

Article

## Characterization of a Phospho-Specific Antibody to the Fcε Receptor γ Chain, Reveals Differences in the Regulation of Syk and Akt Phosphorylation

Ryo Suzuki, Sarah Leach, Barbara Dema and Juan Rivera \*

Laboratory of Molecular Immunogenetics, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Building 10, Room 13C103, Bethesda, MD 20892, USA; E-Mails: suzukir@mail.nih.gov (R.S.); saleach@sas.upenn.edu (S.L.); barbara.dema@nih.gov (B.D.)

\* Author to whom correspondence should be addressed; E-Mail: juan\_rivera@nih.gov; Tel.: +1-301-496-7592; Fax: +1-301-480-1580.

Received: 10 April 2013; in revised form: 20 April 2013 / Accepted: 3 May 2013 /

Published: 13 May 2013

---

**Abstract:** We previously demonstrated that the Fc receptor γ-chain Y<sup>58</sup> (C-terminal tyrosine) is highly susceptible to dephosphorylation; a mechanism that controls the extent of Syk activation and the downstream signaling in mast cells. Here, we explored the importance of the γ-chain Y<sup>47</sup> (N-terminal tyrosine) in mast cell signaling. We generated a highly sensitive and versatile phospho-specific antibody that recognized the phosphorylated Y<sup>47</sup> in various species. Using this antibody, we found that mutation of the FcεRIβ Y<sup>219</sup> to phenylalanine caused a loss in the phosphorylation of the γ-chain Y<sup>47</sup>, consistent with the previously described role of Y<sup>219</sup> in Lyn association with FcεRIβ and subsequent FcεRIγ phosphorylation. These conditions also diminished the tyrosine phosphorylation of Syk and LAT1 but, surprisingly, not the phosphorylation of Akt at T<sup>308</sup>. Mutation of Y<sup>47</sup> or Y<sup>58</sup> of the γ-chain also caused a marked inhibition of Syk and LAT1 phosphorylation, but only the latter mutant showed a reduction in Akt phosphorylation. These findings show that the full phosphorylation of Syk and LAT1 requires the FcεRIβ Y<sup>219</sup> and both Y<sup>47</sup> and Y<sup>58</sup> of the γ-chain. However, T<sup>308</sup> phosphorylation of Akt is largely independent of FcεRIγ Y<sup>47</sup> phosphorylation and of the Lyn-binding site (Y<sup>219</sup>) on the FcεRIβ.

**Keywords:** phospho-specific antibody; mast cell; FcεRI; immunoreceptor tyrosine-based activation motif (ITAM); Akt; Syk

---

## 1. Introduction

Mast cells express high levels of the high affinity receptor for IgE, FcεRI, which is comprised of four non-covalently associated polypeptides, the IgE-binding α-chain, the signal transduction amplifying β-chain, and the signal inducing γ-chain [1,2]. Both the β- and γ-chain contain an immunoreceptor tyrosine-based activation motif (ITAM) that encodes the consensus sequence (D/E)XXYXXLX<sub>7-11</sub>-YXXL(L/I) [3]. While the γ-chain ITAM (Y<sub>47</sub>TGLNTRSQETY<sub>58</sub>ETL) is comprised of the consensus sequence (YxxL-x<sub>7</sub>-YxxL), the β-chain ITAM (Y<sub>219</sub>EELHVV<sub>225</sub>SPIY<sub>229</sub>SEL) has an additional tyrosine (Y<sup>225</sup>: non-canonical) residue between two canonical tyrosine (Y<sup>219</sup> and Y<sup>229</sup>) residues [3]. Aggregation of FcεRI results in the phosphorylation of the tyrosine residues in its β- and γ-chains through association of Lyn kinase (a Src family protein tyrosine kinase) with the β chain of this receptor. Once phosphorylated these tyrosine residues serve as docking sites for recruitment of additional signaling molecules, like Syk kinase [4,5]. Mutational analysis of some of the tyrosine residues in the β- or γ-chain ITAM revealed that they have distinct roles in mast cell signaling [6–9]. For instance, previous studies have shown that Y<sup>219</sup> in the FcεRIβ ITAM is important for Lyn association with this receptor [6]. Our own studies [9] demonstrated that the Y<sup>225</sup> in this same subunit plays an important role in negatively regulating cytokine synthesis and secretion by regulating mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NFκB) activation following antigen (Ag) stimulation. It has long been recognized that, once phosphorylated, the γ-chain ITAM serves as a docking site for Syk kinase and that both Y<sup>47</sup> and Y<sup>58</sup> participate in the docking and activation of Syk kinase [10,11]. However, we recently found [12] that Y<sup>58</sup> (C-terminal tyrosine) serves as a regulator of Syk activation since it is rapidly dephosphorylated limiting the amount of active Syk and thus controlling the extent of mast cell effector responses. These findings demonstrate that the selective phosphorylation/dephosphorylation of ITAM tyrosine residues is not only essential for initiating signaling protein interaction and activation but also participates in controlling the rate and extent of this interaction and activation, thus regulating the extent of a mast cells' response.

For many years it was thought that the FcεRI signaling in mast cells was driven through the initial activity of Lyn kinase alone [13]. The discovery [14] of complementary signaling driven through FcεRI-dependent Fyn kinase activation led to the realization that some signals generated upon receptor engagement were relatively independent of Lyn activity. Thus, the molecular events leading to activation of phosphatidylinositol 3 OH-kinase (PI3K) and Akt (also known as protein kinase B (PKB)) were found to be more dependent on Fyn kinase than on Lyn kinase. Akt is a 56 kDa member of the serine/threonine protein kinase family that plays an important role in cell survival, proliferation, migration, and cell polarity [15–18]. Akt undergoes membrane recruitment through the N-terminus PH domain that binds to phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>), a phospholipid synthesized by PI3K, leading to phosphorylation and activation of Akt. In mast cells, Akt was found to play a role in cytokine production by regulating binding of the NFκB, the nuclear factor of activated T cells (NFAT),

and activator protein 1 (AP-1) to their respective binding sites in the promoter regions of IL-2 and TNF $\alpha$  [19].

Given that Akt plays an essential role in mast cell effector responses and that it is activated upon Fc $\epsilon$ RI engagement in a Fyn kinase dependent manner, how the Fc $\epsilon$ RI $\beta$  and  $\gamma$  ITAM tyrosine's contributed in controlling Akt activation was not clear. In the current study, we set out to investigate if the Fc $\epsilon$ RI $\beta$  and  $\gamma$  ITAMs both contributed to Akt activation and to define which ITAM tyrosine residues were essential. For these studies we generated a novel phospho-specific monoclonal antibody to the phosphorylated  $\gamma$ -chain ITAM Y<sup>47</sup> (ITAM $\gamma$  pY<sup>47</sup>) as this is the dominant phosphorylated site seen upon isolation of the Fc $\epsilon$ RI $\gamma$ . This antibody proved to be a highly sensitive measure of Fc $\epsilon$ RI activation in cells and tissues under various applications and led us to the discovery that Akt phosphorylation upon Fc $\epsilon$ RI engagement is almost solely dependent on the phosphorylation of Y<sup>58</sup> of the Fc $\epsilon$ RI $\gamma$  ITAM.

## 2. Results and Discussion

### 2.1. Generation of a Novel Monoclonal Antibody That Specifically Recognizes the Phosphorylated Fc $\epsilon$ RI $\gamma$ -Chain ITAM Y<sup>47</sup>

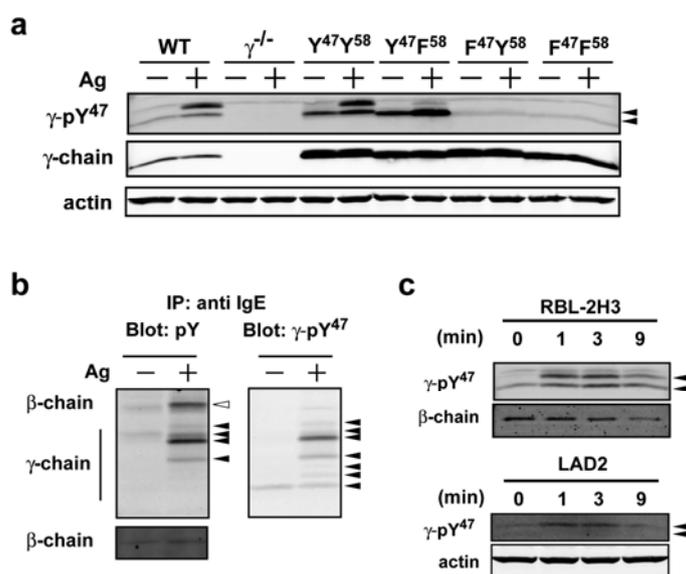
To investigate the role of Fc $\epsilon$ RI phosphorylation in Akt phosphorylation we first developed a phospho-specific monoclonal antibody to the  $\gamma$ -chain ITAM Y<sup>47</sup>. Mice were immunized with an ITAM peptide (as detailed in the Experimental Section) encoding the phosphorylated  $\gamma$ -chain ITAM Y<sup>47</sup> (pY<sup>47</sup>) and six hybridoma clones were successfully established. A phosphorylated peptide-bound ELISA was developed and culture supernatants from the six hybridoma clones were assayed for reactivity to pY<sup>47</sup>. A peptide encoding the non-phosphorylated Y<sup>47</sup> was used as a negative control. Test of culture supernatants (1:10–1:2,430) by ELISA showed that the antibodies produced by all six hybridoma clones were able to recognize the peptide encoding the phosphorylated Y<sup>47</sup> antigen but not the peptide encoding the non-phosphorylated Y<sup>47</sup> (data not shown).

We chose to work with clone 6A5G5 (designated as  $\gamma$ -pY<sup>47</sup> in the following text) based on the sensitivity and selectivity of the observed reactivity by Western blot. Mast cells expressing mutant forms of the Fc $\epsilon$ RI $\gamma$  in which the ITAM tyrosine residues were individually or jointly mutated, were used to verify the specificity of the phospho-specific antibody to phosphorylated Y<sup>47</sup>. When we compared lysates from normal (WT) bone marrow-derived mast cells (BMMCs), lysates derived from Fc $\epsilon$ RI $\gamma$ -deficient ( $\gamma^{-/-}$ ) BMMCs showed no reactivity with the antibody to pY<sup>47</sup> in Western blots (Figure 1a). Upon reconstitution of  $\gamma^{-/-}$  mast cells with wild type (Y<sup>47</sup> Y<sup>58</sup>)  $\gamma$ -chain, antibody recognition of pY<sup>47</sup> was restored (Figure 1a). In contrast, reconstitution with various tyrosine to phenylalanine mutants (YF, FY, FF) resulted in differential recognition of pY<sup>47</sup>, with cell lysates from YF mutant-expressing cells showing antibody reactivity by Western blot analysis whereas lysates from FY and FF mutants had no reactivity (Figure 1a). Since retroviral reconstitution of  $\gamma^{-/-}$  mast cells resulted in overexpression of YY, YF, FY, and FF ITAMs antibody reactivity was intensified in the resting and activated YY and YF-bearing cells relative to WT cells. Nonetheless, this served to more clearly demonstrate the specificity of the antibody for recognition of pY<sup>47</sup> since lysates from cells overexpressing the FY and FF forms of the Fc $\epsilon$ RI $\gamma$  ITAM did not show any detectable antibody

reactivity (Figure 1a). Both WT and YY-expressing mast cells showed two major bands following Ag stimulation. In YF-expressing MCs, one major band was observed in resting mast cells that intensified upon Ag stimulation and was accompanied by the appearance of a weakly reactive upper band comparable to the upper band seen in WT or YY-expressing mast cell lysates (Figure 1a). This data suggested that majority of signal in upper band corresponds to dual phosphorylation at Y<sup>47</sup> and Y<sup>58</sup> and mutation of the latter resulted in an increase in the lower mono-phosphorylated (pY<sup>47</sup>) band of FcεRIγ. We have previously shown that the phosphorylated homodimers of FcεRIγ are detected as multiple bands under non-reducing conditions with differing molecular mass [12,20]. Western blotting of immunoprecipitated FcεRI with an antibody to phosphotyrosine identified the phosphorylated β-chain and at least four major species of phosphorylated FcεRIγ (Figure 1b, left panel) in Ag-stimulated conditions with little or no phosphorylation detected in non-stimulated conditions. Interestingly, the γ-pY<sup>47</sup> antibody blotting of the same stripped and reprobed blot showed a minor reactivity in resting BMMCs, demonstrating that this antibody is more sensitive in detecting phosphorylated FcεRIγ than the widely used anti-phosphotyrosine antibody 4G10 (Figure 1b, right panel). An additional six species of the phosphorylated FcεRIγ were observed following Ag stimulation. Some of the observed species were identical in both phosphotyrosine antibody and γ-pY<sup>47</sup> antibody blots. However, the three species (with the lowest molecular mass) seemed to be detected only by the γ-pY<sup>47</sup> antibody. Peptide microarray data has demonstrated that all phosphotyrosine antibody clones (*i.e.*, 4G10, PY20, p-TYR-100) recognize their target in a sequence-specific context and that this recognition differs for each clone [21]. Our results demonstrate that this novel antibody, γ-pY<sup>47</sup>, recognizes its target sequence in the FcεRIγ with high selectivity and sensitivity, and recognizes more of the multiple molecular mass species (or modifications) of the FcεRIγ than the general anti-phosphotyrosine antibody 4G10.

Given the usefulness of such a reagent to assess the status of FcεRI phosphorylation directly in cell lysates, we examined whether γ-pY<sup>47</sup> antibody could recognize phosphorylation of FcεRIγ Y<sup>47</sup> in mast cells from different species. The similarity of the amino acid sequence of the immunizing peptide among mouse, rat, and human was considerable, with mouse to rat being 92.9% identical and mouse to human being 85.7% identical. Thus, it was possible that the γ-pY<sup>47</sup> antibody might react with rat and human FcεRIγ. As shown in Figure 1c, Western blot analysis showed that the γ-pY<sup>47</sup> antibody reacted with phosphorylated FcεRIγ species in lysates from non-stimulated and Ag-stimulated RBL-2H3 (rat mast cell line) as well as with LAD2 (human mast cell line) cells. Interestingly, the lower molecular mass species in the LAD2 human mast cell line was only weakly detected and because the homology of mouse with human is considerably lower than that of mouse with rat, this may reflect a contextual difference in recognition of Y<sup>47</sup> or that basal phosphorylation is more restrained in this human mast cell line. Regardless, our findings show γ-pY<sup>47</sup> antibody recognizes phosphorylated FcεRIγ Y<sup>47</sup> of mouse, rat and human origin.

**Figure 1.** A novel phospho-specific antibody ( $\gamma$ -pY<sup>47</sup>) that selectively recognizes the phosphorylated Fc $\epsilon$ RI $\gamma$  ITAM Y<sup>47</sup> from mouse, rat, and human origin. **(a)** Murine WT BMMCs or  $\gamma^{-/-}$  BMMCs retrovirally transduced with Fc $\epsilon$ RI $\gamma$  mutants (*YY*, *YF*, *FY*, and *FF*) or control vector ( $\gamma^{-/-}$ ) were sensitized with IgE and stimulated with Ag for 1 min at 37 °C. Whole cell lysates were resolved by SDS-PAGE and immunoblotted with  $\gamma$ -pY<sup>47</sup> antibody under reducing conditions. **(b)** Fc $\epsilon$ RI was immunoprecipitated from whole cell lysates of WT cells (see Experimental Section) and resolved by SDS-PAGE under non-reducing conditions. Immunoblots were analyzed with anti-phosphotyrosine antibody (4G10) or  $\gamma$ -pY<sup>47</sup> antibody. **(c)** Whole cell lysates from stimulated or non-stimulated rat (RBL-2H3) and human (LAD2) mast cell lines were probed for phosphorylation of Fc $\epsilon$ RI with  $\gamma$ -pY<sup>47</sup> antibody.



## 2.2. Use of the $\gamma$ -pY<sup>47</sup> Antibody in Various Applications

The findings show that the  $\gamma$ -pY<sup>47</sup> antibody can selectively, and with high sensitivity, recognize phosphorylated Fc $\epsilon$ RI $\gamma$  in whole cell lysates from various species by Western blot. However, the usefulness of such a reagent for other applications was unclear. Thus, we tested its efficacy in detecting phosphorylated Fc $\epsilon$ RI $\gamma$  in immunocytochemistry, immunohistochemistry, and FACS. Double-immunostaining using the Alexa Fluor 568-labeled IgE and the  $\gamma$ -pY<sup>47</sup> antibody detected with Alexa Fluor 488 conjugated secondly antibody was performed in resting and Ag-stimulated BMMCs (Figure 2a). The BMMCs were then visualized by confocal laser scanning microscopy. In resting BMMCs, Alexa Fluor 568-labeled IgE (red) was uniformly distributed on the plasma membrane (intensity plot: red). Fluorescent signals from the  $\gamma$ -pY<sup>47</sup> antibody (in green) were very weakly localized in the plasma membrane (intensity plot: green). Following Ag-stimulation, Alexa Fluor 568-labeled IgE appeared more clustered and  $\gamma$ -pY<sup>47</sup> antibody staining was more co-localized with these Fc $\epsilon$ RI clusters (Figure 2a, middle panel). Quantitative fluorescence intensity plots across a line section of these images showed that  $\gamma$ -pY<sup>47</sup> antibody staining increased its intensity following stimulation with Ag (as might be expected upon increased Fc $\epsilon$ RI phosphorylation) and co-localized with IgE aggregates (intensity plot: Ag (+)). No obvious immunostaining was observed in BMMC

derived from  $\gamma$  chain-deficient mice (Figure 2a: bottom panel). We further tested the utility of  $\gamma$ -pY<sup>47</sup> antibody for immunohistochemistry using skin tissue from the mast cell-dependent mouse model of passive cutaneous anaphylaxis (PCA). Skin samples from the ear of PCA challenged (5 min) WT mice were stained with Alexa Fluor 488 conjugated  $\gamma$ -pY<sup>47</sup> antibody (Figure 2b). Alexa Fluor 647 conjugated-avidin was used to visualize mast cells in skin tissues [22,23]. As shown in Figure 2b, following PCA challenge, mast cells (a single representative cell is shown in red) was observed in the skin and the staining with  $\gamma$ -pY<sup>47</sup> antibody (green) showed punctate pattern reminiscent of Fc $\epsilon$ RI clusters.

**Figure 2.** The  $\gamma$ -pY<sup>47</sup> antibody detects phosphorylation of Fc $\epsilon$ RI $\gamma$  ITAM Y<sup>47</sup> *in vitro* and *in vivo*. **(a)** Single cell analysis of Fc $\epsilon$ RI $\gamma$  ITAM Y<sup>47</sup> phosphorylation of mast cells stimulated with Ag for 1 min. Distribution of  $\gamma$ -pY<sup>47</sup> antibody was detected with Alexa Fluor 488 conjugated secondly antibody (green) and IgE was conjugated with Alexa Fluor 568 (red). An increased fluorescence intensity of Alexa Fluor 568 conjugated IgE was observed upon Fc $\epsilon$ RI clustering following Ag stimulation. The merged images (yellow) indicate colocalization of  $\gamma$ -pY<sup>47</sup> antibody and Alexa Fluor 568 conjugated IgE. The fluorescence intensity in cross-section of individual cells is shown as an intensity plot. **(b)** Detection of Fc $\epsilon$ RI $\gamma$  ITAM Y<sup>47</sup> phosphorylation in ear skin tissue of WT mice locally sensitized with saline or (anti-DNP) IgE in the contralateral ear. The next day, mice were challenged retroorbitally with Ag. Following a 5-min incubation, mice were euthanized, tissue collected and double whole-mount immunostaining was done with  $\gamma$ -pY<sup>47</sup> antibody (green) and Alexa Fluor 647 avidin (for mast cell staining). Mice sensitized with saline and subsequently challenged with Ag showed no detectable fluorescence with  $\gamma$ -pY<sup>47</sup> antibody in tissues (data not shown). **(c)** Human blood from healthy donors was stimulated with anti-human IgE antibody for 2 min and then cells were fixed and stained for the surface markers, APC-conjugated anti-human CD123, PE-conjugated anti-human CD303, Pacific Blue-conjugated anti-human Fc $\epsilon$ RI $\alpha$ , and PercPCy5.5-conjugated anti-human CD203c, followed by intracellular staining with Alexa Fluor 488 conjugated  $\gamma$ -pY<sup>47</sup> antibody. Mean fluorescence intensity (MFI) for non-stimulated or anti-IgE stimulated (2 min) conditions was 228 and 269, respectively. **(d)**  $\gamma$ -pY<sup>47</sup> antibody can be used to determine the activation status of basophils in human blood from subjects with active SLE. Representative data from 4 healthy and 4 SLE subjects is shown.

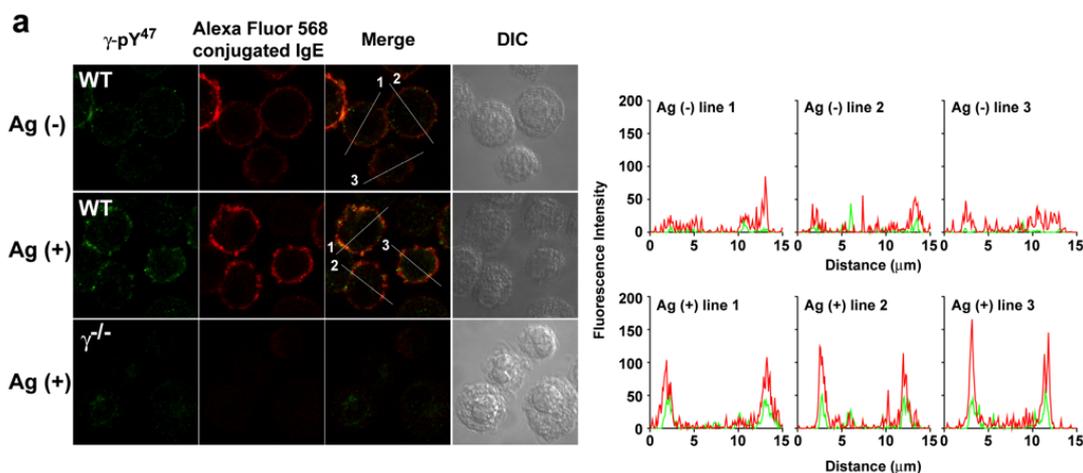
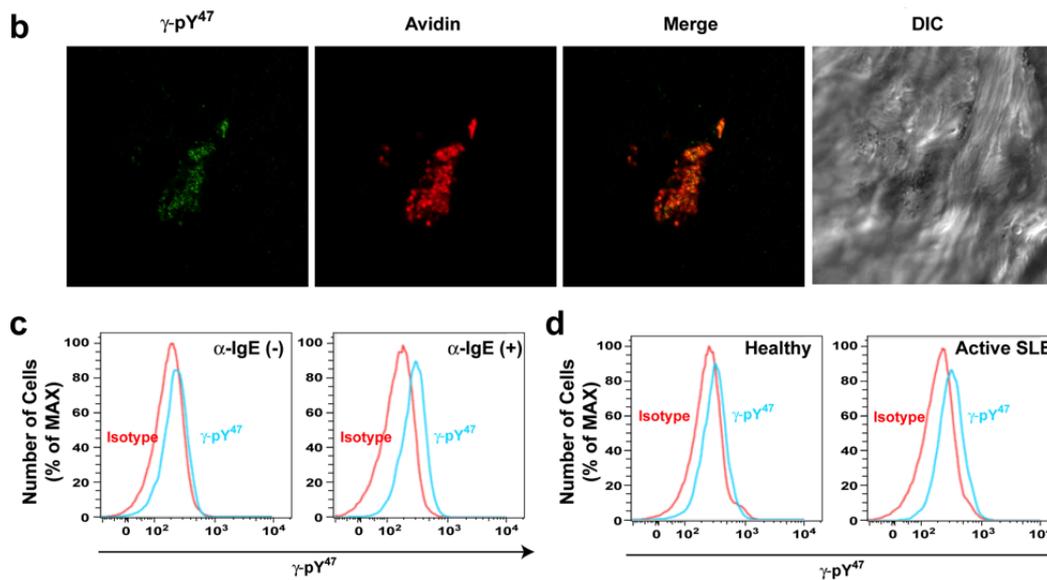


Figure 2. Cont.



Given that our previous findings showed that  $\gamma$ -pY<sup>47</sup> antibody recognized phosphorylated Fc $\epsilon$ RI $\gamma$  from human origin (Figure 1c), we hypothesized that  $\gamma$ -pY<sup>47</sup> antibody might detect the activation status of Fc $\epsilon$ RI in human mast cells or basophils and thus could be useful to analyze such cells in human disease. We previously published that, in systemic lupus erythematosus (SLE), activation of basophils by autoreactive IgE-containing immune complexes serves to amplify the production of autoantibodies and contributes to disease progression and severity [24]. Thus, we first examined whether  $\gamma$ -pY<sup>47</sup> antibody could detect phosphorylated Fc $\epsilon$ RI in human basophil by FACS. Human blood basophils were activated with anti-human IgE antibody for 2 min and fixed and stained with antibodies against different surface marker proteins used to identify human basophils (see Experimental Section). As shown in Figure 2c (left panel),  $\gamma$ -pY<sup>47</sup> antibody staining of non-stimulated human basophils did not differ with isotype control. Following a 2 min stimulation with anti-IgE,  $\gamma$ -pY<sup>47</sup> antibody staining showed a shift in fluorescence intensity relative to isotype control (Figure 2c, right panel). This demonstrated that  $\gamma$ -pY<sup>47</sup> antibody could detect the phosphorylation of Fc $\epsilon$ RI in human basophils following their activation. To test if one could distinguish the phosphorylation status of Fc $\epsilon$ RI in human basophils during disease we tested the reactivity of the  $\gamma$ -pY<sup>47</sup> antibody from healthy donors or donors with active SLE (Figure 2d). Staining of basophils from healthy donors was very similar to isotype control staining (Figure 2d, left panel). However, the staining with  $\gamma$ -pY<sup>47</sup> antibody in basophils from donors with active SLE was considerably higher than that of isotype control or that of the healthy donors (Figure 2d, right panel). These findings demonstrate the utility of the  $\gamma$ -pY<sup>47</sup> antibody in determining the phosphorylation status of Fc $\epsilon$ RI during cell activation or in disease and could be a useful diagnostic tool in allergic and other diseases where IgE antibodies may play a role.

### 2.3. Phosphorylation of Fc $\epsilon$ RI $\gamma$ Y<sup>47</sup> is Dependent on Fc $\epsilon$ RI $\beta$ Y<sup>219</sup>

Our previous work [12] demonstrated that Fc $\epsilon$ RI $\gamma$  Y<sup>58</sup> (C-terminal ITAM tyrosine) is highly susceptible to dephosphorylation. Thus, the aforementioned findings of a predominance in detection of phosphorylation of Y<sup>47</sup> relative to Y<sup>58</sup>, using cells carry mutated Fc $\epsilon$ RI $\gamma$  at these sites (Figure 1a), led

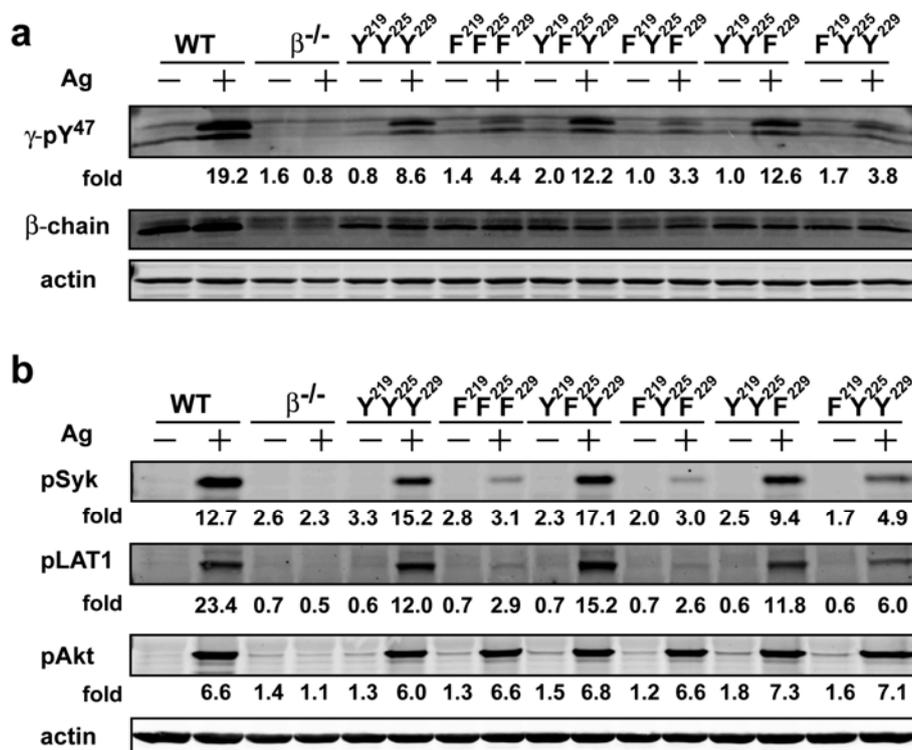
us to further characterize the requirements for phosphorylation of Y<sup>47</sup> and how Y<sup>47</sup> phosphorylation influenced downstream signaling events.

Lyn kinase has been shown to phosphorylate FcεRI [25,26] and associates with the FcεRIβ subunit and the phosphorylation of ITAM Y<sup>219</sup> in FcεRIβ is required for this association [6]. Thus, we investigated the role of the canonical (Y<sup>219</sup> and Y<sup>229</sup>) or noncanonical (Y<sup>225</sup>) tyrosine's of FcεRIβ on the phosphorylation of FcεRIγ Y<sup>47</sup>. Mutation of each tyrosine residue (Y<sup>219</sup> Y<sup>225</sup> Y<sup>229</sup>) individually or in combination to phenylalanine was done. These mutant forms of FcεRIβ were transduced (lentivirus) into BMMCs derived from β-chain deficient (β<sup>-/-</sup>) mice. All transduced BMMCs were more than 95% positive for FcεRI expression on their cell surface with similar fluorescence intensity (data not shown) demonstrating that the expression of the mutant forms of FcεRIβ did not alter receptor expression. FcεRIβ-deficient cells transduced with wild type (YYY) FcεRIβ showed a phosphorylation pattern of FcεRIγ (as detected by the γ-pY<sup>47</sup> antibody) that was similar to the FcεRIγ phosphorylation pattern from WT cells, whereas no detectable phosphorylation was observed in FcεRIβ-deficient cells transduced with a control vector (Figure 3a). Phosphorylation of FcεRIγ Y<sup>47</sup> in YFY or YYF transduced BMMCs was remarkably similar to that of YYY-transduced BMMCs. In contrast, FFF, FYF or FYY transduced cells showed considerable reduction (~40%) of FcεRIγ pY<sup>47</sup> phosphorylation upon Ag stimulation (Figure 3a). Taken together, the findings suggested that the phosphorylation of the FcεRIβ Y<sup>219</sup> (canonical N-terminal tyrosine) played an important role in phosphorylation of the FcεRIγ Y<sup>47</sup>. This finding is consistent with previous studies demonstrating the requirement of Lyn kinase for FcεRI phosphorylation [25] and the importance of FcεRIβ Y<sup>219</sup> for association of Lyn with FcεRI [6]. Because both FcεRIY<sup>47</sup> and Y<sup>58</sup> are required for the full extent of Syk kinase activation in mast cells, we analyzed the extent of Syk phosphorylation at the activation loop Y<sup>519/520</sup> (which when phosphorylated reflects a state of Syk activation) in the cells expressing the different FcεRIβ tyrosine mutants. As shown in Figure 3b, while phosphorylation of Syk Y<sup>519/520</sup> in YFY and YYF mutant was comparable to the YYY expressing cells, FcεRIβ tyrosine mutants expressing FFF or FYF ITAMs showed a marked reduction (~80%) in Syk phosphorylation whereas FYY expressing cells showed a considerable reduction (>60%) in phosphorylation of Syk activation loop Y<sup>519/520</sup>. Thus, these findings showed that the loss of phosphorylation of the FcεRIβ ITAM tyrosine's has a significant impact on Syk activation. Importantly, this also translates to defective molecular signaling further downstream as the extent of LAT1 (linker for activation of T cells) phosphorylation at Y<sup>191</sup> (a downstream target of Syk) was similarly affected (Figure 3b). Moreover, they clearly demonstrate the importance of FcεRIβ ITAM in regulating the phosphorylation of FcεRIγ and further show that the FcεRIβ Y<sup>219</sup> contributes to the extent of Syk and LAT1 phosphorylation following FcεRI engagement.

The findings also raised the question of whether all signaling downstream of FcεRI engagement were similarly regulated by the ITAM tyrosine's of FcεRIβ or FcεRIγ. We previously demonstrated that Fyn kinase initiates complementary signals in mast cells [14], which are less dependent on Lyn, Syk, or LAT1. Our findings showed that Fyn activation was important for the phosphorylation of the adapter molecule Gab2 and the activation of PI3K and the phosphorylation of Akt [14]. In cells expressing the various FcεRIβ ITAM mutants, analysis of Akt phosphorylation on T<sup>308</sup> (the site phosphorylated by the PI3K-dependent kinase 1) demonstrated no marked effect on its phosphorylation (Figure 3b). However, phosphorylation of Akt was clearly dependent on expression of FcεRI because FcεRIβ<sup>-/-</sup> BMMCs (which do express FcεRI on their cell surface [9]) failed to induce Akt T<sup>308</sup>

phosphorylation. Thus, the data suggested that phosphorylation of Akt was likely to be dependent on the FcεRIγ, a hypothesis we subsequently explored.

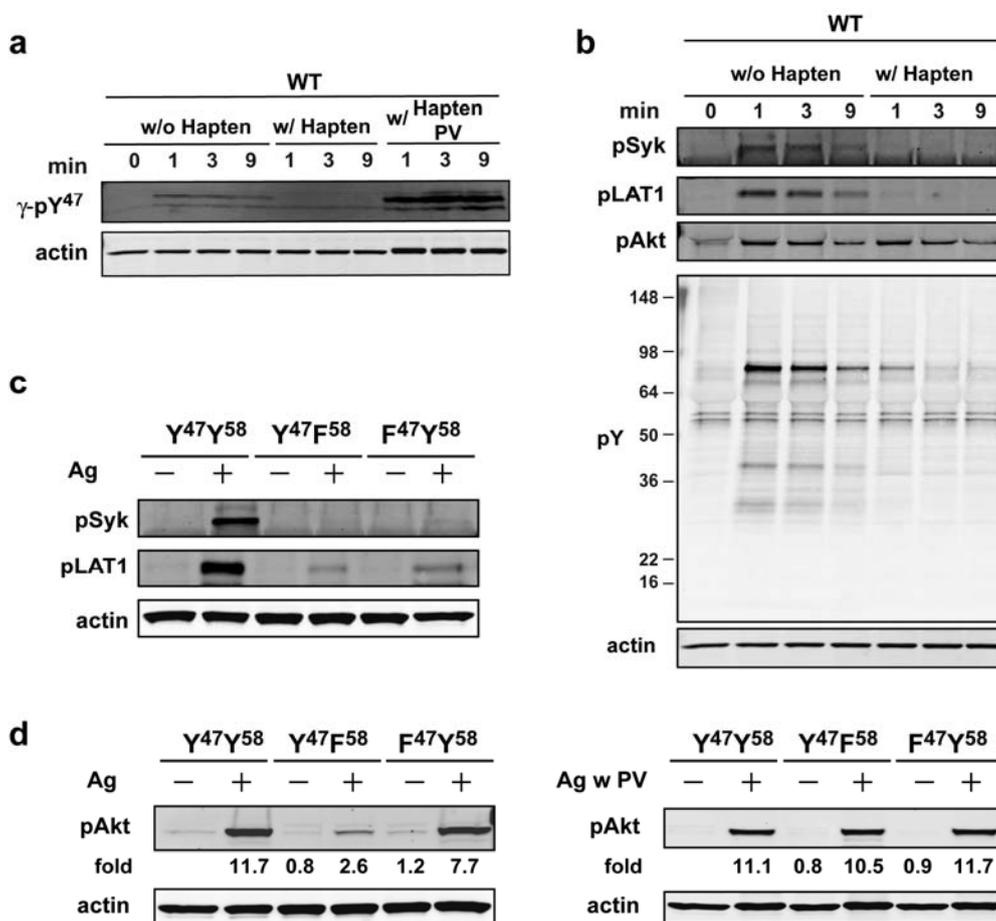
**Figure 3.** The FcεRIβ Y<sup>219</sup> regulates phosphorylation of FcεRIγ ITAM Y<sup>47</sup> and proximal signaling molecules. Whole cell lysates from WT cells were resolved under reducing conditions. (a) Immunoblots were probed with γ-pY<sup>47</sup> antibody. (b) Anti-phosphoSyk antibody (Y<sup>519/520</sup>), anti-phospho LAT1 antibody (Y<sup>191</sup>), and anti-phosphoAkt antibody (T<sup>308</sup>) were used to detect site specific phosphorylation. Fold induction was calculated by densitometry and normalized to the respective protein or to actin.



#### 2.4. Phosphorylation of Akt Is Largely Dependent on the Phosphorylation of FcεRIγ Y<sup>58</sup>

Our subsequent experiments were initially aimed at addressing whether the phosphorylation of Akt was dependent on the continuous aggregation of the FcεRI. As shown in Figure 4a the use of monovalent hapten (DNP-L-Lys), which disrupts the receptor aggregation induced by a multivalent Ag (DNP<sub>30-40</sub>-HSA) [27,28], demonstrated that phosphorylation of FcεRI (as measured with γ-pY<sup>47</sup> antibody) is markedly abrogated by such treatment, however, phosphorylation can be restored when a general inhibitor of tyrosine phosphatases (pervanadate) is added to the cells. This suggested that disaggregation of FcεRI by hapten (30 s after Ag addition) makes the receptor susceptible to dephosphorylation by tyrosine phosphatases. Given this finding, we asked if the phosphorylation of downstream molecules (whether tyrosine or serine/threonine phosphorylated) was similarly affected by disaggregation of FcεRI. As shown in Figure 4b, Syk (Y<sup>519/520</sup>) and LAT1 (Y<sup>191</sup>) phosphorylation was markedly affected by hapten addition whereas that of Akt (T<sup>308</sup>) was largely unaffected. This suggested that phosphorylation of Akt on T<sup>308</sup> was less susceptible to dephosphorylation upon disaggregation of FcεRI.

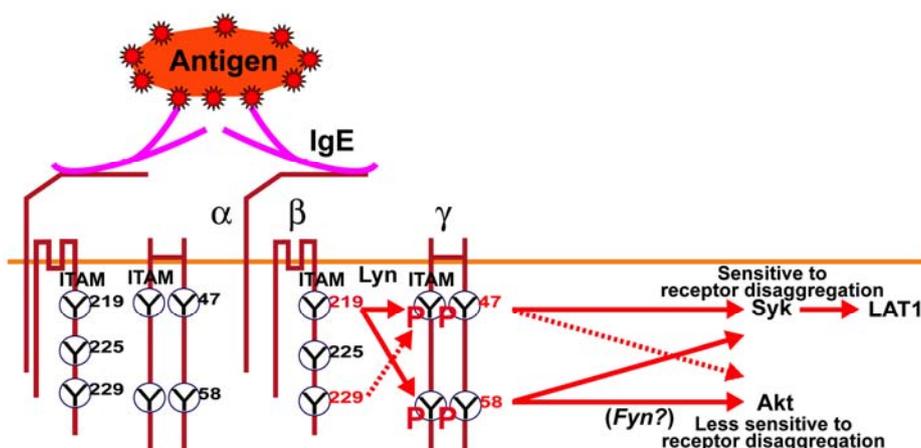
**Figure 4.** Akt phosphorylation is largely initiated by FcεRIγ ITAM Y<sup>58</sup> but once phosphorylated it does not require persistent FcεRI aggregation. **(a)** WT cells were sensitized with IgE and stimulated with Ag. At 30 s of Ag addition hapten (DNP-L-Lys, 100 μM) was added in the presence or absence of pervanadate (PV: 1 mM) and cells were incubated for the indicated time. Whole cell lysates were resolved and immunoblotted with γ-pY<sup>47</sup> antibody. **(b)** Effects of FcεRI disaggregation on proximal signaling proteins as analyzed using indicated antibodies. **(c)** IgE-sensitized BMMCs expressing WT (YY) or mutated (YF, FY) FcεRIγ were stimulated for 1 min with Ag. Whole cell lysate proteins were resolved by SDS-PAGE and immunoblotted with indicated antibodies. **(d)** BMMCs expressing WT or mutant FcεRIγ were sensitized with IgE and were either not stimulated or stimulated with Ag in the presence or absence of pervanadate (PV: 1mM). Proteins in whole cell lysates were resolved and immunoblotted with phospho-specific (T<sup>308</sup>) Akt antibody.



To assess the importance of the individual FcεRIγ ITAM tyrosine's to Akt T<sup>308</sup> phosphorylation we used BMMCs expressing FcεRIγ ITAM tyrosine mutants (Y<sup>47</sup>F or Y<sup>58</sup>F). We first verified that mutation at either of these sites had an impact on Syk and LAT1 phosphorylation as previously described [12]. As shown in Figure 4c, phosphorylation of Syk Y<sup>519/520</sup> and LAT1 Y<sup>191</sup> was dramatically reduced in BMMCs expressing either Y<sup>47</sup> or Y<sup>58</sup> mutant FcεRIγ. In contrast, analysis of Akt T<sup>308</sup> phosphorylation revealed a modest decrease (<35%) in BMMCs expressing the FY mutant form of FcεRIγ, whereas cells expressing the YF form of FcεRIγ showed a marked reduction (~80%) in Akt phosphorylation (Figure 4d, left panel). These findings suggested that initiation of Akt phosphorylation

is primarily dependent on the phosphorylation of FcεRIγ Y<sup>58</sup>. We next investigated whether phosphorylation of FcεRIγ Y<sup>58</sup> in initiating Akt phosphorylation could be by-passed by inhibiting tyrosine phosphatases. As shown in Figure 4d, right panel, treatment of cells carrying the YF mutant form of FcεRIγ with pervanadate, results in the restoration of Akt T<sup>308</sup> phosphorylation demonstrating that the key steps for PI3K-dependent Akt phosphorylation are downstream of FcεRIγ Y<sup>58</sup> phosphorylation. These findings are consistent with the view that FcεRIγ Y<sup>58</sup> is key in initiating FcεRI-dependent Akt phosphorylation (see Model, Figure 5) and this occurs upstream of the key signals (like Fyn kinase) that are essential for the activation of Akt [14]. This is also consistent with the finding that the absence of Lyn, which causes an increase in Fyn kinase activity [29], results in defective FcεRI phosphorylation but enhanced Akt phosphorylation. In contrast, Fyn-deficiency does not affect FcεRI phosphorylation but causes a marked dampening of Akt phosphorylation [14].

**Figure 5.** Schematic model of the regulatory role of FcεRIβ and γ ITAM tyrosine's on downstream signaling in mast cells. Ag-dependent aggregation of IgE antibody-occupied FcεRI on mast cells results in the Lyn kinase-dependent phosphorylation of the FcεRIβ and γ ITAM tyrosine's. The FcεRIβY<sup>219</sup> is important for the full phosphorylation of FcεRIγ and was previously shown to be important for Lyn association with FcεRI [6]. Loss of phosphorylation at FcεRIγ Y<sup>47</sup> or Y<sup>58</sup> had a marked effect on Syk and LAT1 phosphorylation; molecules known to be important for mast cell degranulation. However, Akt phosphorylation (T<sup>308</sup>), whose activity is required for cytoskeletal reorganization and gene expression, was largely unaffected by loss of phosphorylation at FcεRIγ Y<sup>47</sup> whereas loss of phosphorylation at FcεRIγY<sup>58</sup> greatly reducedAkt phosphorylation. This can be overcome by inhibition of tyrosine phosphatases, which fully restored Akt phosphorylation in FcεRIγ Y<sup>58</sup>F expressing mutant mast cells. Previous findings demonstrate that Fyn kinase is critical for Akt phosphorylation, thus we propose that FcεRIγY<sup>58</sup> is an upstream regulator of the Fyn-Gab2-Akt pathway.



### 3. Experimental Section

#### 3.1. Generation of Phospho-Specific Antibody to the Phosphorylated FcR $\gamma$ Y<sup>47</sup> ( $\gamma$ -pY<sup>47</sup>)

Antigen peptide (DAVpYTGLNTRSQETC) was conjugated with KLH as immunogen and mice were immunized with the conjugated peptide. Hybridoma generation was performed by GenScript (Piscataway, NJ, USA). Enzyme-linked immunosorbent assays (ELISAs) were used for primary screening of the resulting hybridomas using culture supernatants. Non-phosphorylated peptide (DAVYTGLNTRSQETC) was used as a negative control.

#### 3.2. Animals

Mice used in this study were wild-type (WT), *B6-Ms4a2<sup>tm1Knt/J</sup>* (*FcR $\beta$ <sup>-/-</sup>*), and *B6.129-Fcer1 $\gamma$ <sup>tm1Rav</sup>* (*FcR $\gamma$ <sup>-/-</sup>*) mice were obtained from Jackson Laboratory or Taconic Farms. Animals were maintained and used according to National Institutes of Health guidelines and a National Institute of Arthritis and Musculoskeletal and Skin Diseases-approved animal study proposal.

#### 3.3. Human Blood Samples

Samples were collected from adult subjects enrolled in a long-term natural history study of SLE. The study was approved by the Institutional Review Board of National Institute of Arthritis and Musculoskeletal and Skin Diseases. All subjects fulfilled the American College of Rheumatology classification criteria for SLE. Control samples were obtained from healthy blood donors. All subjects provided written informed consent.

#### 3.4. General Reagents

Mouse anti-2,4-dinitrophenol (DNP) monoclonal IgE antibody was purified from culture supernatants of the H1-DNP- $\epsilon$ -26.82 hybridoma [30]. DNP<sub>30-40</sub>-HSA, DNP-L-Lysine, sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), and Hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal antibody to Fc $\epsilon$ RI ITAM $\beta$  (clone: JRK) was previously described [31]. Rabbit polyclonal antibody to Lyn and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Syk (Y<sup>519/520</sup>), anti-phospho-Akt (T<sup>308</sup>), and anti-phospho-LAT1 (Y<sup>191</sup>) antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Anti-mouse IgE antibody was purchased from SouthernBiotech (Birmingham, AL, USA). Anti-phosphotyrosine 4G10 mAb was from Upstate Biotechnology (Lake Placid, NY, USA). APC-conjugated anti-human CD123, Pacific Blue-conjugated anti-human Fc $\epsilon$ RI $\alpha$ , and PercPCy5.5 conjugated anti-human CD203c were purchased from Biolegend (San Diego, CA, USA). PE-conjugated anti-human CD303 was purchased from MiltenyiBiotec (Auburn, CA, USA).

#### 3.5. Cell Cultures

Total bone marrow from the femurs of wild-type (WT); *FcR $\beta$ <sup>-/-</sup>*; and *FcR $\gamma$ <sup>-/-</sup>* mice was extracted and used to obtain cultures of bone marrow-derived mast cells (BMMCs). Cells were cultured for 4 weeks in the presence of IL-3 and stem cell factor (PeproTech, Rocky Hill, NJ, USA) [12,20,32].

Cultures were checked periodically for the extent of mast cell differentiation (CD117<sup>+</sup>) and purity by FcεRI expression. Cells were used when cultures achieved ≥95% of the population expressing both markers.

### 3.6. Viral Transduction of BMMCs with Mutant FcεRI Genes

For expression of FcεRIβ mutants, lentivirus gene transduction was used as previously described [20,32]. Viral supernatants were produced by transfecting the packaging cell 293LTV with β-chain mutants in the pLenti6 vector using Lipofectamine 2000 (Invitrogen). Some cells were also mock-transfected with LacZ/pLenti6 vector as a negative control. For expression of FcεRIγ mutants, viral supernatants were produced by transfecting the retroviral packaging cells Phoenix-E with wild type or mutated FcRγ in the pMX-puro vector as previously described [12,20]. Empty pMX-puro vector was used as a negative control. After infection, cells were washed and allowed to grow in IL-3- and SCF-containing medium for 2 days before initiating the selection of transduced cells with 8 μg/mL of blasticidin S (Invitrogen) and 3 μg/mL of puromycin (Invitrogen) for lentiviral- or retroviral-transduced cells, respectively. Following 2 weeks of selection, cells were analyzed for FcεRI expression and used when >95% of the cells expressed this receptor.

### 3.7. Ag stimulation, Immunoprecipitation, and Western Blotting

For Ag-dependent stimulation of BMMCs, cells were sensitized with 1 μg/mL IgE in RPMI 1640 without cytokines at 37 °C for 3 h. Cells were then washed and stimulated at 37 °C with 30 ng/mL Ag (DNP-HSA) or with the indicated concentration in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 20 mM HEPES, 5.6 mM glucose, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 0.05% BSA (pH 7.4)). In some experiments, cells were stimulated with pervanadate (prepared freshly by incubating 100 mM vanadate with 100 mM H<sub>2</sub>O<sub>2</sub>) for 15 min at room temperature, as indicated. For kinetic experiments, Ag-stimulation time varied as indicated. Cell lysates were prepared by incubation of 20 × 10<sup>6</sup> cells in 1 mL BBS buffer containing 1.0% NP-40 and 60 mM Octyl β-d-glucopyranoside (Sigma-Aldrich, St. Louis, MO, USA) on ice for 15 min. Lysates were clarified by centrifugation for 15 min at 14,000 × g at 4 °C and supernatants collected. For Western blotting, the membranes were blocked for 1 h at room temperature with Odyssey blocking buffer (Li-COR Biosciences, Lincoln, NE, USA) diluted two times in PBS. Membranes were then incubated with the indicated primary Abs, followed by secondary Abs linked to infrared fluorescent dyes. After washings with PBS-0.1% Tween-20 (Sigma-Aldrich), the immuno-reactive proteins were visualized with IRDye800-conjugated goat anti-mouse IgG (Rockland Immunochemicals, Boyertown, PA, USA) or Alexa Fluor 680-conjugated goat anti-rabbit IgG (Invitrogen) and detected by an Odyssey infrared imaging system (Li-Cor Biosciences).

### 3.8. Confocal Microscopy

For single cell measurements of phosphorylated FcεRIγITAM Y<sup>47</sup> localization, cells were incubated with Alexa Fluor 568 conjugated IgE and were stimulated with Ag for 1 min. Non-stimulated and Ag-stimulated cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Fixed cells were permeabilized and stained with 0.1% Triton X-100 and

5  $\mu\text{g/mL}$  of  $\gamma\text{-pY}^{47}$  antibody in PBS overnight at 4 °C. Cells were then incubated with Alexa Fluor 488 conjugated secondary antibody for 1 h. Confocal laser fluorescence microscopy, LSM-780 (Carl Zeiss) was used for detection of fluorescence in cells with both Alexa Fluor 488 conjugated secondary antibody and Alexa Fluor 568 conjugated IgE. The fluorescence of Alexa Fluor 488 and Alexa Fluor 568 was detected through a band pass filter (505–530 nm) and a long pass filter (>560 nm), respectively.

For whole-mount immunostaining, the ears of WT mice were sensitized with anti-DNP IgE (75 ng) in the contralateral ear. The next day, mice were retroorbitally challenged with Ag. After 5 min, the mice were euthanized, and the ears were collected. The collected ears were fixed in 1% paraformaldehyde overnight at 4 °C. The tissues were blocked and permeabilized in PBS containing 1% bovine serum albumin and 0.1% Triton X-100 for 1 h, and then incubated with Alexa Fluor 488 conjugated  $\gamma\text{-pY}^{47}$  antibody overnight at 4 °C. Tissues were washed and incubated with Alexa Fluor 647 conjugated avidin for 1 h at 4 °C. After washing with PBS, tissue samples were embedded on a slide using Fluoromount G (SouthernBiotec). The fluorescence of Alexa Fluor 488 and Alexa Fluor 647 was detected through a band pass filter (505–530 nm) and a long pass filter (>650 nm), respectively.

### 3.9. Flow Cytometry

Blood samples from healthy donors or active SLE patients were harvested in heparin-coated tubes. Samples (1 mL) were stimulated for 2 min at 37 °C with anti-human IgE antibody (1:100 dilution: Beckman Coulter). The cells were immediately fixed with 20  $\times$  volumes of pre-warmed BD<sup>TM</sup>Phosphoflow Lyse/Fix Buffer (BD biosciences) and incubated for 10 min at 37 °C and pelleted by centrifugation (500  $\times$  g, 10 min). Cells were then washed in PBS and resuspended in 1 mL of FACS buffer (PBS supplemented with 1.0% BSA, 0.05% NaN<sub>3</sub>). Cells were subsequently processed for extracellular staining with the surface markers: APC-conjugated anti-human CD123, PE-conjugated anti-human CD303, Pacific Blue-conjugated anti-human Fc $\epsilon$ RI $\alpha$ , and PercPCy5.5-conjugated anti-human CD203c. After washing with PBS, cells were permeabilized by adding 200  $\mu\text{L}$  of BD<sup>TM</sup>Phosflow Perm Buffer III (BD biosciences) and incubated for 30 min on ice. Then Alexa Fluor 488 conjugated  $\gamma\text{-pY}^{47}$  antibody was added. Human basophils were identified as Fc $\epsilon$ RI $\alpha$ <sup>+</sup>CD303<sup>-</sup>CD123<sup>+</sup> cells. FACSCanto was used for data acquisition and analysis was performed by FlowJo software (Tree Star, Inc.).

## 4. Conclusions

The findings herein demonstrate the sensitivity and selectivity of a novel phospho-specific antibody to Fc $\epsilon$ RI Y<sup>47</sup>, which showed considerable versatility in various applications. Using this antibody, we found that the Fc $\epsilon$ RI $\beta$  Y<sup>219</sup> has a dominant role in Fc $\epsilon$ RI $\gamma$  phosphorylation (Figure 3a). Both Fc $\epsilon$ RI $\gamma$  Y<sup>47</sup> and Y<sup>58</sup> were found to contribute to the phosphorylation of Syk and LAT1, molecules whose phosphorylation requires sustained Fc $\epsilon$ RI aggregation (Figure 4a–c). In contrast, phosphorylation of Akt is largely independent of Fc $\epsilon$ RI $\gamma$  Y<sup>47</sup> whereas Y<sup>58</sup> is important in initiating the phosphorylation of Akt, which, once phosphorylated, is less sensitive to dephosphorylation following Fc $\epsilon$ RI disaggregation (Figure 4a,b,d). Collectively, these findings demonstrate the utility of the  $\gamma\text{-pY}^{47}$  antibody in

determining the phosphorylation status of FcεRI in cells and tissues and further reveal new insights on the contributory role of the FcεRI ITAM tyrosines in downstream signals in mast cells.

### Acknowledgments

We gratefully acknowledge the support of the Animal Care and Use Program and the Light Imaging Section of the Office of Science and Technology, NIAMS.

### Conflict of Interest

The authors declare no conflict of interest.

### References

1. Rivera, J.; Fierro, N.A.; Olivera, A.; Suzuki, R. New insights on mast cell activation via the high affinity receptor for IgE. *Adv. Immunol.* **2008**, *98*, 85–120.
2. Alvarez-Errico, D.; Lessmann, E.; Rivera, J. Adapters in the organization of mast cell signaling. *Immunol. Rev.* **2009**, *232*, 195–217.
3. Rivera, J.; Gilfillan, A.M. Molecular regulation of mast cell activation. *J. Allergy Clin. Immunol.* **2006**, *117*, 1214–1225.
4. Kihara, H.; Siraganian, R.P. Src homology 2 domains of Syk and Lyn bind to tyrosine-phosphorylated subunits of the high affinity IgE receptor. *J. Biol. Chem.* **1994**, *269*, 22427–22432.
5. Benhamou, M.; Ryba, N.J.; Kihara, H.; Nishikata, H.; Siraganian, R.P. Protein-tyrosine kinase p72<sup>syk</sup> in high affinity IgE receptor signaling. Identification as a component of pp72 and association with the receptor  $\gamma$  chain after receptor aggregation. *J. Biol. Chem.* **1993**, *268*, 23318–23324.
6. On, M.; Billingsley, J.M.; Jouvin, M.H.; Kinet, J.P. Molecular dissection of the FcR $\beta$  signaling amplifier. *J. Biol. Chem.* **2004**, *279*, 45782–45790.
7. Nunomura, S.; Kawakami, Y.; Kawakami, T.; Ra, C. The FcR $\beta$ - and  $\gamma$ -ITAMs play crucial but distinct roles in the full activation of mast cells induced by IgE $\kappa$  and Protein L. *J. Immunol.* **2012**, *188*, 4052–4064.
8. Xiao, W.; Nishimoto, H.; Hong, H.; Kitaura, J.; Nunomura, S.; Maeda-Yamamoto, M.; Kawakami, Y.; Lowell, C.A.; Ra, C.; Kawakami, T. Positive and negative regulation of mast cell activation by Lyn via the FcεRI. *J. Immunol.* **2005**, *175*, 6885–6892.
9. Furumoto, Y.; Nunomura, S.; Terada, T.; Rivera, J.; Ra, C. The FcεRI $\beta$  immunoreceptor tyrosine-based activation motif exerts inhibitory control on MAPK and I $\kappa$ B kinase phosphorylation and mast cell cytokine production. *J. Biol. Chem.* **2004**, *279*, 49177–49187.
10. Chen, T.; Repetto, B.; Chizzonite, R.; Pullar, C.; Burghardt, C.; Dharm, E.; Zhao, Z.; Carroll, R.; Nunes, P.; Basu, M.; *et al.* Interaction of phosphorylated FcεRI $\gamma$  immunoglobulin receptor tyrosine activation motif-based peptides with dual and single SH2 domains of p72<sup>syk</sup>. Assessment of binding parameters and real time binding kinetics. *J. Biol. Chem.* **1996**, *271*, 25308–25315.

11. Kimura, T.; Kihara, H.; Bhattacharyya, S.; Sakamoto, H.; Appella, E.; Siraganian, R.P. Downstream signaling molecules bind to different phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) peptides of the high affinity IgE receptor. *J. Biol. Chem.* **1996**, *271*, 27962–27968.
12. Yamashita, T.; Suzuki, R.; Backlund, P.S.; Yamashita, Y.; Yergey, A.L.; Rivera, J. Differential dephosphorylation of the FcR $\gamma$  immunoreceptor tyrosine-based activation motif tyrosines with dissimilar potential for activating Syk. *J. Biol. Chem.* **2008**, *283*, 28584–28594.
13. Yamashita, T.; Mao, S.Y.; Metzger, H. Aggregation of the high-affinity IgE receptor and enhanced activity of p53/56<sup>lyn</sup> protein-tyrosine kinase. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 11251–11255.
14. Parravicini, V.; Gadina, M.; Kovarova, M.; Odom, S.; Gonzalez-Espinosa, C.; Furumoto, Y.; Saitoh, S.; Samelson, L.E.; O'Shea, J.J.; Rivera, J. Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* **2002**, *3*, 741–748.
15. Chung, C.Y.; Potikyan, G.; Firtel, R.A. Control of cell polarity and chemotaxis by Akt/PKB and PI3 kinase through the regulation of PAKa. *Mol. Cell* **2001**, *7*, 937–947.
16. Datta, S.R.; Brunet, A.; Greenberg, M.E. Cellular survival: A play in three Akts. *Genes Dev.* **1999**, *13*, 2905–2927.
17. Lawlor, M.A.; Alessi, D.R. PKB/Akt: A key mediator of cell proliferation, survival and insulin responses? *J. Cell Sci.* **2001**, *114*, 2903–2910.
18. Manning, B.D.; Cantley, L.C. AKT/PKB signaling: Navigating downstream. *Cell* **2007**, *129*, 1261–1274.
19. Kitaura, J.; Asai, K.; Maeda-Yamamoto, M.; Kawakami, Y.; Kikkawa, U.; Kawakami, T. Akt-dependent cytokine production in mast cells. *J. Exp. Med.* **2000**, *192*, 729–740.
20. Alvarez-Errico, D.; Yamashita, Y.; Suzuki, R.; Odom, S.; Furumoto, Y.; Yamashita, T.; Rivera, J. Functional analysis of Lyn kinase A and B isoforms reveals redundant and distinct roles in Fc $\epsilon$ RI-dependent mast cell activation. *J. Immunol.* **2010**, *184*, 5000–5008.
21. Tinti, M.; Nardoza, A.P.; Ferrari, E.; Sacco, F.; Corallino, S.; Castagnoli, L.; Cesareni, G. The 4G10, PY20 and p-TYR-100 antibody specificity: Profiling by peptide microarrays. *New Biotechnol.* **2012**, *29*, 571–577.
22. Tharp, M.D.; Seelig, L.L., Jr.; Tigelaar, R.E.; Bergstresser, P.R. Conjugated avidin binds to mast cell granules. *J. Histochem. Cytochem.* **1985**, *33*, 27–32.
23. Heib, V.; Becker, M.; Warger, T.; Rechtsteiner, G.; Tertilt, C.; Klein, M.; Bopp, T.; Taube, C.; Schild, H.; Schmitt, E.; *et al.* Mast cells are crucial for early inflammation, migration of langerhans cells, and CTL responses following topical application of TLR7 ligand in mice. *Blood* **2007**, *110*, 946–953.
24. Charles, N.; Hardwick, D.; Daugas, E.; Illei, G.G.; Rivera, J. Basophils and the T helper 2 environment can promote the development of lupus nephritis. *Nat. Med.* **2010**, *16*, 701–707.
25. Pribluda, V.S.; Pribluda, C.; Metzger, H. Transphosphorylation as the mechanism by which the high-affinity receptor for IgE is phosphorylated upon aggregation. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 11246–11250.
26. Eiseman, E.; Bolen, J.B. Engagement of the high-affinity IgE receptor activates *src* protein-related tyrosine kinases. *Nature* **1992**, *355*, 78–80.

27. Kitaura, J.; Song, J.; Tsai, M.; Asai, K.; Maeda-Yamamoto, M.; Mocsai, A.; Kawakami, Y.; Liu, F.T.; Lowell, C.A.; Barisas, B.G.; *et al.* Evidence that IgE molecules mediate a spectrum of effects on mast cell survival and activation via aggregation of the FcεRI. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 12911–12916.
28. Mao, S.Y.; Metzger, H. Characterization of protein-tyrosine phosphatases that dephosphorylate the high affinity IgE receptor. *J. Biol. Chem.* **1997**, *272*, 14067–14073.
29. Hernandez-Hansen, V.; Smith, A.J.; Surviladze, Z.; Chigaev, A.; Mazel, T.; Kalesnikoff, J.; Lowell, C.A.; Krystal, G.; Sklar, L.A.; Wilson, B.S.; *et al.* Dysregulated FcεRI signaling and altered Fyn and SHIP activities in Lyn-deficient mast cells. *J. Immunol.* **2004**, *173*, 100–112.
30. Liu, F.T.; Bohn, J.W.; Ferry, E.L.; Yamamoto, H.; Molinaro, C.A.; Sherman, L.A.; Klinman, N.R.; Katz, D.H. Monoclonal dinitrophenyl-specific murine IgE antibody: Preparation, isolation, and characterization. *J. Immunol.* **1980**, *124*, 2728–2737.
31. Rivera, J.; Kinet, J.P.; Kim, J.; Pucillo, C.; Metzger, H. Studies with a monoclonal antibody to the β subunit of the receptor with high affinity for immunoglobulin E. *Mol. Immunol.* **1988**, *25*, 647–661.
32. Suzuki, R.; Liu, X.; Olivera, A.; Aguiniga, L.; Yamashita, Y.; Blank, U.; Ambudkar, I.; Rivera, J. Loss of TRPC1-mediated Ca<sup>2+</sup> influx contributes to impaired degranulation in Fyn-deficient mouse bone marrow-derived mast cells. *J. Leukoc. Biol.* **2010**, *88*, 863–875.

© 2013 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (<http://creativecommons.org/licenses/by/3.0/>).