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Increased Expression of Genes Linked to FcεRI Signaling and to Cytokine and Chemokine Production in Lyn-Deficient Mast Cells

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Cross-linking the high-affinity IgE receptor, FcεRI, on mast cells activates signaling pathways leading to the release of preformed inflammatory mediators and the production of cytokines and chemokines associated with allergic disorders. Bone marrow-derived mast cells (BMMCs) from Lyn-deficient (Lyn−/−) mice are hyperresponsive to FcεRI cross-linking with multivalent Ag. Previous studies linked the hyperresponsive phenotype in part to increased Fyn kinase activity and reduced SHIP phosphatase activity in the Lyn−/− BMMCs in comparison with wild-type (WT) cells. In this study, we compared gene expression profiles between resting and Ag-activated WT and Lyn−/− BMMCs to identify other factors that may contribute to the hyperresponsiveness of the Lyn−/− cells. Among genes implicated in the positive regulation of FcεRI signaling, mRNA for the tyrosine kinase, Fyn, and for several proteins contributing to calcium regulation are more up-regulated following Ag stimulation in Lyn−/− BMMCs than in WT BMMCs. Conversely, mRNA for the low-affinity IgG receptor (FcγRIIβ), implicated in negative regulation of FcεRI-mediated signaling, is more down-regulated in Ag-stimulated Lyn−/− BMMCs than in WT BMMCs. Genes coding for proinflammatory cytokines and chemokines (IL-4, IL-6, IL-13, CSF, CCL1, CCL3, CCL5, CCL7, CCL9, and MIP1β) are all more highly expressed in Ag-stimulated Lyn−/− mast cells than in WT cells. These microarray data identify Lyn as a negative regulator in Ag-stimulated BMMCs of the expression of genes linked to FcεRI signaling and also to the response pathways that lead to allergy and asthma. The Journal of Immunology, 2005, 175: 7880–7888.

Materials and Methods

**Abs and reagents**

Monoclonal mouse anti-DNP IgE was purified from ascites as described in Liu et al. (14). Biotinylated anti-DNP IgE was prepared using EZ-Link Sulfo-norlormal horse serum-Biotin (Pierce). Biotinylated anti-CD117 (c-kit) mAb and the isotype control, biotinylated rat anti-mouse IgG2B, were purchased from Caltag Laboratories.

**Cell culture and activation conditions**

WT and Lyn knockout mice (12) on a C57BL/6 background were bred in specific pathogen-free facilities in the University of New Mexico Animal Research Facility (Albuquerque, NM). BMMCs were obtained by culturing bone marrows from 8- to 12-wk WT and Lyn−/− mice as described in Hernandez-Hansen et al. (6, 7). Briefly, BMMCs were obtained by culturing mouse bone marrow cells in RPMI 1640 medium, supplemented with
10% FCS (HyClone), 2 mM t-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 55 μM 2-ME, and 1 mM HEPES (complete RPMI medium) and 30% WEHI-3-conditioned medium. Culture reagents were from Invitrogen Life Technologies. Mast cell morphology, granularity, and differentiation were analyzed by toluidine blue-stained cytopsin preparations and flow cytometry as described previously (6). By 6 wk, WT and Lyn+/− mast cells expressed similar levels of FcεRI and c-kit at their surfaces and were morphologically similar with respect to granule content. Microarray experiments were carried out on 6-wk-old mast cells. For stimulation, WT and Lyn+/− BMMCs were sensitized with 1 g/ml anti-DNP IgE overnight (12 h) in complete RPMI medium without WEHI-3 medium. BMMCs were harvested, washed, and stained with trypan blue to verify viability. Viability was >95% for all experimental conditions. BMMCs were resuspended in WEHI-3 RPMI medium and activated by the addition of 10 ng/ml DNP-BSA for 2 or 4 h at 37°C.

Isolation and labeling of mast cell RNA

Total RNA was prepared from resting and activated WT and Lyn+/− BMMCs using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. Further purification of RNA was done using the RNeasy mini kit clean-up protocol (Qiagen). A total of 10 μg of total RNA was converted to cDNA using the Superscript II RNA reverse transcription kit (Invitrogen Life Technologies). Double-strand cDNA was produced with an oligo(dT) primer containing the T7 RNA polymerase site at the 5′ end. The cDNA was labeled with biotinylated nucleotides directly with the ENZO in vitro labeling kit (Affymetrix) to produce antisense RNA. Labeled cRNA was fragmented and purified using Agilent RNA chips (Agilent Technologies). A total of 15 μg of labeled cRNA was used to prime cDNA synthesis for U74A murine Genechips (Affymetrix) containing 12,488 genes and expressed sequence tags (ESTs). A mixture of bacterial RNA (Biobd, bioc, biod, cre) with known concentrations (1, 5, 25, and 100 pM, respectively) was added to each chip hybridization mixture. Chips were washed, stained with PE-streptavidin and read with an HP Gene Array Scanner according to the manufacturer’s instructions.

DNA microarray analysis

All Affymetrix chips were analyzed in the Affymetrix MAS 5.0 software to a target fluorescence of 500 and had scaling factors ranging from 2 to 15, indicating good probe preparation and hybridization. Filtering and statistical analyses of microarray data were performed using Genespring software version 6.0 (Silicon Genetics). For all statistical analyses, the experiment interpretation mode was set to the log of ratios and data were normalized per gene to the median of the appropriate unsupervised duplicate samples. For analysis of differential gene expression between stimulated WT and Lyn+/− BMMCs, data were normalized so that the baseline mRNA expression corresponded to the median of the stimulated WT BMMCs. To evaluate changes in gene expression, data were filtered in Genespring for genes expressing a 3-fold up or down change in expression when compared to the control samples. A Flag filter was applied to exclude those data in which a gene’s expression was absent or marginally present in at least half of the samples based on the Affymetrix algorithm. Finally, the one-way ANOVA filter (p < 0.05) was used to identify statistically significant differences in gene expression between resting and stimulated samples. For generation of heat maps, lists of regulated genes were clustered with the gene tree method by measuring similarity with smooth correlation. Statistical analyses were performed with two-way ANOVA to identify strain and/or treatment interactions for all eight samples. Experiments were performed twice with triplicate samples (cDNA isolated from three separate cultures of BMMCs). Statistical analysis was performed with the Student unpaired t test using GraphPad Prism software.

Cytokine ELISA

Supernatants were collected from resting and activated WT and Lyn+/− BMMCs and analyzed for IL-4 or IL-13 using a two-site sandwich ELISA as described previously (15). Abs specific for IL-4 (11B11, and biotin-BVD6-24G2) were from BD Pharmingen; Abs specific for IL-13 (38213.11, and biotin-BAF413) were from R&D Systems. Capture mAbs were biotinylated ELISA MAbs with a final concentration of 1 μg/ml, and detection Abs were peroxidase-conjugated swine anti-mouse (Kirkegaard & Perry Laboratories). Plates were washed, blocked with 1% BSA in PBS, and incubated overnight at 4°C with samples. Bound cytokines were detected by the addition of biotinylated mAbs followed by streptavidin-HRP (0.625 μg/ml final concentration for IL-13; 2.5 μg/ml final concentration for IL-4) and colloformic substrate (ABTS for IL-4; tetramethylbenzidine (TMB); Sigma) for 30 min. OD405 was determined for IL-13 and OD492 for IL-4. OD492 was read on a microplate reader (Biochrom 2010) at 492 nm, and OD405 was read on a microplate reader (Biorad Diaflex) at 405 nm. Supernatants were tested in triplicate with two concentrations of Abs per sample. Levels of cytokine were calculated using a standard curve generated using rIL-4 (BD Pharmingen) and rIL-13 (R&D Systems). Supernatants were analyzed with commercial ELISA kits for IL-6 (BD Biosciences) and for IL-2 and TNF-α (eBioscience) according to the manufacturer’s instructions. Detection limits for each cytokine assay were calculated as the lowest concentration in the linear portion of the standard curve. Measurements were made in duplicate using cells from three independent cultures of BMMCs.

Results

Different gene expression profiles of resting WT and Lyn+/− BMMCs

Fig. 1 and Table I summarize the principal differences in gene expression between WT and Lyn+/− BMMCs under resting conditions. The data are strikingly similar between the duplicate samples, indicating that the technical procedures from RNA preparation to data acquisition and analysis are highly reproducible. Expression levels for the mRNAs encoding the receptors CXCR4 and insulin-like growth factor 2 receptor (IGF2R) are higher in Lyn+/− BMMCs than in WT BMMCs. In addition, annexin A1 that inhibits PLACA activity in vitro and phospholipase A2 group VII are expressed at higher levels in Lyn+/− BMMCs. Levels of several other mRNAs are consistently lower in Lyn+/− BMMCs.
and Lyn. Eight separate samples representing two separate experiments for
Table I.

To identify other differentially transcribed genes between WT and Lyn deficiency alters the profile of Ag-induced gene expression lack known roles in signal transduction pathways.

FIGURE 1. The absence of Lyn alters the gene expression profile of resting mast cells. Two independent cultures of 6-wk-old WT and Lyn−/− BMMCs were incubated in complete RPMI without growth factors for 16 h. Cells were harvested, their viability was confirmed by trypan blue exclusion, and total mRNA was isolated. Biotinylated antisense cRNA was hybridized to Affymetrix U74A DNA chips for 16 h. DNA chips were washed and scanned with an Affymetrix scanner. Data were normalized so that the level of baseline mRNA expression corresponded to the median of unstimulated WT BMMCs. The heat map represents 14 genes that were up- or down-regulated by a factor of 3 or greater and was generated using a stringent filter scheme that incorporated the ANOVA filter. Each row corresponds to a single gene and each column represents an independent condition. Location of the gene and common name are provided. Changes in gene expression correspond to the color scale shown.

compared with WT BMMCs; in general, the down-regulated genes lack known roles in signal transduction pathways.

Lyn deficiency alters the profile of Ag-induced gene expression

To identify other differentially transcribed genes between WT and Lyn−/− BMMCs, we compared the gene expression profiles of eight separate samples representing two separate experiments for each of four conditions: resting and Ag-stimulated from both WT and Lyn−/− cells. Two-way ANOVA was used to identify genes whose expression levels were significantly altered based on kinase status (±Lyn), treatment (±Ag), or on the interaction of both parameters (kinase status and treatment). Through this analysis, we identified 501 gene products with a Benjamini-Hochberg adjusted p value <0.05, indicating their expression is statistically different due to the absence of Lyn, the presence of Ag, or the interaction of both parameters. Fig. 2 provides two alternative graphical representations of these results.

The data on 501 gene products are shown in Fig. 2A as a heat map that compares all eight samples, normalized to the median of the two unstimulated WT samples. The striking similarity between the duplicate samples is again evident. Gene expression is moderately altered between unstimulated samples and is greatly altered in Ag-stimulated samples, with almost as many genes showing decreased expression (blue) as increased expression (red).

The Venn diagram (Fig. 2B) displays the numbers of these gene products whose p values are influenced by each parameter independently (kinase status or treatment) and also by the effect that each parameter has on the other. From a total of 145 genes that display a kinase effect, 27 are significant due to the kinase effect only while 2 genes displayed a kinase effect and an interaction effect (Fig. 2B, left circle). Another 470 genes displayed a treatment effect (Fig. 2B, right circle). Of those, 85 genes are affected by both kinase status and treatment but each factor affects gene expression independently of one another. As shown in Fig. 2B, lower circle, there are 31 total genes that have p values <0.05 for each of the tests: kinase effect, interaction effect, and treatment effect. In addition, 14 genes are significant due to the treatment effect and interaction effect but not the kinase effect. Two genes that are not significant in either the kinase test or treatment test alone had a p value <0.05 for the interaction effect.

Table II organizes a subset of these 501 gene products into functional categories and shows their fold change in expression after FcεRI cross-linking in both WT and Lyn−/− cells. A complete list of these genes is available at (www.cellpath.unm.edu).

Among genes involved in tyrosine kinase-coupled signaling, levels of Fyn mRNA are increased in both WT and Lyn−/− BMMCs after FcεRI cross-linking, with the increase being greater

Table I. Significantly changed genes between unstimulated WT and Lyn−/− mast cells

| Common Name | Description | GenBank | Fold Change in Lyn−/− Cells |
|-------------|-------------|---------|-----------------------------|
| Receptors   |             |         |                             |
| CXCR4       | Chemokine (CXC motif) receptor 4 | Z80112 | 13.5                        |
| IGF2R       | Insulin-like growth factor 2 receptor | U04710 | 4.4                         |
| Lipid metabolism |         |         |                             |
| PLA2G7      | Phospholipase A2 group VII | U34277 | 4.7                         |
| Calcium regulation |       |         |                             |
| ANXA1       | EST, Annexin A1 | AV003419 | 5.1                        |
| ANXA1       | Annexin A1 (phospholipase A2 inhibitor activity) | M69260 | 13.5                        |
| Proteolysis and peptidolysis |       |         |                             |
| EGFBP1      | Epidermal growth factor binding protein type 1 | M17979 | 11.8                        |
| KLK9        | Major epidermal growth factor binding protein | M17962 | 5.6                         |
| Defense response |     |         |                             |
| CD24A       | M1/69-J11d heat stable antigen | M58661 | 0.26                        |
| Transcription |          |         |                             |
| KROX-24     | Zinc finger protein | M28845 | 0.32                        |
| EST         | EST         | AW123567 | 0.22                      |
| ARCNI       | Archain 1   | A1853439 | 0.12                      |
| 1810009A16Rik | EST         | AV086272 | 0.19                      |
| D6Ertd365e  | EST         | AA796868 | 0.25                      |
| Others      | Overexpressed in testicular tumors | L29441 | 0.23                      |

* Data were normalized to the median baseline expression of unstimulated WT cells and analyzed with the one-way ANOVA filter. One-way ANOVA analysis established that the fold change in Lyn−/− cells compared to WT cells was statistically significant (p < 0.05) for each mRNA.
in Lyn<sup>−/−</sup> cells (Table II). In contrast, mRNA expression for the low-affinity IgG receptor FcγRIIB is significantly reduced after Ag stimulation in both WT cells and Lyn<sup>−/−</sup> cells, with the extent of down-regulation being greater in the Lyn<sup>−/−</sup> BMMCs. Recent studies implicate Fyn in Lyn-independent signaling in activated mast cells (2) and FcγRIIB in the negative regulation of FceRI signaling (16, 17). Thus, both observations may be relevant to the hyperresponsiveness of Lyn<sup>−/−</sup> BMMCs. Several genes implicated in calcium regulation, including the type I inositol-1,4,5-trisphosphate receptor (Ins(1,4,5)P<sub>3</sub> receptor 1), sarco(endo)plasmic reticulum calcium ATPase 2 (SERCA2), and sphingosine kinase-1, are also significantly up-regulated in Ag-stimulated Lyn<sup>−/−</sup> BMMCs compared with control cells and could contribute to cellular hyperresponsiveness.

A consistent trend of higher up-regulation in activated Lyn<sup>−/−</sup> mast cells extends to other genes that may contribute to signaling pathway activities. These include genes involved in G protein-coupled signal transduction and cellular retinoic acid-binding protein II (CRABP2) (Table II). Additionally, expression of mRNA for at least one transcription factor, the NF-ATc isoform, is up-regulated in Lyn<sup>−/−</sup> mast cells. In contrast, phospholipid scramblase 2 (PLSCR2) mRNA is only up-regulated 2.1-fold in Lyn<sup>−/−</sup> mast cells compared to 17.1-fold in WT cells. The most striking differences noted between the two cell types is in the expression of cytokine and chemokine genes that are to be up-regulated upon FceRI aggregation in mast cells and basophils. In general, cytokine and chemokine gene expression is the same in WT and Lyn<sup>−/−</sup> BMMCs under resting conditions. As an exception, the expression of CXCR4 is increased in unstimulated Lyn<sup>−/−</sup> mast cells (Table I). Following FceRI cross-linking (Table II), mRNA levels for the Th2 cytokines IL-4 and IL-13 are up-regulated 18.2- and 81-fold in Lyn<sup>−/−</sup> BMMCs, respectively, compared to 0.7- and 8.7-fold in WT mast cells. In addition, mRNA levels for all chemokines of the CC family, CCL1, CCL3, CCL4 (MIP1β), CCL5 (RANTES), CCL7, and CCL9 (MIP1γ) are significantly higher in activated Lyn<sup>−/−</sup> mast cells than in WT cells. As an exception, mRNA for the platelet-derived growth factor-inducible protein (CCL2) is up-regulated in both cell types to similar levels upon FceRI cross-linking.

Validation of microarray data

Microarray data were validated for selected genes by either real-time quantitative PCR or ELISAs. Fig. 3 shows the mRNA expression of Fyn, IL-13, and actin relative to that of unstimulated WT cells. Fyn and IL-13 mRNA expression increased in activated BMMCs (WT, 4.9 ± 0.9-fold; Lyn<sup>−/−</sup>, 8.7 ± 0.2-fold, mean ± SEM, n = 6; p < 0.05) and (WT, 5.9 ± 2.4-fold; Lyn<sup>−/−</sup>, 36.5 ± 3.0-fold; mean ± SEM, n = 6, p < 0.005), respectively (Fig. 3, A and B). In contrast, there was no significant difference in actin mRNA expression after cross-linking FceRI (WT, 1.2 ± 0.1-fold; Lyn<sup>−/−</sup>, 1.2 ± 0.2-fold, mean ± SEM, n = 6; p > 0.05) (Fig. 3C). Standard curves generated from the indicated cDNAs showed a quantitative relationship between cDNA copy number and fluorescence signal intensity (data not shown).

Fig. 4 shows that cytokines are increased at the protein level in Ag-stimulated Lyn<sup>−/−</sup> mast cells. Lyn<sup>−/−</sup> BMMCs secrete significantly more IL-4 than control cells at 4 h after FceRI cross-linking (Fig. 4A). Similarly, FceRI-mediated release of both IL-6 and IL-13 is significantly higher in Lyn<sup>−/−</sup> BMMCs at 2 and 4 h of activation (Fig. 4B and C).

Previous studies suggest that both IL-2 and TNF-α production are increased in Ag-stimulated Lyn<sup>−/−</sup> BMMCs (5). Both Ag-stimulated WT and Lyn<sup>−/−</sup> cells secrete very little IL-2, however, we note a slight increase in IL-2 production in Lyn<sup>−/−</sup> BMMCs after 4 h of Ag stimulation compared with WT mast cells (Fig. 4D). We confirmed that Lyn<sup>−/−</sup> mast cells secrete more TNF-α at both 2 and 4 h of activation than WT cells (Fig. 4E).

Discussion

The cross-linking of IgE-FceRI complexes with multivalent Ag activates mast cell signaling pathways leading to the release of a wide range of proinflammatory cytokines, chemokines, and other
mediators associated with allergic responses. Activated mast cells release IL-4, IL-13, and other Th2 cytokines that enhance the IgE response by stimulating the differentiation of Th2 lymphocytes and promoting class switching to IgE in B cells, leading to increased production of IgE and resensitization of FceRI on mast cells (reviewed in Ref. 18).

Previously, we showed that BMMCs from Lyn−/− mice divide faster than WT cells in response to growth-promoting cytokines (IL-3 and stem cell factor) and undergo less apoptosis when cytokine is withdrawn (6). We and others (2, 5, 7, 8, 19) have also shown that Lyn−/− BMMCs are slow to initiate responses to FceRI cross-linking but are deficient in the termination of signaling, resulting in prolonged biochemical responses (receptor phosphorylation, the activation of AKT, phospholipase Cγ, ERK, and other signaling enzymes) and exaggerated physiological responses (calcium mobilization, secretion, cytokine production, and integrin activation). Resolving previous controversy (reviewed in Refs. 6 and 7), these results now provide a consensus view of Lyn as both a kinetic accelerator and negative regulator of FceRI signaling in mast cells. Previous work has also provided partial insight into the mechanism of the hyperresponsiveness of Lyn−/− BMMCs. Specifically, increased basal and Ag-induced Fyn activity is thought to contribute to signal initiation in Lyn-deficient cells (7, 8), while the loss of Ag-induced SHIP activation provides at least a partial explanation for the failure of signal termination in Lyn−/− cells (7).

Previous groups have used microarray analyses to explore transcriptional profiles induced by FceRI cross-linking in normal and secretion-impaired rodent and human mast cells and basophils (20–26). Here, microarray analysis was used to discover new properties of both unstimulated and Ag-stimulated Lyn-deficient BMMCs that might provide further insight into their hyperresponsive phenotype.

Relatively few genes showed strongly different expression levels between unstimulated WT and Lyn−/− BMMCs and none could be clearly linked to the enhanced early responses (calcium mobilization, integrin activation, degranulation, and others) to FceRI cross-linking. The chemokine receptor, CXCR4, is a possible exception. CXCR4 was strongly up-regulated in Lyn−/− BMMCs. Ligation of G protein-coupled receptors can prime mast cells for increased FceRI-mediated degranulation (27).

In contrast, expression of 501 genes was increased or decreased at least 3-fold after 4 h of FceRI cross-linking. Of these

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**Table II. Differentially regulated genes between resting and Ag-stimulated WT or resting and Ag-stimulated Lyn−/− mast cells**

| Common Name | Gene Description | GenBank Acc. No. | Fold Change WT (+Ag) | Fold Change Lyn−/− (+Ag) |
|-------------|------------------|------------------|----------------------|------------------------|
| Cytokine or chemokine | | | | |
| IL-4 | Interleukin 4 | X03532 | 0.7 | 18.2 |
| IL-6 | Interleukin 6 | X54542 | 5.6 | 22 |
| IL-13 | Interleukin 13 | M23504 | 8.7 | 81 |
| TNF-α | Tumor necrosis factor alpha | D84196 | 13.4 | 12 |
| M-CSF | Macrophage colony-stimulating factor | M21952 | 1.9 | 3.4 |
| GM-CSF | Granulocyte macrophage colony-stimulating factor | X03020 | 0.9 | 43 |
| IL-7R | Interleukin-7 receptor | M29697 | 1.4 | 3.6 |
| CCL1 | Secreted T cell protein, CCL1 | M23501 | 10 | 102 |
| CCL2 | Platelet-derived growth factor-inducible protein | M19681 | 2.7 | 2.5 |
| CCL3 | TCR L2G25B protein | J04491 | 6.5 | 31 |
| CCL5 | RANTES | AF065947 | 2.9 | 79 |
| CCL7 | Cytokine gene | X70508 | 11 | 65 |
| CCL9 | Macrophage inflammatory protein-1 γ | U49513 | 3.7 | 34 |
| MIP1β | Macrophage inflammatory protein-1 β | X62502 | 2.3 | 8.6 |
| CCR1 | MIP1-α/RANTES receptor | U29678 | 2 | 7.4 |
| XCL1 | Lymphotoxin | U15607 | 0.5 | 31 |
| Signal transduction | | | | |
| Fyn | p59^fyn | M27266 | 5.7 | 9.7 |
| CISH | Cytokine SH2-containing protein | D89613 | 2.2 | 5.9 |
| FCγRIIB | Low affinity IgG receptor IIB | M31312 | 0.4 | 0.1 |
| Calcium regulation | | | | |
| CDH2 | Neural cadherin (N-cadherin) | M31131 | 4 | 3.4 |
| SERCA2 | Sarco(endo)plasmic reticulum calcium ATPase | AF029982 | 3.5 | 7.7 |
| CAM1 | Calmodulin | M19381 | 3.1 | 3.1 |
| ITPR1 | Inositol 1,4,5-triphosphate receptor type 1 | X15373 | 2.2 | 5.8 |
| PCD6 | Inositol 1,4,5-triphosphate receptor type 1 | M21530 | 1.2 | 3.6 |
| SPHK1A | Sphingosine kinase 1 a | AF068748 | 1.1 | 33 |
| PLSCR2 | Phospholipid scramblase 2 | AF015790 | 17.1 | 2.1 |
| G-protein signal transduction | | | | |
| CREB1 | Early growth response 2 | U77461 | 4.4 | 5.5 |
| RGS5 | Retinally abundant regulator of G-protein signaling | U94828 | 1.9 | 12 |
| RAMP2 | Receptor activity modifying protein 2 | A250490 | 11 | 7 |
| Transcription factors | | | | |
| EGR2 | Early growth response 2 | M24377 | 39 | 32 |
| MYC | c-myc | L00039 | 9.8 | 8.7 |
| NF-ATC1 | Transcription factor NF-ATc isoform a | AF087434 | 1.8 | 6.2 |
| Others | | | | |
| LGALS3 | Mac-2 Ag | X16834 | 4.5 | 4.2 |
| SCD1 | Stearyl-CoA desaturase | M21285 | 35 | 19 |
| CRABP2 | Cellular retinoic acid-binding protein II | M35523 | 12.8 | 77 |

* Data were normalized to the median baseline mRNA expression of either unstimulated WT mast cells or unstimulated Lyn−/− cells. The genes listed were selected from a list of 501 genes that resulted from analysis by two-way ANOVA.
Increased Fyn activity, perhaps linked in part to delayed activation of Csk-binding protein (Cbp), in Lyn<sup>−/−</sup> BMMCs (7, 8), these data support the hypothesis that increased signaling through the recently discovered Fyn-mediated pathway can contribute to the persistent hyperresponsiveness of Lyn<sup>−/−</sup> BMMCs.

Conversely, FceRI cross-linking induced a greater down-regulation of mRNA for the IgG receptor, FcyRIIB, in Lyn-deficient than in WT BMMCs. FcyRIIB is well-recognized as a negative regulator of FceRI signaling when the two receptors are co-cross-linked. The mechanism involves the recruitment of the inositol phosphatase, SHIP, to the membrane via its association with phosphorylated ITIMs present in FcyRIIB (16, 17). Recent evidence that SHIP-deficient BMMCs degranulate spontaneously and are hyperresponsive to FceRI cross-linking suggests that SHIP also plays a constitutive role in the down-regulation of signaling (28). In this case, reduced levels of FcyRIIB in Lyn-deficient BMMCs may help to maintain the reduced levels and activity of membrane-associated SHIP and the elevated levels of membrane phosphatidylinositol 3,4,5-trisphosphate (PI (3,4,5)P<sub>3</sub>) characteristic of these cells (7).

Among the cytokines expressed after 4 h of Ag stimulation in BMMCs, our data show that levels of mRNA and protein for IL-4, IL-6, and for IL-13 are all significantly higher in Lyn<sup>−/−</sup> than in WT BMMCs. Both IL-4 and IL-13 proteins are elevated in the lungs of asthmatic patients, and are thought to be central regulators of this disease. In mice, recent studies suggest that IL-13 may be more directly involved in mediating allergic responses than IL-4 (29–32). Thus, Ag-exposed Lyn-deficient BMMCs clearly develop a cytokine profile consistent with a predisposition to allergy and asthma.

We failed to see greater increases in TNF-α and IL-2 mRNA levels in Lyn<sup>−/−</sup> cells than in WT mast cells. However, ELISAs showed that Lyn<sup>−/−</sup> BMMCs produce higher amounts of TNF-α and IL-2 protein than WT cells. These results are consistent with previously published data (5). The discrepancies between mRNA levels and TNF-α and IL-2 protein production may be attributed to mRNA instability. We also did not observe robust production of IL-2 in either WT or Lyn<sup>−/−</sup> BMMCs as was reported by Kawakami et al. (5). These different results very likely reflect differences in the time that mast cells were stimulated: our measurements were made after 4 h of activation, while the previous group made measurements after 20 h. Likewise, Nishizumi and Yamamoto (19) reported that Lyn deficiency does not affect the production of cytokines (IL-4, IL-5, IL-6, TNF-α, TNF-β) when BMMCs are stimulated with Ag for 2.5 h. We, too, found rather little differences in IL-4 or IL-6 production when WT and Lyn<sup>−/−</sup> BMMCs were stimulated for 2 h, even though the differences were substantial after 4 h (Fig. 4).

Among the chemokines, mRNAs coding for CCL1, CCL3 (MIP1α), CCL4 (MIP1β), CCL5 (RANTES), CCL7 (MCP-3), and CCL9 (MIP1y) are all up-regulated in Ag-stimulated Lyn<sup>−/−</sup> BMMCs compared with WT BMMCs. The greater induction of both RANTES and its receptor CCR1 may contribute to the hyperresponsiveness of Lyn<sup>−/−</sup> BMMCs via a potential autocrine signaling mechanism. Previous studies have demonstrated a central role for chemokines in mediating multiple aspects of the asthmatic response. Chemokines induce B cell Ab class switching (33). In addition, IL-13 is a potent inducer of chemokines (eotaxins) in airway epithelial cells (31, 34) and current models suggest that coordinated interactions between IL-13 and chemokines are importantly involved in the pathogenesis of asthma (35).

**FIGURE 3.** Transcription of IL-13 and Fyn genes in WT and Lyn<sup>−/−</sup> mast cells is differentially regulated by Ag stimulation. Quantitative detection of Fyn (A), IL-13 (B), and actin (C) mRNA transcripts in resting and activated WT or Lyn<sup>−/−</sup> BMMCs. The copy number of the target gene was normalized using the average copy number of GAPDH as an internal standard. Data were then normalized so that the level of baseline mRNA expression corresponded to the median of unstimulated WT BMMCs. Data are presented as the mean fold induction ± SEM obtained from two independent experiments, each performed in triplicate. Mean values significantly different from WT levels are indicated: *, p < 0.05; **, p < 0.01; ***, p < 0.005. Student’s t test.

Among genes for signaling proteins, FceRI cross-linking induced a greater up-regulation of the tyrosine kinase, Fyn, and of a series of genes implicated in calcium regulation in Lyn-deficient than in WT BMMCs. In combination with earlier evidence for genes, more were up-regulated than down-regulated. In general, the extent of up-regulation was greater in Lyn<sup>−/−</sup> than in WT BMMCs. Thus, there is strong potential for a transcriptional component to the enhanced late responses (cytokine and chemokine production and others) to FceRI cross-linking in the Lyn-deficient cells.

Among genes for signaling proteins, FceRI cross-linking induced a greater up-regulation of the tyrosine kinase, Fyn, and of a series of genes implicated in calcium regulation in Lyn-deficient than in WT BMMCs. In combination with earlier evidence for
Increased expression of cytokine and chemokine mRNA is likely the consequence of increased activity of transcription factors. Our data show that mRNA coding for at least one transcription factor, the cytoplasmic NF-AT (NF-ATC, also known as NFATC1 and NFAT2) is induced 3-fold more in Lyn/H11002/H11002 BMMCs than in WT BMMCs (Table II). In addition, Ag-stimulated Lyn/H11002/H11002 BMMCs express slightly higher levels of NF-kB (Lyn/H11002/H11002, 1.6-fold; WT, 0.8-fold), and slightly lower levels of c-Jun/activator protein-1 (AP-1) (Lyn/H11002/H11002, 2-fold; WT, 4-fold). Studies of the phosphorylation and/or activation of these transcription factors are needed to know if increased levels are linked to increased activities of transcription factors.

The linkage between increased FcεRI signaling and increased gene transcription in Lyn/H11002/H11002 BMMCs is not known with certainty. However, we note that biochemical studies published previously linked reduced SHIP activation (discussed above) to increases in AKT and Ras/MAPK pathway activities (7, 28). Ag-stimulated Lyn/H11002/H11002 BMMCs express slightly higher levels of NF-kB (Lyn/H11002/H11002, 1.6-fold; WT, 0.8-fold), and slightly lower levels of c-Jun/activator protein-1 (AP-1) (Lyn/H11002/H11002, 2-fold; WT, 4-fold). Studies of the phosphorylation and/or activation of these transcription factors are needed to know if increased levels are linked to increased activities of transcription factors.

The 33-fold up-regulation of sphingosine kinase 1 in Ag-stimulated Lyn/H11002/H11002 BMMCs may also contribute to increased chemokine production. Sphingosine kinase is activated upon FcεRI cross-linking in RBL-2H3 cells and mast cells and is linked to calcium mobilization (39–41). Additionally, the product of sphingosine kinase, sphingosine-1-phosphate (S1P), acts as a ligand for G-protein coupled chemokine receptors (42). S1P levels are increased in bronchoalveolar lavage fluid from lungs of asthmatics after challenge with allergen (43) and can lead to a heightened production of chemokines in RBL-2H3 mast cells (44).

Our results in Lyn/H11002/H11002 BMMCs differ from results obtained in a recent study of gene expression in Lyn-deficient DT40 chicken B cells, where the absence of Lyn led to down-regulation of numerous genes encoding proteins involved in BCR signaling.
proliferation, control of transcription, immunity/inflammation response, and cytoskeletal organization (45). One major difference between Lyn-deficient DT40 cells and BMMCs is that the Lyn+/− DT40 cells have no other Src kinase family members, whereas the Src kinase, Fyn, increases in both activity (7) and transcript levels (Fig. 3, Table II) in Ag-stimulated Lyn−/− BMMCs.

In conclusion, the FcεRI-mediated activation of Lyn+/− BMMCs results in greater increases in mRNAs encoding proteins in the FcεRI signaling pathway, greater decreases in mRNAs encoding a negative regulator of signaling and greater increases in mRNAs encoding Th2 cytokines and chemokines and key transcription factors than occur in control cells. All of these differences are likely to contribute to the hyperresponsiveness of Lyn−/− mast cells and to the greater predisposition of Lyn−/− mice to the allergic phenotype as indicated by the higher numbers of skin and peritoneal mast cells, higher serum IgE levels, increased levels of circulating histamine, and increased in vivo expression of surface FcεRI on the mast cells of Lyn+/− mice in comparison with WT littermates (6, 9, 10, 12, 19). Recently, Beavit et al. (46) confirmed that Lyn+/− mice develop severe, persistent asthma suggesting a role for Lyn as an important negative regulator of Th2 immune responses. Overall, our analysis suggests a key role for Lyn in setting the thresholds for mast cell signaling and response pathways, thus determining predisposition to allergic responses.

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Disclosures

The authors have no financial conflict of interest.

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