

CUTTING EDGE

Cutting Edge: Genetic Variation Influences FcεRI-Induced Mast Cell Activation and Allergic Responses¹

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*Mast cell responses are influenced by a diverse array of environmental factors, but little is known about the effect of genetic background. In this study, we report that 129/Sv mice had high levels of circulating IgE, increased expression of the high-affinity receptor for IgE (FcεRI), and greater sensitivity to anaphylaxis when compared with C57BL/6 mice. Bone marrow-derived mast cells (BMMCs) from 129/Sv mice showed more robust degranulation upon the engagement of FcεRI. Deficiency of the Src family kinase Lyn enhanced degranulation in 129/Sv BMMCs but inhibited this response in C57BL/6 cells. C57BL/6 *lyn*^{-/-} BMMCs had reduced expression of the Src family kinase Fyn, and increasing its expression markedly enhanced degranulation. In human mast cells the silencing of Lyn or Fyn expression resulted in hyperdegranulation or hypodegranulation, respectively. The findings demonstrate a genetic influence on the extent of a mast cell's response and identify Fyn kinase as a contributory determinant. The Journal of Immunology, 2007, 179: 740–743.*

The plasticity of a mast cell (MC)³ has long been recognized and the environmental milieu of the tissue in which the MC resides is a known contributory factor (1). MCs mature in the peripheral tissue, and the organs they inhabit have considerably different environments. It is thought that this adaptability provides the MC with the needed flexibility to respond appropriately to an immune challenge in the context of the particular tissue where it resides (2).

Although the granule protein composition (tryptases) of MCs was previously demonstrated to be different in 129/Sv versus C57BL/6 mice (3), one less well-studied facet is how MC responsiveness is influenced by genetics. It is well recognized that both genes and environment are key in the development of allergy and asthma (4). It is thought that the skewing of im-

mune responses toward a Th2 phenotype is requisite (5). However, in a Th2 setting individuals with similar levels of high affinity, allergen-specific IgE Abs that react to the same allergen can, nonetheless, respond differently to a challenge (6). These genetically determined divergent responses are also manifest in mouse models of MC function. In this respect, we and others have reported (7, 8) that deficiency in the Src family protein tyrosine kinase Lyn results in increased MC responsiveness upon engagement of FcεRI, whereas other studies demonstrate (9–11) no change in responsiveness or decreased responsiveness in the absence of Lyn. We hypothesized that these differences might be determined by a difference in the MC responsiveness of the two commonly used mouse strains (129/Sv and C57BL/6).

Our findings demonstrate that the in vivo and in vitro responses of MCs from the 129/Sv or C57BL/6 backgrounds differed markedly. The findings may well explain the conflicting observations of the various aforementioned studies on the effects of Lyn deficiency in MC degranulation.

Materials and Methods

Abs and reagents

Abs and reagents used in this study have been described elsewhere (7, 12, 13). Biotinylated human IgE was prepared as described (14). Streptavidin was purchased from Sigma-Aldrich. Secondary Abs were previously described (13).

Mice, cell cultures, activation, lysates, immunoprecipitation, and immunoblots

Congenic wild-type (WT) 129/SvJ and C57BL/6J mice (8–12 wk of age) and their Lyn-deficient counterparts (129/Sv-Lyn^{tm1Sor}/J and B6.129S4-Lyn^{tmSor}/J; henceforth referred to as 129/Sv *lyn*^{-/-} and C57BL/6 *lyn*^{-/-} mice, respectively) were obtained from The Jackson Laboratory and used in accordance with National Institutes of Health guidelines and a National Institute of Arthritis and Musculoskeletal and Skin Diseases-approved animal study proposal. The bone marrow-derived MC (BMMC) cultures, lysate preparations, immunoprecipitations, and immunoblots used have been described elsewhere (7, 12, 13). Human MCs (HuMCs) were developed from CD34⁺ cells as described (15). FcεRI stimulation of HuMCs (sensitized with biotinylated IgE) was done with the indicated concentrations of streptavidin. Lysates were prepared and proteins identified as described (16).

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Received for publication April 26, 2007. Accepted for publication May 11, 2007.

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¹ This research was supported by the Intramural Research Programs of the National Institute of Arthritis and Musculoskeletal and Skin Diseases and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (NIH) and NIH Grants AI 059638 (to J.J.R.) and AI067254 (to S.C.). N.C. was supported by the Fondation pour la Recherche Médicale de France.

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³ Abbreviations used in this paper: MC, mast cell; BMMC, bone marrow-derived MC; HuMC, human MC; PLC, phospholipase; shRNA, short hairpin RNA; WT, wild type.

Lentivirus expression and short hairpin RNA (shRNA) vector construction and gene transduction

The construction of the lentiviral vectors carrying active and catalytically inactive forms (K296R) of Fyn kinase and shRNA for human Fyn and Lyn silencing as well as the gene transduction strategy was essentially as previously described (16). The sense and antisense oligonucleotide sequence for the targeting of Lyn (GenBank accession nos. M16038 and M79321) and Fyn (GenBank accession nos. M14333, S74774, and BC032496) shRNA were as follows: 5'-CACCGACG GAGGTTT CAGACTGAACGTGTGCTGTCCGTTTAGTTT GAAACC TCTGTC-3' (Lyn sense), 5'-AAAAGACAGAGGTTTCAAACCTAAACGT GTGCTGTCCGTT CAGTCTGAAACCTCCGTC-3' (Lyn antisense), 5'-CACCACGGGAGGTTTCGAGTCGAACGTGTGCTGTCCGTTTGAT TGTGAACCTCCCGT-3' (Fyn sense), and 5'-AAAACGGGAGGTTCA CAATCAAACGACAGCACACGTTTCGACTGCGAACCTCCCGT-3' (Fyn antisense).

Measurement of cytosolic calcium, degranulation, cytokine production, eosinophil migration, and anaphylaxis

Calcium measurements on fura-2-loaded cells were previously described (16). Release (degranulation) of the granule marker β -hexosaminidase was assayed as previously described (7, 12). For cytokine secretion, a multiplex array was performed by a commercial source (Linco/Millipore). Eosinophil migration assays were performed essentially as previously described (17). Passive systemic anaphylaxis was performed as described (7) with a minor modification. Rat monoclonal anti-IgE (100 μ g; BD Biosciences) was used to challenge the mice to

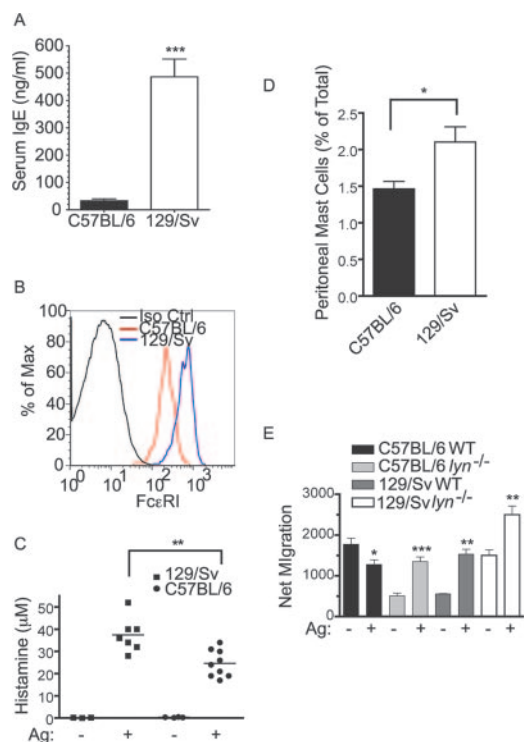


FIGURE 1. Differences in the Th environment and MC responses of C57BL/6 and 129/Sv mice. *A*, Serum IgE levels of unchallenged 129/Sv and C57BL/6 mice were measured by ELISA (***, $p < 0.001$). *B*, FACS analysis of FcεRI expression on peritoneal MCs for the indicated genotypes. Isotype controls were identical for both genotypes. *C*, Plasma histamine levels after anaphylactic challenge of indicated mice using anti-IgE (100 μ g). Significance was determined by one-way ANOVA (**, $p < 0.001$). *D*, C57BL/6 ($n = 6$) and 129/Sv ($n = 9$) mice differ in the percentage of peritoneal MCs. Mast cells were distinguished on the basis of c-KIT⁺FcεRI⁺ by flow cytometry. Significance was determined by one-way ANOVA (*, $p < 0.03$). *E*, C57BL/6 and 129/Sv BMMCs differ in their abilities to recruit eosinophils, which are enhanced by 129/Sv *lyn*^{-/-} BMMCs. Supernatants from unstimulated (–) or IgE/Ag (Ag)-stimulated (+) BMMCs of the indicated mouse strains were used. Data shown are mean $\pm$ SEM from 3–4 samples/condition. Significance was determined by a Student t test relative to unstimulated (–) samples (*, $p < 0.05$; **, $p < 0.008$; ***, $p < 0.0008$).

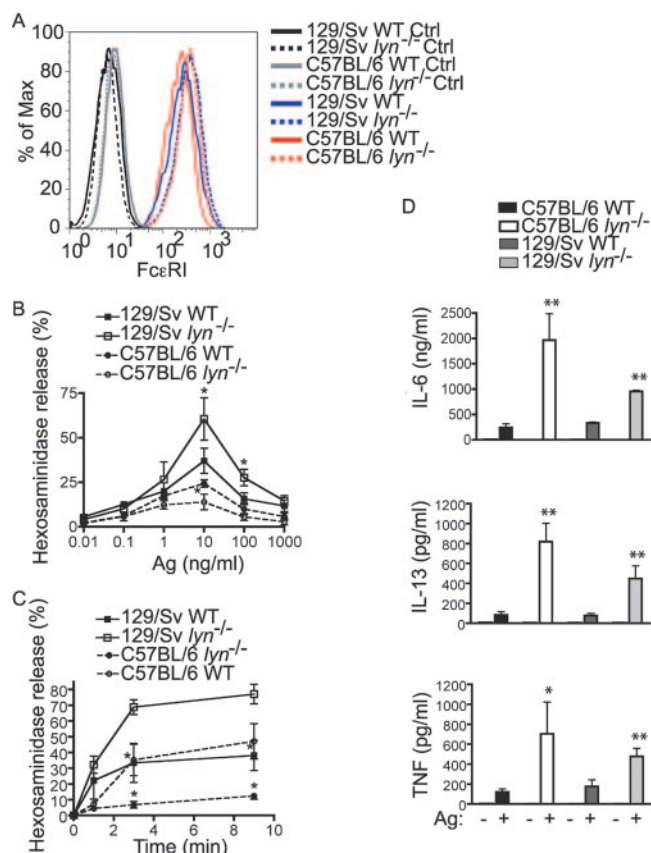


FIGURE 2. Degranulation, but not cytokine secretion, differs for C57BL/6 and 129/Sv BMMCs. *A*, FACS analysis of BMMC FcεRI expression for the indicated genotypes. Ctrl, Control. *B*, Degranulation of BMMCs from the indicated genotypes at the shown doses of Ag was determined by hexosaminidase release. Net release at 15 min is shown. Data are mean $\pm$ SD. Significance (*, $p < 0.05$ to < 0.01) was determined by a Student t test for each genotype in a comparison of *lyn*^{-/-} with respect to WT. *C*, Kinetics of BMMC degranulation for the indicated genotypes measured as described above using 50 ng/ml Ag. Data are mean $\pm$ SEM. Significance was determined as above (*, $p < 0.01$). *D*, The amount of IL-6, IL-13, and TNF secreted from BMMCs of the indicated genotypes into the medium before and 4 h after Ag challenge (10 ng/ml) was measured in a multiplex format. Data shown are mean $\pm$ SD from five experiments. Significance of *lyn*^{-/-} with respect to WT was as follows: *, $p < 0.05$; and **, $p < 0.005$.

circumvent the possible inhibition of the binding of Ag-specific IgE used to sensitize the mice because of the high levels of circulating IgE in 129/Sv mice.

Results and Discussion

Infectious challenge of C57BL/6 or 129/Sv mice has revealed a skewing of immune responses toward Th1 or Th2, respectively (18). To assess whether this skewing was evident under non-challenge conditions, we evaluated the amount of circulating IgE and peritoneal MC FcεRI expression for both, as these are known to increase in a Th2 environment. 129/Sv mice had markedly increased serum IgE levels and increased FcεRI expression on their MCs relative to C57BL/6 mice (Fig. 1, *A* and *B*). Anaphylactic challenge (Fig. 1 *C*) showed increased MC responsiveness in 129/Sv mice, demonstrating that the Th2 skewing predisposed these mice to enhanced in vivo allergic responses. The percentage of MCs in the peritoneum of 129/Sv WT mice was also significantly higher than that of C57BL/6 WT mice (Fig. 1 *D*), and FcεRI-activated 129/Sv BMMCs

showed an increased capacity to recruit eosinophils when Lyn expression (129/Sv *lyn*^{-/-}) was lost (Fig. 1E).

The difference between the *in vivo* responses suggested that the increased expression of FcεRI, because of high IgE levels in 129/Sv vs C57BL/6, was a dominant factor in the enhanced response of 129/Sv mice. To test whether other cell-intrinsic factors might play a role, we assessed the degranulation response of *in vitro* cultured WT and Lyn-deficient (*lyn*^{-/-}) BMMCs. Fig. 2A shows that cultured BMMCs did not differ in FcεRI expression. However, as shown in Fig. 2, B and C, there was a marked difference in the extent and kinetics of the degranulation response of BMMCs from the two genetic backgrounds. 129/Sv BMC showed a trend toward a more robust degranulation response than C57BL/6 cells with a more rapid kinetics, having a *t*_{1/2} of <1 min vs a *t*_{1/2} of >2 min, respectively. Strikingly, Lyn deficiency had opposing effects on the degranulation response, with BMMCs from 129/Sv mice showing a significant enhancement (~2-fold, *p* < 0.02; *n* = 5) in degranulation whereas those from C57BL/6 mice showed a significant decrease (~2-fold, *p* < 0.05; *n* = 5). Kinetic analysis (Fig. 2C) revealed that these differences could not be overcome within 9 min poststimulation. No significant differences were observed for cytokine secretion (IL-6, IL-13, or TNF) in WT 129/Sv vs C57BL/6 BMMCs (Fig. 2D) and Lyn deficiency in both genetic backgrounds caused significant (*p* < 0.01; *n* = 5) enhancement of cytokine secretion, thus demonstrating an unambiguous role for Lyn kinase in the negative regulation of this response.

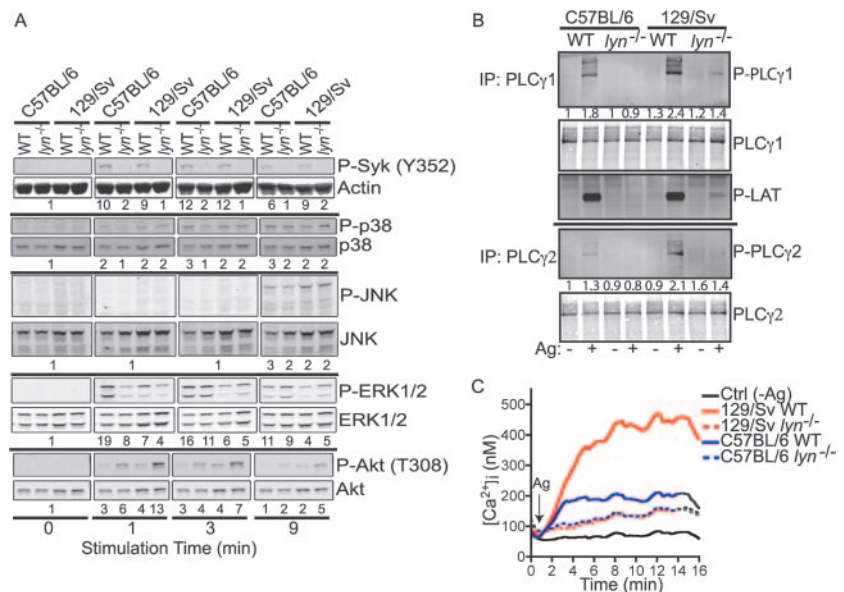
To explore what other factors underlie the observed differences in the extent of the degranulation responses of 129/Sv and C57BL/6 BMMCs, we performed an analysis of the phosphorylation state of a number of proteins activated upon FcεRI engagement (Fig. 3A). No significant differences were found in the phosphorylation of Syk, p38 MAPK, and JNK, whereas enhanced phosphorylation of ERK was observed in C57BL/6 WT relative to 129/Sv WT BMMCs. Lyn deficiency made little difference in the phosphorylation of p38MAPK and JNK, consistent with our prior finding of a role for Fyn in their activation (13). In contrast, Lyn deficiency caused loss of Syk phosphorylation in both 129/Sv and C57BL/6 BMMCs, whereas Akt

phosphorylation was modestly increased in C57BL/6 *lyn*^{-/-} BMMCs but markedly increased in those from 129/Sv *lyn*^{-/-} mice. Phosphorylation of phospholipase C (PLC) γ₁ and PLCγ₂ as well as that of PLCγ₁-associated LAT (linker of activated T cells) was also enhanced in 129/Sv background, and it is noteworthy that phosphorylation of these proteins was still observed, although reduced, in 129/Sv *lyn*^{-/-} but not in C57BL/6 *lyn*^{-/-} BMMCs (Fig. 3B). Analysis of the calcium response revealed a 2-fold difference (from 200 to ~400 nM) in the FcεRI-induced intracellular calcium concentrations of C57BL/6 and 129/Sv WT BMMCs, respectively (Fig. 3C). Lyn-deficiency caused the loss of calcium responses in both backgrounds, with a slow rise in calcium as previously noted (9).

In the course of this work we discovered that C57BL/6 BMMCs expressed considerably less Fyn kinase than BMMCs derived from 129/Sv mice (Fig. 4A). Thus, we explored the possibility that this difference might contribute to the loss of degranulation in C57BL/6 *lyn*^{-/-} BMMCs vs the hyperdegranulation observed in 129/Sv *lyn*^{-/-} cells. Fig. 4B shows that ectopic expression of Fyn in C57BL/6 *lyn*^{-/-} BMMCs restored degranulation. Three independent experiments using individual cultures showed a marked restoration of degranulation with a trend toward enhanced degranulation when compared with C57BL/6 WT BMMCs achieving significance (*p* < 0.05) at high Ag concentration. The restored degranulation was associated with restoration of the calcium response (Fig. 4C) to the level of the intracellular calcium concentrations observed in 129/Sv WT BMMCs (Fig. 3C). Thus, these experiments demonstrated that differences in the expression of Fyn kinase contributed to the enhanced degranulation response of 129/Sv vs C57BL/6 BMMCs. This suggests that both Lyn and Fyn are required for normal calcium responses and that the ratio of Lyn:Fyn expression may be important in determining the action of Fyn kinase and its role in degranulation.

The relevance of these conflicting findings on the degranulation in the two Lyn-deficient mouse strains was explored in cultured CD34⁺-derived HuMCs. Fig. 4D shows that the selective silencing of Fyn and Lyn expression in HuMCs caused suppressed and enhanced degranulation, respectively. These

FIGURE 3. Differences in the signaling and calcium responses of WT and Lyn-deficient BMMCs from C57BL/6 and 129/Sv mice. *A*, Phosphorylation of signaling proteins in BMMCs from WT or Lyn-deficient BMMCs. Immunoblots were performed using the indicated phospho (P)-specific Abs on proteins from unstimulated cells (0 min) or cells stimulated with Ag (50 ng/ml). Equal protein loading was determined with an Ab to actin or to the respective protein. One representative of four individual experiments is shown. Numbers are the mean fold increase in phosphorylation (for all experiments, *n* = 4) as determined by densitometric quantitation (using NIH Image J software). *B*, Phosphorylation (P-) of PLCγ₁ and PLCγ₂ was determined by a phosphotyrosine immunoblot of immunoprecipitated (IP) protein. PLCγ₁-associated phospho-LAT was also detected. Numbers represent mean fold increase in phosphorylation (for all experiments, *n* = 3) as determined by densitometry. *C*, Calcium response of the indicated genotypes using fura 2 fluorometry. IgE-sensitized cells were stimulated with Ag (20 ng/ml) as indicated by the arrow and calcium responses were monitored for the indicated time. One representative of three experiments is shown. Ctrl, Control.



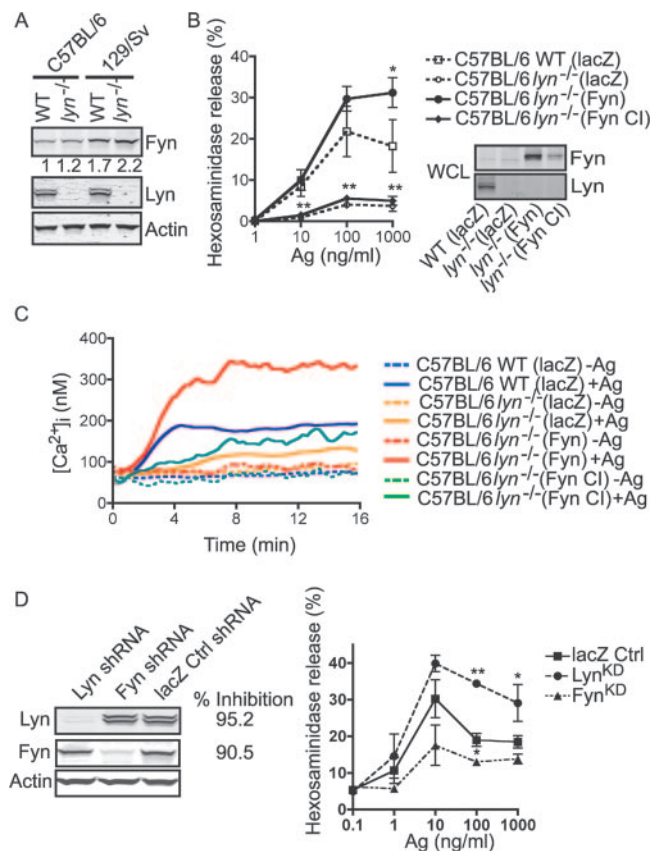


FIGURE 4. Fyn kinase expression is reduced in C57BL/6 BMMCs and Fyn contributes to degranulation in mouse and human MCs. *A*, Cell lysates from the indicated genotypes were probed with Abs to Fyn and Lyn. Equal protein loading is indicated by the actin immunoblot. One representative of three experiments is shown. Numbers are the relative quantitation of Fyn with respect to WT C57BL/6 BMMC for all experiments. *B*, Degranulation of WT and Lyn-deficient C57BL/6 BMMCs from control lacZ-transduced, Fyn-transduced, or Fyn catalytically inactive (Fyn Cl)-transduced cells. *Inset* shows ectopic Fyn expression levels for one representative experiment. Degranulation was measured by β -hexosaminidase release using the indicated Ag doses. Data are mean $\pm$ SD from three individual experiments. Significance was determined by a Student *t* test relative to WT (lacZ) responses (*, $p < 0.05$; **, $p < 0.005$). *C*, Calcium response of the indicated transduced genotypes using fura 2 fluorometry. IgE-sensitized cells were stimulated or not with Ag (20 ng/ml) and monitored for the indicated time. One representative of four experiments is shown. *D*, CD34⁺-derived HuMCs were transduced with shRNA to silence Lyn (Lyn^{KD}) and Fyn (Fyn^{KD}) expression or with control (Ctrl) shRNA (lacZ). Immunoblots with Abs to Fyn, Lyn, and actin reveal the relative expression of these proteins in the transductants. Transduced HuMCs were sensitized with biotinylated IgE and stimulated with the indicated concentrations of streptavidin (Ag) for 30 min. Degranulation was measured by hexosaminidase release. Data are means $\pm$ SD from five individual experiments. Significance is relative to WT cells (*, $p < 0.05$; **, $p < 0.001$).

findings demonstrate that Fyn has a positive role in degranulation whereas Lyn is a negative regulator of degranulation in HuMCs.

Our findings provide several important lessons. First, MC responses are clearly altered by the genetic makeup of the cells. We find that while the in vivo environment (high IgE levels and Fc ϵ RI expression) influences MC responsiveness (anaphylaxis), the level of Fyn expression is also a likely contributor. Second, mouse neonates of various genetic backgrounds (including C57BL/6) were shown to have a Th2 skewing of immune responses (19), suggesting that C57BL/6 mice, unlike 129/Sv or BALB/c mice, develop a Th1 skewing in later life that may re-

sult from genetic and environmental influences. This brings into question what is an appropriate genetic background for in vivo allergic models. Third, the findings suggest that the apparent controversy over whether Lyn kinase plays a negative regulatory role in MC degranulation likely results from the varying genetic makeup of the mice or cells under study. Importantly, in HuMCs we find that Fyn and Lyn have a positive and negative influence, respectively, on this response. Finally, our findings further warn that a phenotype observed in only one genetic mouse strain may have relevance to humans.

Acknowledgments

We thank the Flow Cytometry Unit of the Office of Science and Technology of National Institute of Arthritis and Musculoskeletal and Skin Diseases for their contribution.

Disclosures

The authors have no financial conflict of interest.

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