106 PHA treated lymphocytes was incubated with 2.5 μg/ml PI-PLC (Sigma-Aldrich) in 1 ml of PBS for 30 min at 37°C in 5% CO2, and washed twice with PBS.

ELISA for detecting PIBF binding to IL-4R

During an overnight incubation at 4°C, 96-well microtiter plates were coated with 1 and 0.5 μg/ml soluble recombinant human IL-4Rα (R&D Systems) in 50 mM carbonate buffer (pH 9.6). All further incubations were performed at 37°C. Plates were washed three times and free binding sites were blocked with PBS containing 0.05% Tween, 0.5% gelatin, and 0.1% BSA for 60 min. Logarithmic dilutions (0.01–1 μg/ml) of PIBF or rIL-4 in PBS (pH 7.4) were incubated on the plates for 60 min. The plates were washed three times and incubated with 1/1000 diluted biotin-conjugated anti-PIBF IgG or 1/1000 diluted goat anti-IL-4 for 60 min. Following three further washing cycles, biotin-conjugated anti-PIBF IgG was reacted with 1/1000 diluted streptavidin-biotin-HRP for 30 min, while anti-IL-4 Ab was reacted with 1/2000 diluted HRP-labeled anti-goat IgG for 60 min. The reaction was developed with O-phenylenediamine (FLUKA; Sigma-Aldrich) and stopped by adding 50 μl of 4 M H2SO4. Absorbance was read at 490 nm.

Determination of receptor binding of PIBF by flow cytometry

One million PBL from healthy volunteers were incubated with 2 μg/ml FITC-conjugated PIBF in the presence of increasing concentrations (0–200 μg/ml) of unlabeled PIBF or monoclonal anti-IL-4Rα Ab for 30 min at 4°C. The cells were washed in PBS, fixed in 0.5% formalin and analyzed by flow cytometry, using a FACSCalibur flow cytometer equipped with a 488-nm excitation laser with the CellQuest software program (both from BD Biosciences).

Confocal microscopy

One million PBL from healthy volunteers were incubated with 5 μg of FITC-conjugated PIBF for 20 min at 37°C. A total of 1 × 106 cells was plated on poly-r-lysine-coated slides and incubated at 37°C for further 10 min. After washing, the plates were treated with 1% TCS and fixed with freshly prepared 3% paraformaldehyde in PBS at 10 min at room temperature. After washing, the plates were incubated with 0.5 μg of monoclonal anti-IL-4Rα or 2 μl of PE-labeled mouse anti-CD45RA Ab for 45 min at each) in TBS-Tween (pH 7.4), the blots were incubated at room temperature for 45 min with 1/2000 diluted HRP-labeled anti-rabbit IgG, and then washed six times (10 min each) in TBS-Tweed. Ab binding was detected using an ECL kit (PerkinElmer) according to the manufacturer’s instructions.

EMSA supershift

Nuclear extracts were prepared as described by Xu and Cooper (23). Lymphocytes were washed twice in ice-cold PBS and resuspended in 10 volumes of buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, protease inhibitors (complete, Mini EDTA-plates; Boehringer Mannheim), phosphatase inhibitors (Phosphatase Inhibitor Cocktail; Sigma-Aldrich) and placed on ice for 10 min. After vigorous vortexing, nuclei were collected by centrifugation in microcentrifuge at 10,000 rpm for 10 s, resuspended in 2 volumes of buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitors, and phosphatase inhibitors and incubated on ice for 20 min. After centrifugation (10,000 rpm for 10 s) supernatants were collected and the protein content of the extracts was determined, aliquoted, and stored at −80°C. For the shift assay, STAT6 binding probes (5′-TCGACTTCCCCAAAGACAGCA-3′) and their reverse complementary pairs were incubated at 80°C for 10 min in annealing buffer containing 100 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA and allowed to cool down slowly to room temperature. Double-stranded oligonucleotide was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega) according to the manufacturer’s protocol. Binding was performed at room temperature for 30 min in 20 μl volume, containing 300 ng of poly(deoxyinosinic-deoxyctydilic acid), 100 mM NaCl, 0.25 mM EDTA, 1 mM HEPES (pH 7.9), and 20 fmol 32P-labeled oligonucleotide probe. For supershift assays the reaction mixtures were preincubated with 1 μl of rabbit anti-human STAT6 IgG (2 μg/ml) for 1 h at 4°C before adding the 32P-labeled oligonucleotide. Samples were separated on 3.5% acrylamide gel with 0.5 × Tris-borate-EDTA buffer. The gel was dried and exposed to x-ray film.
room temperature. Cells were washed twice and incubated with 2 µl of PE-labeled rat anti-mouse IgG2A and IgG2B or PE-labeled rat anti-mouse IgG1 for 30 min at room temperature. Cells were washed twice and the slides were mounted with DABCO (Sigma-Aldrich). To control the specificity of capping formation, all steps were also performed at 4°C. The slides were analyzed with a Bio-Rad confocal microscope with ×100 objective, using laser excitation at 473 nm with filters 580 ± 16 nm for PE and 522 ± 17.5 nm for FITC. Images were analyzed using the Adobe Photoshop 7.0 software.

RNA interference

Oligonucleotides were hand-designed to interfere exclusively with STAT6 mRNA (Ambion). As a negative control, the same nucleotides were scrambled to form a nongenomic combination (controlled by basic local alignment search tool). Oligonucleotide sequences used for STAT6 RNA interference were target sequence for STAT6 AAG CAG GAA GAA CTC AAG TTT and target sequence scrambled AAA CGA GAG TGT TAT AAC TGT.

Cells were washed twice with OptiMEM (Invitrogen Life Technologies). Oligonucleotides were dissolved in RNase-free water, annealed according to the guidelines provided by the manufacturer, and incubated for 20 min at room temperature with Oligofectamine (Invitrogen Life Technologies). This mixture was added dropwise to the cells, until a concentration of 66 nM was reached. After 4 h incubation at 37°C, DMEM containing 30% FCS was added to cultures. Western blots for STAT6 expression were performed 24 h subsequent to small interfering (si)RNA transfection. STAT6 expression was markedly reduced, whereas STAT2 and STAT4 expressions were not affected (see Fig. 2A).

Lymphocyte cultures

Intact lymphocytes, those treated with scrambled oligonucleotides and those after RNA interference were adjusted to 10^6 cells/ml in RPMI 1640 with 10% FCS, and cultured for 48 h at 37°C, 5% CO2 in a humidified atmosphere together with 0.2 or 10 µg/ml PIBF or without PIBF. Supernatants were harvested for cytokine determination. Lymphocytes were lysed for Western blot analysis.

Cytometric bead arrays

IL-10, TNF-α, and IFN-γ concentrations were determined by Cytometric Bead Array (CBA; BD Biosciences or Bender MedSystems). Supernatants were incubated with labeled capture beads and detection reagent for 3 h in the dark at room temperature, and analyzed with flow cytometer (FACS-Calibur; BD Biosciences for BD arrays or alternatively Galaxy; DakoCytomation) by using the respective CBA Analysis software (BD Biosciences) and Bender MedSystems software.

Statistical analysis

The two-tailed Student’s t test was used for statistical evaluation of the data. Differences were considered significant if the value for p ≤ 0.05.

Results

PIBF activates STAT6

To test whether the previously described cytokine effects of PIBF were mediated by the Jak/STAT pathway, cytoplasmic fractions of PIBF-treated or untreated human PBL were separated on SDS-PAGE, blotted to nitrocellulose membranes, and reacted with anti-phospho-STAT6 Abs. Controls included the lysate of E. coli that had undergone the same purification procedure as the recombinant PIBF, as well as isotype controls. Similar to IL-4, PIBF induced STAT6 phosphorylation, which was inhibited by a neutralizing anti-PIBF IgG (Fig. 1A). To clarify whether PIBF induces nuclear translocation of phosphorylated STAT6 dimers, nuclear extracts prepared from PIBF-treated cells, and those from IL-4-treated as

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** PIBF activates STAT6. A, PIBF treatment induces STAT6 phosphorylation. Cytoplasmic fractions of untreated human PBL, and those treated for 20 min with PIBF or PIBF plus anti-PIBF IgG, were separated on SDS-PAGE, blotted to nitrocellulose membrane, and reacted with anti-phospho-STAT6 Abs. B, PIBF treatment induces nuclear translocation of STAT6. Nuclear extracts from untreated human PBL, and those treated for 20 min with PIBF or IL-4 were hybridized with radioactive labeled STAT6 binding specific oligonucleotide probes 5’-TCGACTCTCCAAAGAACAGCA-3’ and their reverse complementary pairs. The samples were separated by nondenaturing PAGE and the bands were detected by autoradiography. Supershift was performed with anti-STAT6 Ab. C, Time-dependence of STAT6 phosphorylation. STAT6 phosphorylation was tested on lysates of lymphocytes incubated with 200 ng/ml PIBF (b), IL-4 (a), or progesterone (c) for varying periods. D, Concentration dependence of STAT6 activation by PIBF. STAT6 phosphorylation was tested on lysates of lymphocytes incubated for 20 min with different concentrations of PIBF. Western blots were reacted with anti-phospho-STAT6 (top) or anti-STAT6 Ab (bottom).
well as untreated lymphocytes were hybridized with labeled STAT6 binding, specific oligonucleotide probes and their reverse complementary pairs. PIBF induced nuclear translocation of STAT6 in lymphocytes. The specificity of the reaction was verified with a supershift assay. This result is based on anti-STAT6 IgG-STAT6 forming high molecular mass complexes, which migrate slower than STAT6 alone.

In the presence of anti-STAT6 IgG, a supershifted complex appeared in the extract of PIBF-treated lymphocytes. This band was not detectable when the irrelevant anti-NF-κB Ab was used (Fig. 1B).

To investigate the concentration- and time-dependence of PIBF effects, STAT6 phosphorylation was tested on lysates of lymphocytes incubated with different concentrations of PIBF or with 200 ng/ml PIBF for varying periods. Tyrosine phosphorylated STAT6 appeared as early as 1 min after addition of IL-4 (Fig. 1C, a) or PIBF (Fig. 1C, b) to the cells, whereas a 24-h continuous presence of progesterone was required for the same effect (Fig. 1C, c). PIBF in concentrations from 10 ng to 10 μg/ml exerted a concentration-dependent effect on STAT6 phosphorylation (Fig. 1D). Our earlier data show that during normal pregnancy PIBF concentrations in urine reach 100–300 ng/ml, serum PIBF levels of the same women being usually higher. In patients with malignant tumors, PIBF levels are more variable (100–1000 ng/ml in urine). Based on these data, we chose 200 ng/ml for further treatments as a concentration relevant to in vivo biological situations.

**FIGURE 2.** STAT6 is required for the cytokine effects of PIBF. The effect of PIBF on cytokine production by STAT6-deficient (siRNA) lymphocytes. Intact lymphocytes, those treated with scrambled oligonucleotides, and those after RNA interference were adjusted to 10^6 cells/ml in RPMI 1640 with 10% FCS and cultured for 48 h at 37°C, 5% CO2 in a humidified atmosphere together with 0.2 or 10 μg/ml PIBF, or without PIBF. Cytokines were determined from the supernatants by cytometric bead assay. For controlling the effect of possible LPS contamination in recombinant PIBF, parallel lymphocyte samples, and those after RNA interference were adjusted to 10^6 cells/ml in RPMI 1640 with 10% FCS and cultured for 48 h at 37°C, 5% CO2 in a humidified atmosphere together with 0.2 or 10 μg/ml PIBF, or without PIBF. Cytokines were determined from the supernatants by cytometric bead assay. For controlling the effect of possible LPS contamination on cytokine production. These had no effect on IL-10 and IFN-γ production, although they induced a slightly increased TNF-α production. The data shown are corrected for E. coli lysate control. Data are the mean ± SEM of nine individual experiments. Significantly different (*, p < 0.05) values from controls are shown. A. The efficiency and specificity of STAT6 depletion by siRNA. Western blots of lysates from siRNA-treated cells were reacted with anti-STAT6, anti-STAT4, or anti-STAT2. A representative experiment is shown. B. The effect of PIBF on IL-10 production. C. The effect of PIBF on TNF-α production. D. The effect of PIBF on IFN-γ production.

**FIGURE 3.** PIBF-induced activation of the STAT6 pathway is mediated by IL-4R. STAT6 signaling is initiated by the engagement of IL-4R. Assuming that PIBF might be a ligand of IL-4R, the STAT6 activating effect of PIBF was tested in lymphocytes pretreated with blocking concentrations of anti-IL-4R or anti-IL-13R Ab. The blocking of the IL-13R had no effect, but the use of a blocking anti-IL-4R mAb counteracted PIBF-induced STAT6 activation (Fig. 3A). This was, however, not due to the inhibition of the binding of PIBF to IL-4R because we could not demonstrate PIBF binding to IL-4R by ELISA (Fig. 3B), nor did anti-IL-4R treatment prevent PIBF binding to its own receptor (Fig. 3C). STAT6 phosphorylation by PIBF was not affected by simultaneous anti-IL-4 treatment (Fig. 3D). PIBF induces phosphorylation of the IL-4Rα-associated Jak1 (Fig. 4A), but not that of the γ-chain-associated Jak3 (Fig. 4B), and the former effect is inhibited by anti-IL-4R Ab (Fig. 4A).

Both PIBF and IL-4 treatment resulted in activation of SOCS-3, and blocking of IL-4R abolished the effect (Fig. 5A). IL-12-induced SOCS-1 activation was inhibited by PIBF, and the latter effect was abolished by blocking IL-4R (Fig. 5B). SOCS-3 inhibits IL-12 signaling, which explains the finding that IL-12-induced STAT4 phosphorylation disappeared after PIBF treatment in the absence, but not in the presence of anti-IL-4Rα Ab (Fig. 5C). Treatment of the cells with anti-IL-12 Ab inhibited STAT4 phosphorylation, suggesting that the freshly isolated PBL were under IL-12 effect (Fig. 5D).
**FIGURE 3.** PIBF-induced STAT6 activation is mediated by IL-4R. A. The effect of IL-4R block on PIBF-induced STAT6 phosphorylation. Lysates of untreated human lymphocytes, and those treated for 20 min with PIBF, PIBF and anti-IL-4Rα, or PIBF and anti-IL-13R Ab, are reacted with anti-phospho-STAT6 (top) or anti-STAT6 (bottom) Ab. B. Binding PIBF to IL-4R ELISA plates coated with IL-4Rα were reacted with increasing concentrations of PIBF or IL-4, and subsequently with anti-PIBF and anti-IL-4, or anti-IL-4 and anti-IL-13R Ab, and those treated for 20 min with PIBF, PIBF and anti-IL-4, IL-4, IL-4 plus anti-IL-4, or anti-IL-4 alone are reacted with anti-phospho-STAT6 (top) or anti-STAT6 (bottom) Ab.

Functional association of PIBF receptor and IL-4R with PIBF receptor as a GPI-anchored protein

Our data suggest that the PIBF receptor might form a heterodimer with IL-4Rα to activate the STAT6 pathway. Confocal microscopy analysis of PE anti-IL-4Rα and FITC PIBF-labeled cells revealed a cocapping of the two receptors, when PIBF labeling was done at 37°C (Fig. 6A, top panel), whereas PE anti-CD45 and FITC-PIBF did not colocalize (data not shown). Six cocapping experiments on PBL from different healthy individuals under the same conditions gave similar results. Typically cocapping was observed on 18% of the cells, and 1.7% of lymphocytes reacted with IL-4Rα-PE only, whereas the ratio of single FITC PIBF-positive cells was negligible.

No capping was seen in cells incubated at 4°C with the Abs (Fig. 6A, bottom panel).

Because IL-4Rα was a requirement for PIBF signaling, we tested whether PIBF receptor was a GPI-anchored protein. Digesting the putative anchoring region with PI-PLC resulted in a loss of STAT6 activation by PIBF, whereas the same effect of IL-4 remained unaltered (Fig. 6B).

**Discussion**

In this study we suggest the existence of a new type of IL-4R, in which the α-chain of the IL-4R is complemented by the GPI-anchored PIBF receptor. The treatment of human PBL with low concentrations of PIBF results in immediate phosphorylation and nuclear translocation of STAT6. STAT6 activation is dependent on ligation of IL-4R (21). This implies that for activating STAT6, PIBF needs to interact with IL-4R. Indeed, the STAT6 activating effect of PIBF was lost after blocking the IL-4R α-chain by a specific Ab. In contrast, we could not demonstrate a direct binding of PIBF to IL-4Rα, nor could we prevent PIBF from binding to its own receptor by anti-IL-4R treatment. The possibility that the PIBF Ab binds to a region within PIBF that is required for interaction with the IL-4R, thus competing for the same site on PIBF, can be ruled out because in the ELISA we used a polyclonal anti-PIBF IgG, which recognizes multiple epitopes on the PIBF molecule. Furthermore, the assay performed in a reverse order (solid phase bound PIBF reacted with IL-4Rα, revealed with anti-IL-4Rα) gave similarly negative results. Previously we showed (6) that supernatants from spleen cells activated in the presence of PIBF produce significantly more IL-4 than those in the absence of PIBF. The possible scenario in which instead of PIBF, PIBF-induced IL-4 would bind to the IL-4R and phosphorylate STAT6, can also be excluded. As short as a 1 min incubation with PIBF is sufficient to induce the phosphorylation of STAT6. This is too short an interval for gene induction plus the synthesis of IL-4. Another argument against this concept is that treatment of the cells with anti-IL-4 Abs did not inhibit, nor even reduce the effect of PIBF on STAT6 phosphorylation. The blocking of the IL-13R (which consists of an IL-13 binding IL-13R α-chain (25, 26) and IL-4Rα (27) does not exert a similar effect, suggesting that the IL-4R α-chain is needed for the STAT6-inducing effect of PIBF.

Because PIBF does not directly bind to IL-4R, but STAT6 phosphorylation is inhibited by anti-IL-4R Ab, we investigated the effect of IL-4R block on Jak phosphorylation in PIBF-treated cells.

**FIGURE 4.** PIBF phosphorylates Jak1, but not Jak3. A. The effect of PIBF treatment and IL-4R blocking on phosphorylation of Jak1. Lysates of untreated human lymphocytes, and those treated for 20 min with PIBF, IL-4, or PIBF and anti-IL-4Rα Ab cells, are reacted with anti-phospho-Jak1 (top) or anti-Jak1 (bottom) Ab. B. The effect of PIBF treatment on phosphorylation of Jak3. Lysates of untreated human lymphocytes, and those treated for 20 min with IL-4 or PIBF, are reacted with anti-phospho-Jak3 (top) or anti-β-actin (bottom) Ab.
PIBF-induced Jak1 phosphorylation was inhibited by anti-IL-4R Ab treatment, whereas the blocking of IL-13R had no effect.

STAT4 was phosphorylated in untreated cells, and this did not change upon addition of IL-12. In this study we used freshly isolated lymphocytes from healthy individuals, which produce IL-12. The treatment of the cells with anti-IL-12 Ab abolished STAT4 phosphorylation, thus phosphorylation of STAT4 in untreated lymphocytes could be due to IL-12 already present in the system. The STAT4 activating effect of IL-12 was counteracted by PIBF in the absence, but not in the presence of anti-IL-4R Ab. This implies that effects of PIBF (both the positive ones on STAT6 and the negative ones on STAT4) are dependent on the IL-4R/IL-13R-chain.

Ligand binding of IL-4R leads to phosphorylation of Jak1. Jak then phosphorylates STATs, which move into the nucleus and activate the transcription of, among others, genes coding for the SOCS proteins. IL-12/STAT4 and IL-4/STAT6 pathways are under negative feedback regulation by SOCS proteins. SOCS-3 is induced by IL-4 signal, then it binds to IL-12R and inhibits IL-12 signaling (28). Thus, IL-12-induced STAT4 activation is inhibited in Th2 cells that express high levels of SOCS-3, whereas IL-4/STAT6 signaling is constitutively activated in Th2 cells, but not in Th1 cells, with high SOCS-1 expression (16). In our hands, similar to IL-4, PIBF treatment induced SOCS-3 activation, whereas the IL-12-induced SOCS-1 expression disappeared after PIBF or IL-4

PIBF SIGNALING VIA IL-4Rα

FIGURE 5. PIBF induces SOCS-3 and inhibits SOCS-1 induction as well as STAT4 phosphorylation via IL-4Rα. A, The effect of PIBF and anti-IL-4Rα Ab on SOCS-3 induction. Lysates of untreated human lymphocytes, and those treated for 20 min with PIBF, IL-4, PIBF and anti-IL-4Rα, IL-4 and anti-IL-4Rα, or IL-12, are reacted with anti-SOCS-3 (top) or anti-β-actin (bottom) Ab. B, The effect of PIBF and anti-IL-4Rα Ab on IL-12-induced SOCS-1 induction. Lysates of untreated human lymphocytes, and those treated for 20 min with IL-12, IL-12 and PIBF, IL-12 and IL-4, IL-12 and PIBF and anti-IL-4Rα, IL-12 and PIBF and anti-IL-13R, or IL-12 and IL-4 and anti-IL-4Rα Ab are reacted with anti-SOCS-1 (top) or anti-β-actin (bottom) Ab. C, The effect of PIBF and anti-IL-4Rα Ab on STAT4 phosphorylation. Lysates of untreated human lymphocytes, and those treated for 20 min with PIBF, IL-12, IL-12 and PIBF, IL-12 and PIBF and anti-IL-4Rα, or anti-IL-4Rα Ab, are reacted with anti-phospho-STAT4 (top) or anti-STAT4 (bottom) Ab. D, The effect of anti-IL-12 Ab treatment on STAT4 phosphorylation in freshly isolated PBL. Lysates of untreated human PBL, and those treated for 20 min with anti-IL-12 or IL-12, are reacted with anti-phospho-STAT4 (top) or anti-STAT4 (bottom) Ab.

FIGURE 6. The PIBF receptor is a GPI-anchored protein and forms a heterodimer with IL-4Rα. A, Colocalization at 4°C (bottom) and cocapping at 37°C (top) of IL-4Rα and PIBF receptor. View of FITC PIBF (a), PE anti-IL-4Rα (b), and merged (c) are presented. For capping (top), lymphocytes were incubated with FITC-conjugated PIBF for 30 min at 37°C, fixed and reacted with monoclonal anti-IL-4Rα for 45 min at room temperature, followed by incubation with PE-labeled rat anti-mouse IgG2A and IgG2B or PE-labeled rat anti-mouse IgG1 for 30 min at room temperature. Fluorescence and transmission views are merged to show the cellular localization of the receptors. B, PI-PLC treatment results in a loss of STAT6 activation by PIBF. Cell lysates of PI-PLC digested PIBF, IL-4-treated and control untreated (marked as 0) as well as nondigested cells are reacted with anti-phospho-STAT6 (top) or anti-STAT6 (bottom) Ab.
FIGURE 7. PIBF acts on the cytokine balance via a novel type of IL-4R. A, In addition to the classical IL-4R, in which IL-4R α-chain dimerizes with the common γ-chain (1) or the α-chain (2) of the IL-13R, IL-4R α-chain forms a heterocomplex with the PIBF receptor (3). B, Upon ligation, the PIBF receptor associates with IL-4Rα, blocking of the latter suspended all IL-4R-dependent effects of PIBF. Furthermore, with confocal microscopy we demonstrated not only colocalization, but under appropriate conditions, also cocapping of IL-4Rα and the PIBF receptor. Therefore the hypothesis was put forward that, following ligation, the PIBF receptor might either form a heterodimer with the α-chain of the IL-4R, or the engaged PIBF receptor associates with the complete IL-4R, allowing PIBF to activate the STAT6 pathway. To date because of the lack of a PIBF receptor-specific Ab, coccapsulation of the two receptors has not been performed. However, the finding that PIBF treatment induces the phosphorylation of Jak1 (associated with the IL-4R α-chain), but not that of Jak3, which is associated with the α-chain, provides strong indirect evidence for the former and against the latter concept.

Our findings raise the question, why IL-4Rα is needed for PIBF signaling. A plausible explanation would be that the PIBF receptor itself does not possess an intracellular domain; therefore it uses that of IL-4Rα. Several proteins are anchored to membranes via a posttranslational lipid modification, the GPI anchor. Ligation of these proteins by Abs results in signal transduction, despite the fact that these molecules have no transmembrane or intracellular domains. Their signaling capacity is due to the association of these molecules with putative transmembrane proteins that can signal via conventional mechanisms (30).

Testing the hypothesis that the PIBF receptor was a GPI-anchored protein, we digested the anchoring region with PI-PLC. After this treatment, IL-4 was still able to activate STAT6, but PIBF failed to do so, suggesting that a GPI-anchored protein was involved in PIBF signaling. GPI deficiency causes female infertility (12), but not that of Jak3, which is associated with the IL-4Rα-chain (2) of the IL-13R, IL-4Rα-chain dimerizes with the common γ-chain (1) or the α-chain (2) of the IL-13R, IL-4R α-chain forms a heterocomplex with the PIBF receptor (3). B, Upon ligation, the PIBF receptor associates with IL-4Rα, blocking of the latter suspended all IL-4R-dependent effects of PIBF. Furthermore, with confocal microscopy we demonstrated not only colocalization, but under appropriate conditions, also cocapping of IL-4Rα and the PIBF receptor. Therefore the hypothesis was put forward that, following ligation, the PIBF receptor might either form a heterodimer with the α-chain of the IL-4R, or the engaged PIBF receptor associates with the complete IL-4R, allowing PIBF to activate the STAT6 pathway. To date because of the lack of a PIBF receptor-specific Ab, coccapsulation of the two receptors has not been performed. However, the finding that PIBF treatment induces the phosphorylation of Jak1 (associated with the IL-4R α-chain), but not that of Jak3, which is associated with the α-chain, provides strong indirect evidence for the former and against the latter concept.

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The concept that both IL-4Rα and PIBF receptor are required for PIBF signaling is supported by the following: 1) anti-IL-4Rα does not prevent binding of PIBF to its receptor, suggesting that PIBF receptor and IL-4Rα are separate entities; 2) digesting the GPI anchor abolishes PIBF-driven signaling, thus a GPI-anchored protein is required for PIBF signaling; and 3) anti-IL-4Rα Ab inhibits PIBF-induced STAT6 phosphorylation in intact cells showing that PIBF cannot signal via its own receptor without the involvement of IL-4Rα. Taken together, the GPI-anchored PIBF receptor is required, but not sufficient, for PIBF signaling.

Our data suggest the existence of a novel IL-4R (Fig. 7A) in which upon ligation, the PIBF receptor combines with IL-4Rα (Fig. 7B) and induces Jak1 phosphorylation, which in turn, activates STAT6. In STAT6-deficient cells, both the Th2 cytokine-inducing and Th1 cytokine-inhibiting effects of PIBF were reduced. PIBF-induced SOCS-3, through binding to the IL-12R, inhibits STAT4 phosphorylation (Fig. 7C) and Th1 responses.

Disclosures
The authors have no financial conflict of interest.

References