Lyn and Fyn function as molecular switches that control immunoreceptors to direct homeostasis or inflammation

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Immunoreceptors can transduce either inhibitory or activatory signals depending on ligand avidity and phosphorylation status, which is modulated by the protein kinases Lyn and Fyn. Here we show that Lyn and Fyn control immune receptor signaling status. SHP-1 tyrosine 536 phosphorylation by Lyn activates the phosphatase promoting inhibitory signaling through the immunoreceptor. By contrast, Fyn-dependent phosphorylation of SHP-1 serine 591 inactivates the phosphatase, enabling activatory immunoreceptor signaling. These SHP-1 signatures are relevant in vivo, as Lyn deficiency exacerbates nephritis and arthritis in mice, whereas Fyn deficiency is protective. Similarly, Fyn-activating signature is detected in patients with lupus nephritis, underlining the importance of this Lyn–Fyn balance. These data show how receptors discriminate negative from positive signals that respectively result in homeostatic or inflammatory conditions.
The immune system is controlled by a finely tuned network of regulatory mechanisms that maintain homeostasis or can initiate inflammatory responses. An important aspect of regulation comprises immunoreceptor tyrosine-based activation motifs (ITAM)-containing immunoreceptors, such as the T-cell receptors (TCR) and B-cell receptors (BCR), Fc receptors (FcR). ITAMs are defined by two consecutive Yxx[L/I] sequences separated by 6 to 12 amino acids, and are present in the cytoplasmic domains of several transmembrane adapter molecules, such as the common γ subunit of FcR (FcγR), the Igα and Igβ subunits of the BCR, the γ, δ, ε, and ζ subunits of the TCR-associated CD3 complex, and in FcγRIIA. Cellular responses after FcγR triggering depend on ligand avidity. Receptor clustering mediated by high avidity ligand interaction induces phosphorylation on ITAM tyrosine residues by membrane-anchored and receptor-associated Src-family kinases (SFK). Phosphorylated ITAMs are docking sites for recruitment of the tyrosine kinases Syk or Zα70, which launch inflammatory responses and restore homeostasis. However, in case of dysregulation or chronic stimulation, ITAM signal can also result in autoimmune and inflammatory diseases. Both in innate and adaptive immunity, the activation of ITAMs-bearing immune receptors is actively counteracted by the action of ITIM-bearing inhibitory receptors such as FcyRIIB with the ITIM being defined by a single [I/V/L/S]xYxx[L/V] sequence. This regulation generally involves co-aggregation of inhibitory and targeted activated receptors and is promoted through recruitment of relevant phosphatases such as Src homology region 2 domain-containing inositol 5’ phosphatases (SHIP-1 and SHIP-2). In addition to this inhibitory feedback targeting co-aggregated activated receptors, a continuously active inhibitory mechanism generated by ITAM-bearing receptors following low avidity interactions has been described that acts towards a whole array of activating receptors without the requirement for co-aggregation. This mechanism has been named inhibitory ITAM (ITAMI) and is thought to be involved in the maintenance of homeostasis. Various FcRs, such as FcγRI, FcγRIIA, and FcγRIIB can function as such bi-functional receptors to trigger inhibitory signals, a property that can be exploited to reduce the susceptibility to autoimmune and inflammatory diseases.

**Fig. 1** Differential regulation of FcR-ITAM signals by Lyn and Fyn. a After induction of FcγRIIA-ITAMI or ITAM signalling in THP-1-CD14+ FcγRIIA+ cells transfected with indicated siRNAs, immunoprecipitation (IP) and immunobots (IB) were performed with indicated Abs. Quantification of the indicated band using ImageJ software relative to total corresponding protein levels in cell lysates (see Supplementary Fig. 1) is indicated at the bottom of each panel, representing one out of at least three experiments. b Modulation of LPS-mediated IL-8 production by Lyn and Fyn during FcγRIIA-ITAMI induction. THP-1-CD14+ FcγRIIA+ cells transfected with indicated siRNAs were stimulated for indicated time points to induce either ITAMI or ITAM signals followed by stimulation with LPS (10 ng/ml) for 1h. Then, supernatant was collected for cytokine measurement. c Modulation of IL-8 production by Lyn and Fyn during FcγRIIA-ITAMI induction for 18h. Data are presented as the mean ± s.e.m. ***P < 0.001; Student’s unpaired t-test.
and dendritic cells, is associated with a variety of pathologies. Together, these evidences support an important role of SHP-1 in the maintenance of immune homeostasis.

SFKs, such as Lyn and Fyn, are implicated in the initiation of ITAM-receptor-mediated signaling. These kinases are responsible for ITAM phosphorylation upon receptor aggregation leading to Syk recruitment initiating further signal propagation via downstream effectors such as PI3-kinase and phospholipase C-γ. In B cells, Lyn was reported to have both positive and negative roles in BCR-mediated signaling. Aged Lyn-deficient mice have high levels of serum immunoglobulins (including autoantibodies) and their B cells are hyper-responsive to IL-4 and, downstream effectors such as PI3-kinase and phospholipase C-γ, are downregulated Fyn or Lyn expression as concomitant negative roles in BCR-mediated signaling. Lyn and Fyn differentially regulate FcR-ITAM signals in order to address whether SFKs play a role in the switch between ITAMI and ITAM signals following FcR engagement. This ITAM switch involves a capacity to differentially control SHP-1 by shifting its phosphorylation status. These signaling signatures are confirmed in inflammatory and auto-immune diseases involving an imbalance between ITAM and ITAMI signals.

**Results**

Lyn and Fyn differentially regulate FcR-ITAM signals. In order to address whether SFKs play a role in the switch between ITAMI or ITAM signaling, we first downregulated Fyn or Lyn expression in representative human monocytic cell lines by a siRNA strategy. The cells were then stimulated for ITAMi signaling by divalent targeting, or for ITAM signals by multivalent crosslinking of FcγRIIA or FcεRI as described previously. The ITAMI molecular signature was characterized by transient Syk recruitment followed by stable and prolonged SHP-1 recruitment. This required the presence of Lyn but not Fyn. In contrast, multivalent crosslinking of these receptors inducing an ITAM activation signal molecular signature with a stable recruitment of Syk (but not SHP1), required the recruitment of Fyn (Fig. 1a and, Supplementary Figs. 1a and 2a, left panel). Fyn silencing had no effect on ITAMI signaling but reversed the ITAM to an ITAMi function and coordination of Lyn and Fyn in the control of ITAM signaling following FcR engagement.
signature by the recruitment of SHP-1 to the receptors despite their multivalent crosslinking (Fig. 1a and, Supplementary Figs. 1 and 2a, middle panel). Lyn silencing completely abolished the ITAMi signaling without affecting the ITAM activation signal (Fig. 1a and, Supplementary Figs. 1 and 2a, right panel). Syk silencing did not alter the recruitment of Lyn or Fyn to the FcγRIIA (Supplementary Fig. 3a) but affected SHP-1 recruitment under ITAMi conditions as previously shown. Functional consequences of individual SFK silencing were then evaluated. Lyn but not Fyn was essential for the ITAMi-dependent
Fig. 4 Lyn-SHP-1Y536 axis protects mice against lethal nephritis. a Survival curves, b proteinuria, and c serum blood urea nitrogen (BUN) after NTN induction. d Rabbit IgG deposit quantification by immunohistochemistry (IHC) (top panels), haematoxylin & eosin (H&E), and fibrosis Masson’s stain (middle panels) of kidney sections from one representative out of nine mice. Immunostaining for CD11b+ and F4/80+ cells in kidney sections of the indicated mouse lines (bottom panels). e Relative gene expression of indicated cytokines assessed by q-PCR of independent kidney tissue RNA samples. *P < .05, **P < .01, ***P < .001; Mann–Whitney test. Non significant, ns. Data are from at least five mice per group. f Representative photomicrographs of glomeruli stained for phalloidin and p-Syk-Alexa 647. g Representative photomicrographs of glomeruli stained for phalloidin, p-SHP-1Y536-Alexa 647, and p-SHP-1S591-Alexa 488. Scale bars: 200 μm.
FcyRII-mediated inhibition of LPS-induced IL-8 production (Fig. 1b and Supplementary Fig. 2b). By contrast, Fyn but not Lyn was essential for ITAM-dependent cell activation as measured by IL-8 production after multivalent engagement of FcyRIIA (Fig. 1c) or FcεRI (Supplementary Fig. 2c). To address whether other SFK could compensate for ITAM signals, we first silenced both Lyn and Fyn expression in transiently monocytic cell line THP-1-CD14<sup>-</sup>-FcyRIIA<sup>+</sup> This abolished both the ITAMi-signal-inhibiting heterologous TLR4 receptor and the ITAM-mediated IL8 production following crosslinking of FcyRIIA (Supplementary Fig. 4a–c). This was confirmed by Western blot analysis of tyrosine phosphorylated proteins in THP-1 whole cell lysates after ITAMi or ITAM induction (Supplementary Fig. 4g, h) ruling out a compensation by other SFK of Lyn/Fyn absence. In agreement, silencing expression of Hck, Fgr, or both, had no significant effect on ITAMi and ITAM signaling (Supplementary Fig. 4d–h). These results reveal opposing roles of the SFK Fyn and Lyn, transducing, respectively, activating or inhibitory signals depending on the type of ligand interaction.

Distinct SFKs differentially regulate BCR-ITAM signals. To investigate whether other Lyn/Fyn-associated ITAM-bearing receptors could also deliver such opposite signals, divalent or multivalent targeting of BCR were performed using anti-CD79a F(ab′2)2 fragments alone or complexed with anti-κ light chain antibodies in representative lymphocytic cell lines expressing siRNA for Lyn or Fyn. Similar to FcRs, divalent targeting of BCR resulted in typical ITAMi molecular signatures, while multivalent crosslinking led to the expected molecular signature of ITAM activation signal (Fig. 2a and Supplementary Fig. 1b). Furthermore, in agreement with the results obtained with FcRs, BCR-mediated ITAMI signals required Lyn, whereas Fyn was essential for ITAM activation signals. Since Syk is recruited with SHP-1 during the ITAMI signaling, we knocked-down Syk expression by using siRNA to determine its role in SHP-1 phosphorylation under conditions of FcεRIIA-ITAM induction in the presence or absence of ERK, PKC, and PI3K inhibitors<sup>30, 31</sup>. Whereas PI3K and PKC inhibitors completely blocked both SHP-1<sup>SS91</sup> and PKC phosphorylation, the ERK inhibitor had no effect (Fig. 3c and Supplementary Fig. 5b). In addition, both PI3K and PKC inhibitors favored SHP-1<sup>Y536</sup> phosphorylation under conditions of FcεRIIA-ITAM activation signaling, and this preference required the presence of Lyn (Fig. 3c and Supplementary Fig. 5b). Interestingly, silencing of the PKC<α> isofrom also abrogated SHP-1<sup>SS91</sup> phosphorylation under conditions of FcεRIIA multimeric aggregation and favored SHP-1<sup>Y536</sup> phosphorylation (Fig. 3d), which was Lyn-dependent (Fig. 3e). However, PKC<α> silencing had no effect on the phosphorylation status of SHP-1 observed in the absence of Lyn (Fig. 3f). Moreover, PI3K inhibition or PKC<α> silencing did not impair Syk activation following FcεRIIA-ITAM induction in the presence or absence of Lyn or Fyn (Supplementary Fig. 3c). Together, these results indicate that during ITAM-induced activation signals, Fyn inactivates SHP-1 through phosphorylation of the SS91 involving a PI3K–PKCα axis, thereby blocking its activation by Lyn. Our results demonstrate that Lyn is crucial to maintain ITAMi-mediated homeostasis, whereas Lyn is essential for ITAM-mediated cell activation by inducing the PI3K–PKCα signaling axis that inactivates SHP-1 during inflammatory responses for most immunoreceptors.

SFKs differentially control SHP-1 phosphorylation. To address the mechanism by which Lyn regulate ITAMi signaling, we took advantage of our findings that Fyn deletion reverses ITAM activation signals into ITAMi signals to explore a possible link between SHP-1 and Fyn under ITAM-activating configuration. Although Fyn silencing resulted in an inhibitory signal generated by multivalent targeting of FcyRIIA, the silencing of both SHP-1 and Fyn abolished it (Fig. 3a and Supplementary Fig. 5a), indicating that Fyn abrogates an SHP-1-mediated inhibitory signal. Previously, phosphorylation of SHP-1 on Y536 and SS91 residues has been associated with its activation and inactivation, respectively<sup>27, 28, 29</sup>. Hence we analyzed whether Lyn and Fyn could control SHP-1 function through differential phosphorylation of this phosphatase. Stimulation of bone marrow-derived macrophages (BMDM) from FcyRIIA<sup>18</sup> (R131 isoform) mice<sup>29</sup> under ITAMI conditions showed that Lyn induced SHP-1 Y536 phosphorylation. By contrast, receptor multivalent aggregation induced a Lyn-dependent SHP-1 SS91 phosphorylation (Fig. 3b). Under these stimulation conditions if Fyn was absent, a Lyn-dependent Y536 phosphorylation of SHP-1 was observed instead, thus mimicking an ITAMi signal (Fig. 3b). To understand how Fyn, a tyrosine kinase, could promote SHP-1 serine phosphorylation, we performed FcyRIIA-ITAM multivalent aggregation in the presence or absence of ERK, PKC, and PI3K inhibitors<sup>30, 31</sup>. Whereas PI3K and PKC inhibitors completely blocked both SHP-1<sup>SS91</sup> and PKC phosphorylation, the ERK inhibitor had no effect (Fig. 3c and Supplementary Fig. 5b). In addition, both PI3K and PKC inhibitors favored SHP-1<sup>Y536</sup> phosphorylation under conditions of FcεRIIA-ITAM activation signaling, and this preference required the presence of Lyn (Fig. 3c and Supplementary Fig. 5b). Interestingly, silencing of the PKC<α> isofrom also abrogated SHP-1<sup>SS91</sup> phosphorylation under conditions of FcεRIIA multimeric aggregation and favored SHP-1<sup>Y536</sup> phosphorylation (Fig. 3d), which was Lyn-dependent (Fig. 3e). However, PKC<α> silencing had no effect on the phosphorylation status of SHP-1 observed in the absence of Lyn (Fig. 3f). Moreover, PI3K inhibition or PKC<α> silencing did not impair Syk activation following FcεRIIA-ITAM induction in the presence or absence of Lyn or Fyn (Supplementary Fig. 3c). Together, these results indicate that during ITAM-induced activation signals, Fyn inactivates SHP-1 through phosphorylation of the SS91 involving a PI3K–PKCα axis, thereby blocking its activation by Lyn. Our results demonstrate that Lyn is crucial to maintain ITAMi-mediated homeostasis, whereas Lyn is essential for ITAM-mediated cell activation by inducing the PI3K–PKCα signaling axis that inactivates SHP-1 during inflammatory responses for most immunoreceptors.

Opposing control of SHP-1 activity regulates inflammation. To investigate the functional role of Lyn and Fyn in the regulation of ITAM signals in vivo, we employed a mild immune-complex nephrotoxic nephritis (NTN) model via administration of a rabbit anti-mouse glomerular basement membrane (anti-GBM) serum. A mild NTN model was chosen because FcyRIIA<sup>Tg</sup> and Lyn-deficient mice have a pre-established autoimmune phenotype<sup>23, 25, 29</sup>. Consistent with the above-described role of Lyn in maintaining ITAMi-mediated immune homeostasis, i.p. administration of the NTN serum led to a severe acute nephritis associated with high mortality at day 7 in Lyn-deficient FcyRIIA<sup>Tg</sup> recipients, whereas mice deficient for Lyn or Fyn and Fyn-deficient FcyRIIA<sup>Tg</sup> mice did not develop significant disease despite similar glomerular rabbit antibody deposits and no significant differences in mouse IgG anti-rabbit IgG responses (Fig. 4a and Supplementary Fig. 6a). Renal disease development in Lyn-deficient FcyRIIA<sup>Tg</sup> mice was characterized by a marked increase in urinary protein (Fig. 4b) and blood urea nitrogen concentration (BUN) (Fig. 4c). They also exhibited severe renal injury involving extensive mesangial and capillary (subendothelial or even intracapillary) deposits associated with mild mesangial and endocapillary plus extra-capillary proliferation (Fig. 4d and Supplementary Fig. 6b–d). Glomerular lesions were characterized...
by an intense macrophage infiltrate and cytokine production (Figs. 4d, e and Supplementary Fig. 6e). These effects involved ITAM activation signaling, as demonstrated by in situ phosphorylation of the Y525 residue in Syk (Fig. 4f). This was associated with a strong in situ phosphorylation of SHP-1 on the inhibitory S591 residue but not on Y536 (Fig. 4g, middle panels). Moreover, no involvement of SHIP-1-mediated inhibitory signaling by macrophage FcγRII was observed in situ after NTN induction in FcγRIIA mice, as glomerular immunofluorescence stainings with anti-phospho SHP-1 antibody were completely negative (Supplementary Fig. 7). By contrast, NTN induction in Fyn-deficient FcγRIIA mice led to in situ phosphorylation of SHP-1 on Y536 but failed to induce S591 phosphorylation, and this was associated with no renal inflammation despite the presence of antibody deposits on the glomerular membrane (Fig. 4g, bottom panels). Interestingly, the same observations were made in 8-week-old FcyRIIA mice (Fig. 4g, top panels). These results suggest that the absence of Fyn in vivo may protect against autoimmune disease development favoring the activating phosphorylation of SHP-1 on tyrosine residue.

To investigate the aggravating role of Fyn in inflammatory disease development, we took advantage of another autoimmune disease model, namely the collagen antibody-induced arthritis (CAIA) using two transgenic animals expressing either hFcγRIIA and hFcεRI. The aggravating role of Fyn was highlighted by the absence of lesions in both Fyn-deficient FcyRIIA and Fyn-deficient FcεRI mice despite similar serum levels of injected mouse anti-collagen antibodies (Fig. 5a–f). By contrast, Lyn-deficient FcyRIIA and Lyn-deficient FcεRI mice showed enhanced arthritis development characterized by leukocyte...
To determine whether targeting ITAMi could have a therapeutic value, we treated transgenic animals expressing human FcγRIIA or FcαRI for ITAMi signaling with anti-FcγRII F(ab′)2 or monomeric hIgA, respectively, as previously described. ITAMi induction prevented disease development, and this protection required the presence of Lyn but not Fyn (Fig. 5d–f). We next addressed the role of the different phosphorylated forms of kinases and phosphatases in

infiltration (Fig. 5a–f). To determine whether targeting ITAMi could have a therapeutic value, we treated transgenic animals expressing human FcγRIIA or FcαRI for ITAMi signaling with anti-FcγRII F(ab′)2 or monomeric hIgA, respectively, as previously described. ITAMi induction prevented disease development, and this protection required the presence of Lyn but not Fyn (Fig. 5d–f). We next addressed the role of the different phosphorylated forms of kinases and phosphatases in
joints of arthritic FcγRIIA/Iγ mice. While Lyn protected the host against autoimmunity by inducing a constitutive phosphorylation of SHP-1/S591 residue, Fyn favored autoimmunity by inactivation of SHP-1 through the phosphorylation of the SHP-1/S591 residue associated with induction of Syk Y525 phosphorylation (Fig. 6a–c). Moreover, induction of ITAMI by anti-FcγRIIA targeting was associated with in situ detection of Lyn-dependent pSHP-1/S536, but not pSHP-1/S591 (Fig. 6a–c). Altogether, these results support that non-redundant SFKs are crucial in dictating ITAMI/ITAM balance that controls homeostasis as well as inflammatory and autoimmune disease development.

**Fyn-SHP-1/S591 axis is linked to lupus nephritis activity.** To examine whether Fyn-mediated inhibitory SHP-1/S591 phosphorylation were associated with immune complex-mediated
disease via the FcγRIIA in patients with a given inflammatory disease, we analyzed blood leukocytes from untreated patients with lupus nephritis at different stages of renal involvement morphologically classified as class IV-A (severe nephritis with immune deposits and leukocyte infiltration) and pure class V (membranous immune deposits only). As shown in Fig. 7a, b, pSHP-1S591 and pPKCα were exclusively observed in patient cell lysates, and were not associated with FcγRIIA. Consistent with the role of Lyn/SHP-1 axis in homeostasis, Lyn and pSHP-1S591 were strongly associated with FcγRIIA in healthy individuals only, underlining the inhibitory ITAM homeostatic phenotype, whereas Fyn and Syk were exclusively associated with FcγRIIA in patients highlighting the deleterious role of ITAM signaling in this inflammatory disease. Moreover, tissue analysis of phosphorylated phosphatases in situ of renal biopsies from untreated patients with lupus nephritis at different stages of disease show that the phosphorylation status of SHP-1S591 residue was linked to disease activity in proximity of phalloidin+ renal cells (Fig. 7c). No phosphorylation of SHP-1Y536 was detected on patient kidney biopsies. However, in control experiments, pSHP-1Y536 intracellular immunofluorescence staining was observed in THP-1 cells after FcγRIIA-ITAMi induction using the same antibody and, inversely, pSHP-1S591 was only detected after FcγRIIA-ITAMi induction (Supplementary Fig. 8). As some pSHP-1S591-positive glomerular areas were negative for phalloidin, we next examined leukocyte markers for pSHP-1S591 co-expression in biopsies from three patients with lupus nephritis with high disease activity. Figure 7d shows a representative biopsy revealing that significant areas of pSHP-1S591 positivity were located in CD68+ cells but also in CD68- cells. These results indicate that macrophages, which are present in the inflamed glomeruli express inactive SHP-1 in their cytosol. Moreover, in situ phosphorylation of SHP-1S591 but not of S591, was associated with spontaneous severe nephritis in 1-year-old anti-autoimmune R131 FcγRIIA mice (Supplementary Fig. 9a–f). This end-stage chronic kidney disease was characterized by enhanced body weight, increased BUN levels, glomerular IgG deposits, sclerotic glomeruli, fibrosis, and kidney interstitial infiltration by CD11b +, F4/80 + and CD3 + cells and increased expression of mRNA coding for proinflammatory cytokines (Supplementary Fig. 9a–e). In this model pSHP-1S591 was detected in situ, whereas pSHP-1Y536 was detected in the kidney of WT mice (Supplementary Fig. 9f). Altogether, these results support that the type of the phosphorylation of SHP-1 (Y536 vs. S591 residues) determines the shift between the healthy and autoimmune status and can be used as novel biomarkers for lupus disease activity.

Discussion

Here we characterize the mechanism by which SFKs and SHP-1 control the balance between the activating and inhibitory ITAM signaling. Initially described for their activating function, evidences have accumulated that FCεRs bearing an ITAM motif could generate opposite signals, known as ITAMI. Yet, a black box remains in regard to the inhibitory signaling mechanism involved. In this study, we show that low valency aggregations of BCR by anti-CD79a F(ab')2 fragments induced an ITAMI signal characterized by the recruitment of the phosphatase SHP-1, whereas highly multivalent aggregation induced a conventional ITAM activation signal extending the concept that dual ITAM functionality of BCR depends on the valency of the ligand. CD79a-mediated ITAMI inhibitory signals by the BCR is in agreement with previously described pathways to maintain B-cell anergy in which chronic B-cell stimulation results in ITAM monophosphorylation inducing an inhibitory signaling circuit involving SHIP-1 and Dok-1.33 Interestingly, monophosphorylation of FcγRIIA ITAM is also associated with ITAMI signals but with SHP-1, rather than SHIP-1, recruitment31. Whether the number of BCR adapters containing monophosphorylated ITAM is involved in the differential recruitment of phosphatases remains unclear. Our results may also explain a previous observation in which SHP-1 was found to be implicated in the regulation of BCR signaling and in the maintenance of tolerance as B cell-targeted ablation of SHP-1 led to lupus-like disease39. Taken together, these findings support the conclusion that immune receptors associated with an ITAM motif, including antigen receptors, can play a dual role as they can induce either activating (ITAM) or inhibitory (ITAMI) signaling depending on ligand avidity.

Our study further demonstrates that single receptors switch between ITAM and ITAMI signals using distinct SFKs fulfilling opposite signaling functions, such as described here for FcεR and BCR. Upon divalent targeting of immunoreceptors Lyn, but not Fyn, is recruited to the receptor, leading to ITAM partial phosphorylation on tyrosine, as we previously reported for FcγRIIA,11 and of SHP-1 recruitment. In contrast, upon multivalent crosslinking of FcγRIIA, Fyn but not Lyn, plays a crucial role in FcγRIIA-ITAM-mediated cytokine production. This Lyn/Fyn switch is not affected by other SFK such as Hck and Fgr. Interestingly, it has been shown that FcyRs can mediate a well-established ITAM-dependent function, the phagocytosis, in the absence of Lyn, Hck, and Fgr34, 35. Our data highlight that Lyn and Fyn are essential for ITAM responses as silencing of both SFK render the cell unresponsive for FcγRIIA activating or inhibitory stimuli.

The mechanism by which Lyn and Fyn alternatively switch ITAM-bearing receptor function relies on their control of SHP-1 phosphorylation status. It is now clear that SHP-1 phosphorylation at Y536 induces a conformational change easing SHP-1 recruitment through its SH2 domains to phosphotyrosine residues, thereby lifting the inhibition of the phosphatase domain by its N-SH2 domain36. Of note, the two SH2 domains of SHP-1 can cooperate to bind phosphotyrosines on adjacent molecules37, a configuration expected to be that of ITAMI. This recruitment would enable ITAMI-mediated SHP-1 phosphatase activity to inactivate signal effectors mobilized by heterologous receptors38. In contrast, multivalent crosslinking of immunoreceptors results in the recruitment of the SFKs Lyn and Fyn to the receptor leading to full phosphorylation of the ITAMs. These would serve as “docking” sites for Syk preventing SHP-1 recruitment, which is associated with its phosphorylation on S591 in the cytosol. Thus, Fyn but not Lyn redirects SHP-1 towards its inactivated form allowing simultaneously full ITAM phosphorylation and receptor activation signaling by Syk. As Fyn is a tyrosine, but not a serine, kinase we explored the mechanism involved. SHP-1 is constitutively associated with PKCα27. Upon cell activation, PKCα phosphorylates SHP-1 on S591 in its C terminus thereby negatively regulating the activity of this phosphatase27. Using a specific inhibitory and siRNA strategy, we found that a Fyn–PI3K–PKCα axis induced the phosphorylation of SHP-1 on S591 upon crosslinking of FcγRIIA in the presence of Fyn, despite the recruitment of Lyn. Since S591 phosphorylation on SHP-1 keeps the phosphatase in a closed conformation39, our results suggest that while S591 residue is phosphorylated by Fyn, the Y536 residue is inaccessible to Lyn. Moreover, this study demonstrates also that the absence of Fyn favors the phosphorylation of SHP-1 on Y536 in the presence of Lyn, despite the extensive crosslinking of FcγRIIA. These results indicate that the selective absence or inhibition of Fyn may abolish inflammation during autoimmune and proinflammatory processes. Whether Fyn–PI3K–PKCα axis is involved in...
previously described Fyn-dependent humoral responses remains to be demonstrated.

R131H FcyRIIα polymorphism has a strong correlation with the pathogenesis, development and increased susceptibility to several autoimmune diseases, in particular systemic lupus erythematosus (SLE) and rheumatoid arthritis. Interestingly, similar to Lyn-deficient mice, R131H-FcyRIIα mice develop spontaneously autoimmunity at old age, with a complex spectrum of symptoms similar to those found in human rheumatoid arthritis (erosive pannus) and SLE (antinuclear antibodies, glomerulonephritis with immune complex deposition in the renal basement membrane, pneumonitis and non-erosive arthritis). Several studies have reported that Lyn, but not Fyn, controls IgG-mediated or IgE-mediated systemic anaphylaxis. Our data elucidate the role of these SFKs in the development of autoimmune and proinflammatory disease. Indeed, Fyn and Lyn deletion had opposing effects on the regulation of inflammation observed in R131H-FcyRIIα mice. In the mild model of NTN, ablation of Fyn had no effect on young mice, but that of Lyn induced lethal nephritis associated with the activation of Syk and in situ phosphorylation of SHP-1 on Y591. It is unlikely that SHIP-1 plays a negative role in this model (previously described in ref. 44). All mice were of C57BL/6 strain, female gender between 8–10 weeks old. All mice carrying the FcyRIIα transgene were used as heterogeneous animals. Mice were bred and maintained at the mouse facilities of the Bichat Medical School campus. All experiments were performed in accordance with the French Council of Animal Care guidelines and national ethical guidelines of INSERM Animal Care Committee (Animal Use Protocol number 75-1596).

NTN Mouse Model. NTN was induced by i.p. injection (200 µl/20 g body weight) of rabbit anti-mouse GBM in 8–10-week-old mice. Briefly, mice were injected with the following parameters (urinary proteins and BUN), histological and immunohistological parameters were studied.

CAIA model. Arthritis was induced as described, using the Arthritis-CIA Rat Arthritisogenic Monoclonal Antibody kit (Chondrex, Inc.). Mice were injected i.v. with anti-CA Ab cocktail (Day 0) followed by LPS (i.p.) 3 days later. Animals were then treated i.p. with 10 mg/20 g body weight of 500 µg human IgA (purchased from Biomedicals)/20 g body weight or 100 µg AT-10 (F(ab')2; irrelevant mAb F(ab')2 (clone 320) for 10 days at 2-day intervals. The first dose was administered 2 days prior to anti-CA Ab cocktail injection. Paw thickness was measured with a pocket gauge. On day 10, animals were sacrificed and hind paws and knees were fixed in formalin or snap-frozen.

Methods. Seventeen healthy individuals were studied. The SLE group was composed of 10 patients attending at the Bichat’s Hospital specialist nephrology unit between July 2014 and January 2016 meeting at least four ACR SLE criteria presenting with active disease with nephritis proven by kidney biopsy (5 at class IV and 5 at class V) and in whom peripheral blood by venepuncture was obtained immediately prior to immunosuppressive therapy administration. All patients were female with age varying between 25 and 42. Ethical approval for this study was obtained from the Bichat Hospital Local Research Ethics Committee and informed consent was obtained from all patients enrolled. Data collection and analyses were performed anonymously.
immunoprecipitation and immunoblotting. Cells (5 × 10^6 to 10^6) were solubilized in RIPA lysis buffer containing 1% Nonidet P-40, 0.1% sodium deoxycholate (SDS) as described. For immunoprecipitation, cell lysates were incubated with 2 µg/ml of IV.3 anti-FcγRIIA, A77 anti-FcRII or ZTL-4 anti-CD79a mAbs and immunoprecipitated overnight at 4 °C with Protein G-Sepharose (GE Healthcare). Samples were resolved by SDS polyacrylamide gel electrophoresis (10%), transferred to nitrocellulose membranes and immunoblotted with rabbit antibodies followed by goat anti-rabbit IgG (1:200; GE Healthcare) coupled to horseshad peroxidase. Membranes were developed by enhanced chemical luminescence treatment (Amersham Biosciences). All uncit Western blots are available in Supplementary Fig. 10.

Enzyme-linked immunosorbent assay. IL-8 was measured in the supernatants of stimulated cells using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer’s instructions. Anti-type I and II collagen mouse IgG antibodies were measured by an ELISA kit with TMB according to the manufacturer’s instructions (Chondrex, Inc, Catalog #2036 T).

Real-time PCR. RNA purification from homogenized kidneys was performed by using RNAaes (Eurobio). cDNA was obtained by reverse transcription using Moloney murine leukemia virus (Invitrogen). Samples were analyzed by real-time PCR with Taq Man Gene Expression Master Mix (Applied Biosystem). Primers were purchased from Eurofins. Gene quantification was performed using a Chrom o4 Real-Time PCR Detection System (Bio-Rad Laboratories). Data were normalized to β-Chrom o4 Real-Time PCR Detection System (Bio-Rad Laboratories). Data were normalized to β-actin values. For primers and probe sequences see Supplementary Table 1.

siRNA transfections. Experiments were performed using predesign HP GenomeWide (Qiagen, Courtaboeuf, France) siRNAs. For targets DNA sequences and siRNAs sense and anti-sense see Supplementary Table 2. Single-strand and antisense RNA nucleotides were annealed to generate an RNA duplex µnM of each siRNA tested and 2 µl of each siRNA transfections

Histological and immunofluorescence analyses. For the kidney, paraffin-embedded sections 4 µm in thickness were stained with PAS for morphological analysis. For immunohistochemistry, frozen kidney sections were incubated with biotinylated antibodies against rabbit IgG or monoclonal antibodies anti-mouse CD11b, anti-mouse F4/80, anti-mouse CD3, and anti-mouse Ly6G (Becton Dickinson) or biotinylated antibodies against rabbit IgG or monoclonal antibodies anti-mouse IgG (Eurobio). Sections were incubated with streptavidin-peroxidase. Membranes were developed by enhanced chemical luminescence treatment (Amersham Biosciences). All uncit Western blots are available in Supplementary Fig. 10.

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Statistical analysis. All data were expressed as mean ± SEM. Statistical significance between two groups was examined by the Student’s t-test or the Mann–Whitney test, while the one-way and two-way analysis of variance (ANOVA) with Bonferroni’s, Holm–Sidak’s, or Newman–Keuls multiple comparisons test was used to evaluate multiple groups. Sample sizes were higher than five per group. P-values of 0.05 were considered significant; values <0.05 are indicated in the figure legends.

Data availability. The data that support the findings of this study are available from the corresponding authors on request.

Received: 10 October 2016 Accepted: 12 June 2017

Published online: 15 August 2017
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Acknowledgements
This work was supported by grants from ANR (MIEN-2009 and BLANC International-2012) and from LabEx Inflamx (ANR-11-IDEX-0005-02). S.B.M. was supported by a grant from the French Foundation ARC (PDF2010001037). R.C.M. is supported by < Equipe > program of the Fondation pour la recherche médicale (FRM). We thank Marieke Heineke and Lorredana Saveau critical reading of this manuscript, and Julie Bex and Ewan Boedec for animal care. We are indebted to the patients and healthy volunteers who participated in the study.

Author contributions
S.B.M. and R.C.M. designed experiments. S.B.M. performed experiments. A.M., H.F., D.T.-B., L.D. and C.B. contributed to experiments. E.D. provided human samples and performed analyses of morphological data. S.B.M. and R.C.M. wrote the manuscript. P.L., M.B., U.B. and N.C. reviewed and edited the manuscript.

Additional information
Supplementary Information accompanies this paper at doi:10.1038/s41467-017-00294-0.

Competing interests: The authors declare no competing financial interests.

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