Lyn limits cytokine responsiveness of plasma cells to restrict their accumulation.

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Abstract

Plasma cell homeostasis is essential for maintaining immunological memory, while limiting disease. Plasma cell survival relies on extrinsic factors, the limited availability of which determines the size of the plasma cell population. Here we examine the plasmacytosis in autoimmune-prone, Lyn-deficient mice and demonstrate that this develops in a cell-intrinsic manner independently of inflammation. We show that the tyrosine kinase Lyn attenuates STAT3 signalling in response to IL-6 and STAT5 in response to IL-3 in two newly identified plasma cell signalling pathways. Thus in the absence of Lyn, survival is improved, allowing plasma cells to establish themselves in niches in excess numbers. This work identifies Lyn as a key regulator of survival signalling in plasma cells, limiting plasma cell accumulation and autoimmune disease susceptibility.

Introduction

Antibodies bind and neutralise pathogens and their products, and are secreted by plasma cells that persist for years or decades to maintain immunological memory. Plasma cells are the descendants of B cells, normally stimulated to differentiate following recognition of the B cell's specific antigen in concert with appropriate inflammatory or co-stimulatory signals. Plasma cells, having received a complement of differentiation signals, are licensed to enter the pool of cells that is long-lived, although entry into and persistence in this population is limited [1]. In the normal situation, the plasma cell population is maintained at a number that supports humoral immunity while preventing excessive production of immunoglobulin. Plasma cell hyperplasia occurs in plasma cell-associated malignancies and antibody-mediated autoimmune diseases such as systemic lupus erythematosus (SLE). The source of autoantibody in SLE is mainly non-cycling, long-lived plasma cells that are resistant to therapeutic ablation [2, 3, 4]. Thus, understanding the pathways upon which plasma cells rely for survival is potentially of great clinical value.

Plasma cells require MCL1, a pro-survival protein of the BCL2 family, for their persistence [5], and signals that promote *Mcl1* expression, such as the BCMA ligand APRIL, are thus important for plasma cell survival [6, 7, 8]. Other molecules contribute to plasma cell survival, such as CXCL12 secreted by stromal cells, the cytokines IL-4, IL-5 and IL-6, adhesion molecules including CD44 and ICAM1, and CD28, the ligand for the CD80/86 co-stimulatory molecules [9, 10, 11, 12]. None of these factors, however, is singly necessary for plasma cell survival and it is likely multiple factors combine to promote adequate amounts of MCL1 and thus survival [5]. The pathways that lead to *Mcl1* transcription and the mechanisms that limit the intensity of signal transduction in plasma cells are not well defined.

Here we have used a model of plasma cell hyperplasia and autoimmunity, the Lyn-deficient mouse [13, 14], to define signalling pathways that contribute to the accumulation of long-lived plasma cells and to identify the regulatory mechanisms that keep this in check. In mice lacking Lyn, plasma cells accumulate to high numbers, with older $Lyn^{-/-}$ mice containing up to 20 times more plasma cells than normal in their spleens [13, 15]. Excess $Lyn^{-/-}$ plasma cells are supported regardless of the autoimmune status of the mice as IL-6- or MyD88-deficient $Lyn^{-/-}$ double deficient mice and $Lyn^{-/-}$ Btk^{lo} mice develop plasmacytosis despite showing few signs of inflammation and/or autoimmune disease [16, 17, 18]. Therefore, the ability of plasma cells to accumulate in excess is at least partially

cell-autonomous and occurs independently of inflammatory signals present as a result of autoimmunity.

Here, we present data showing that Lyn is expressed in plasma cells, and maintains a regulatory threshold preventing excessive signalling in response to IL-6, a known plasma cell survival factor, and IL-3, a cytokine we have found to also promote plasma cell survival. In the absence of Lyn, signal transduction is augmented in response to IL-6 and IL-3 and as a result, long-lived plasma cells survive in excess.

Results

Long-lived plasma cells accumulate in the absence of Lyn

A clear manifestation of the Lyn-deficient phenotype is hyper-IgM and an accompanying plasmacytosis [13, 14, 16]. To investigate the basis of this plasma cell overrepresentation, we introduced the Blimp-1^{gfp} allele onto the Lyn-deficient background, allowing the frequency of plasma cells (PC) to be assessed by flow cytometry in relation to both location and age (Fig. 1). In Lyn-deficient Blimp-1^{gfp} mice, while PC accumulated in BM with kinetics similar to control (WT) mice (Fig. 1A), splenic plasma cells accumulated more rapidly in the first 10 weeks of life in the absence of Lyn (Fig. 1B), which may have been indicative of enhanced production or persistence. Thus we next sought to determine whether the Lyn-deficient plasmacytosis involved excess production of short-lived PCs, or the accumulation of long-lived PC, possibly due to altered survival. The cells that accumulated in adult *Lyn*$^{-/-}$ spleens expressed high levels of GFP, the surrogate marker for Blimp-1 (Fig. 1C), indicating that they were potentially a more mature, long-lived PC type. WT mice maintain equivalent populations of PCs expressing low and high levels of GFP as they age (Fig. S1 and Fig. 2A) [19]. Excess plasma cells were apparent as early as five weeks of age in *Lyn*$^{-/-}$ spleens at which time Blimp-1^{int} and Blimp-1^{hi} plasma cells were equally represented (Fig. 1C), although as the mice aged, the Blimp-1^{hi} population predominated in the *Lyn*$^{-/-}$ spleen (Fig. 1C, Fig. 2A and Fig. S1). The preponderance of Blimp-1^{hi} PC together with in vitro B cell differentiation experiments showing Lyn-deficient B cells were not prone to excessive differentiation (Fig. S2), suggested that the plasmacytosis in Lyn-deficient mice was due to the accumulation of long-lived PC. BrdU labelling experiments, measuring the appearance of newly formed PC, supported this conclusion with PC turnover in *Lyn*$^{-/-}$ BM and spleen being proportionately lower than in controls (Fig. 1D). Overall we concluded that the accumulation of plasma cells in *Lyn*$^{-/-}$ mice was not necessarily due to excess formation of PC but potentially to intrinsic defects that resulted in enhanced survival once the PC were formed. This suggested that Lyn-deficient PC can subvert the normal restrictions limiting accumulation within the long-lived PC compartment.

Loss of Lyn causes B cell-intrinsic plasma cell hyperplasia

To determine whether the role of Lyn within PC was B cell-autonomous or required a Lyn-deficient environment, we created chimeric mice by BM reconstitution. We used 4 groups of mixed BM donors comprising WT and μMT (1:4); *Lyn*$^{-/-}$ and μMT (1:4); WT and *Lyn*$^{-/-}$ μMT (1:4); and *Lyn*$^{-/-}$ and *Lyn*$^{-/-}$ μMT (1:4). In each case, B cells could only arise from the non-μMT

bone marrow donor, developing within an essentially WT or *Lyn*$^{-/-}$ haematopoietic environment arising from the μMT or *Lyn*$^{-/-}$ μMT donor (Fig. 2A). The mice were analysed 15 weeks after reconstitution for spleen size, B cell and PC representation. In mice reconstituted such that their B cells were *Lyn*$^{-/-}$, irrespective of the genotype of the remaining haematopoietic cells, smaller spleens with fewer B cells were observed relative to those mice in which B cells were WT-derived (Fig. S3). In spite of this, PC representation in the spleens of mice with Lyn-deficient B cells was significantly increased compared to those with WT B cells, irrespective of the Lyn status of other haematopoietic cells (Fig. 2, A and B). Furthermore, Blimp-1^{hi} PC dominated the *Lyn*$^{-/-}$ PC populations in spleen, compared to the more equal distribution of Blimp-1^{hi} and Blimp-1^{int} when B cells were of WT origin (Fig. 2A). Interestingly, the plasmacytosis in the presence of B cell lymphopenia that developed in mice reconstituted with *Lyn*$^{-/-}$ B cells recapitulated that characteristic of intact *Lyn*$^{-/-}$ animals, indicating this was also a B-cell intrinsic property (Fig. 2C). These findings demonstrated that the accumulation of *Lyn*$^{-/-}$ PC was B cell-intrinsic and did not require Lyn deficiency within other haematopoietic cells, many of which contribute to PC survival [20, 21, 22, 23, 24, 25].

Lyn regulates the phenotype of plasma cells

Differences in PC persistence may result from different sensitivity to those signals governing survival and localisation, which in turn may reflect differences in the expression of surface receptors for ligands to which PCs are known to respond. We therefore examined whether the phenotype of *Lyn*$^{-/-}$ PC differed from that of controls. Expression of CXCR4 was elevated on *Lyn*$^{-/-}$ PC from the BM and spleen (Fig. 3). However, expression of CXCR4 was not elevated on *Lyn*$^{-/-}$ *Il6*$^{-/-}$ PC, suggesting this change is a result of the inflammatory environment (Fig. S4). Expression of FcγRIIB and CD98 were also higher on *Lyn*$^{-/-}$ PC in spleen, whereas increased expression of CD84 was restricted to Blimp-1^{hi} PC in spleen (Fig. 3). Conversely, *Lyn*$^{-/-}$ PC had reduced expression of CD44 in both BM and spleen, whereas reduced CXCR3 expression was limited to spleen PC. MHC class II, while reduced on *Lyn*$^{-/-}$ Blimp-1^{hi} BM plasma cells, was increased on Blimp-1^{int} PC in spleen, potentially reflecting existing, higher expression on *Lyn*$^{-/-}$ splenic B cells (Fig. 3). No difference in Fas expression was noted between PC subsets of the mice in either location (Fig. 3). Thus several cell surface markers associated with PC localisation and/or survival were found to be altered in the absence of Lyn. While some of these changes in phenotype may reflect an altered capacity for PC survival, they did not preclude potentially altered responses of Lyn-deficient PC to cytokine-derived survival signals.

Transduction of cytokine survival signals in plasma cells is regulated by Lyn

Several cytokines have been identified as mediators of PC survival. Given the B-cell intrinsic nature of the Lyn-deficient PC expansion, we next considered whether Lyn might act to limit the signalling downstream of such survival stimuli within plasma cells. Stimulation with IL-6 or IL-3 in vitro resulted in significantly enhanced survival of purified Lyn-deficient PC compared to those from WT (Fig. 4, A-D). This improved responsiveness was selective in that *Lyn*$^{-/-}$ PC did not show enhanced survival on exposure to APRIL (Fig. 4, A and B), IL-21, BAFF or Flt3L (Fig. S5), indicating that Lyn did not regulate signal transduction downstream of all pro-survival cytokines in PC. IL-3 has not been reported as a plasma cell survival factor, but experiments using BM from IgE-deficient mice cultured for 5d or 9d with or without IL-3 showed higher IgG1 in the supernatant of cultures in the presence of IL-3, suggesting that IL-3 may in fact act directly on plasma cells [20]. We examined both WT and *Lyn*$^{-/-}$ plasma cells for expression of CD123, the IL-3Rα chain, and found it to be present on both, with more on Blimp1^{hi} than Blimp1^{int} PC, irrespective of genotype (Fig. S6A). Basophils were gated as a positive control and had more CD123 than PCs, and B cells were found to be negative (Fig. S6B).

We next assessed signal transduction downstream of IL-6 or IL-3 exposure, to determine if this was altered by the absence of Lyn. IL-6-induced STAT3 phosphorylation in WT PC peaked 30 minutes after IL-6 exposure and returned to near baseline by 120 minutes (Fig. 4E). While STAT3 phosphorylation in IL-6-stimulated Lyn-deficient PC was similar in kinetics to WT, peaking at 30 minutes and returning to near baseline by 120 minutes, the magnitude of the response was significantly greater (Fig. 4E). The hyper-responsiveness of *Lyn*$^{-/-}$ PC was independent of prior exposure to IL-6 as plasma cells from both *Lyn*$^{-/-}$ and *Lyn*$^{-/-}$*Il6*$^{-/-}$ mice showed near identical STAT3 responses to IL-6 (Fig. 4E). Thus the elevated IL-6 in *Lyn*$^{-/-}$ mouse serum did not underlie the PC hyper-responsiveness to IL-6 [16]. IL-6 hyper-responsiveness was restricted to Lyn-deficient PC; it was not apparent in Lyn-deficient plasmablasts derived in vitro (Fig. 4F), indicating a role for Lyn in the final stages of PC differentiation in vivo. Given the enhanced survival of *Lyn*$^{-/-}$ PC in the presence of IL-3 (Fig. 4, A-D), we next measured the kinetics of STAT5 phosphorylation in WT and *Lyn*$^{-/-}$ PC following exposure to IL-3. Ex vivo Lyn-deficient PC showed appreciable amounts of phospho-STAT5 before stimulation, which increased further after IL-3 exposure, peaking at thirty minutes before returning to pre-stimulation levels by 60 minutes (Fig. 4G). By comparison, WT PC contained lower amounts of phospho-STAT5 on isolation, which increased only

marginally over the course of the experiment (Fig. 4G). Thus, Lyn negatively regulates STAT signalling in PC following IL-6 and IL-3 stimulation.

In general, phosphorylation of STAT molecules following cytokines binding to their receptors requires activation of receptor associated JAK kinases. To confirm that the development of plasmacytosis in Lyn-deficient mice depended on JAK activity, we treated Lyn-deficient mice for four consecutive days with AZD1480, a competitive ATP-inhibitor with preferred specificity for JAK1 and JAK2 [26] or the corresponding vehicle. We took the dramatic reduction in thymus cellularity in AZD1480 treated mice to indicate that the compound was effective in blocking cytokine signalling (Fig. S7). Importantly, plasma cell numbers in the spleens of AZD1480 treated Lyn-deficient animals also were significantly reduced compared to vehicle-treated mice (Fig. S7A). Spleen cellularity, including that of B-cell cells, also diminished following AZD1480 treatment, indicating a not unexpected, general sensitivity to AZD1480 (Fig. S7, B and D). Consistent with the in vivo effects, in vitro treatment of WT and Lyn-deficient PC with AZD1480 prior to addition of IL-6 blocked all STAT3 phosphorylation (Fig. S7E).

Lyn participates in plasma cell signalling

The data presented so far support a plasma cell-intrinsic role for Lyn in regulating signal transduction from receptors for at least two PC survival factors. To confirm that Lyn was expressed and active in plasma cells, we measured *Lyn* mRNA content by qPCR in Blimp-1^{hi} and Blimp-1^{int} plasma cells isolated from WT spleen. *Lyn* mRNA was detected in PCs by qPCR (Fig. 5A). Confirming this, Lyn protein was present in Blimp-1^{int} and Blimp-1^{hi} PC (Fig. 5B). To measure activity of the Lyn kinase present in PC, we used an antibody specific for phospho-SrcY416 as a way of estimating Src family kinase (SFK) activity in plasma cells and to determine whether this changed following cytokine stimulation. As this antibody failed to detect active SFK in lysate from Lyn-deficient PC, in contrast to the signal generated with lysate from WT PC, we concluded that Lyn was the dominant, active SFK in PC (Fig. 5C). Furthermore, Lyn appeared to be constitutively phosphorylated on the active tyrosine in PC as evidenced by the signal in unstimulated plasma cells and, over the time course of these experiments, this signal did not increase following addition of IL-6. Exposing WT PC to IL-3 increased modestly the amount of active Lyn in these cells (Fig. 5C). Finally, to determine if Lyn activity was required at the time of exposure to cytokines to inhibit the induction of pSTAT, isolated WT PC were incubated with the general SFK inhibitor PP2 for 15 min prior to the addition of IL-6. No discernible difference in the amount of pSTAT3 in the PP2 treated or control PC was detected despite the concentration of PP2

used being sufficient to inhibit the appearance of pERK following BCR ligation on B-cells (Fig. S8).

Discussion

Here we have demonstrated that the tyrosine kinase Lyn regulates pathways by which plasma cells respond to cytokines that result in their survival. This is the first characterisation of a negative regulator of such cytokine signalling events in primary plasma cells. We speculate that the elevated signal intensities apparent when Lyn is absent enhance plasma cell accumulation and thus the expansion of the long-lived population. Furthermore, experiments presented here using chimeric mice demonstrate the importance of Lyn in the B-cell intrinsic regulation of plasma cell formation, as plasma cells lacking Lyn accrued abnormally regardless of Lyn expression in other haematopoietic cells. The capacity for persistence of these plasma cells is also independent of disease, as excess plasma cells persist in *Lyn*$^{-/-}$ mice in which inflammation has been removed by various mechanisms including deletion of *Il6* or *MyD88* [16, 17, 18, 27]. Finally, we have identified IL-3 as being capable of promoting plasma cell survival in a Lyn-regulated manner.

Plasma cell survival is considered to be dependent on their localisation in specialised environments comprising cytokines and stromal cell-derived ligands including CXCL12, CXCL9 and CD44 ligands. While *Lyn*$^{-/-}$ plasma cells in the BM and spleen express significantly more CXCR4, up-regulation was not observed on *Lyn*$^{-/-}$ plasma cells from an IL-6-deficient background, and is thus unlikely to be a mechanism of plasmacytosis, which occurs even in the absence of IL-6 [16]. The *Lyn*$^{-/-}$ splenic plasma cell population had moderately lower expression of CXCR3, possibly reflecting the smaller proportion of these cells that are derived from IgG-switched B cells [28, 29]. CD44 was also lower on plasma cells lacking Lyn, which may indicate a diminished dependence on adhesion signals for the survival of these cells. It is also interesting that expression of FcγRIIB was moderately raised on Lyn-deficient splenic plasma cells, ligation of which can induce plasma cells apoptosis [30]. We speculate that this increased expression does not reflect enhanced apoptosis of Lyn-deficient plasma cells, as they accumulate in a stable, long-lived population.

IL-6 is considered to be an important component of the plasma cell survival niche [9] and plasma cells have been shown to stimulate niche availability by inducing IL-6 secretion by stromal cells [10]. IL-6 enhances plasmacytic survival [9], and while *Il6* knockout mice do not show a plasma cell phenotype, this may be due to redundancy in cytokine signalling, with several cytokines able to signal through gp130 and activate STAT3 in the absence of IL-6 [31]. Over-expression of *Il6* supports greater plasma cell numbers [32] and the development of extraosseous plasmacytoma [33], suggesting that elevated IL-6 signalling can effect plasma cell survival in a similar way to that

observed in the Lyn-deficient mice. Here we have shown that IL-6 stimulation induces STAT3 phosphorylation in primary plasma cells, and Lyn limits the magnitude of the IL-6 signal. We have also identified IL-3 as a cytokine able to mediate plasma cell survival in a dose-dependent manner by a mechanism that is regulated also by Lyn activity. While plasma cells have not previously been found to be responsive to IL-3, Lyn is known to regulate IL-3 responsiveness in other circumstances, specifically in mast cells [34]. Lyn-deficient mast cells are hyper-responsive to IL-3, showing enhanced expansion from BM precursors, increased proliferation, reduced apoptosis upon IL-3 withdrawal and enhanced phosphoinoside-3 kinase and mitogen-activated protein kinase signalling but through a STAT independent process [24, 34]. While IL-3 plays an important role in basophil expansion in vivo [35] and basophils have been linked both to plasma cell survival [20] and to the plasmacytosis in Lyn-deficient mice [25], the results presented here demonstrate that IL-3 can act directly on plasma cells with no requirement for additional cell types and therefore independent of IL-3 signalling in basophils.

Treatment of mice with AZD1480, a JAK1/2 inhibitor, resulted in the approximate halving in PC number in the spleens of Lyn-deficient mice. However, compared with the greater than 90% loss of plasma cells following induced, cell-intrinsic deletion of the gene encoding the pro-survival protein Mcl-1 [5], indicates that cytokine signalling via JAK1/2 in plasma cells is not necessarily the major means of maintaining Mcl-1 expression in established PC in Lyn-deficient mice. Indeed, while the reduction in plasma cell number is significant - especially so in the context of reducing autoantibody production – it is in keeping with the overall reduction in spleen cellularity, indicating that plasma cells are not uniquely sensitive to this inhibition. We speculate that these soluble cytokines are critical at the point at which PC are established, such that once stromal cell support has been engaged, PC are able to survive in the absence of JAK1/2 dependent signalling. Thus the role of Lyn may be critical at the point at which plasma cells are established as long-lived within a niche, but, having reached that stage, other signals can maintain survival in the majority of PC in the absence of cytokine signalling.

While we are yet to determine the mechanism by which Lyn activity regulates signalling through STAT3 or STAT5 in plasma cells, it may be by recruiting and/or activating a phosphatase in a manner reminiscent to the recruitment of SHP-1 in activated B-cells [15, 36]. Interestingly, the role of Lyn in this process in PC is unlikely to be activated by exposure to the cytokines as SFK inhibition in WT PC immediately prior to cytokine exposure did not confer hyper-responsiveness to STAT

signalling in these cells. We would suggest rather that Lyn operates to establish and then maintain a regulatory circuit in PC that precedes exposure to cytokines and that the components of this circuit are stable such that the necessarily limited pre-exposure to PP2 was insufficient to materially alter its composition. The cytokine hyper-responsiveness of *Lyn-/-* cells is restricted to in vivo plasma cells, as in vitro derived plasmablasts, generated with CD40L, IL-4 and IL-5, phosphorylated STAT3 in response to IL-6 equally, irrespective of the presence or absence of Lyn, as was the case in the activated B cells in the same cultures. This is an important distinction as it indicates that Lyn is active in PC themselves and not in their precursors. Finally, the dysregulation of survival signals in $Lyn^{-/-}$ plasma cells did not extend to APRIL stimulation, which is another important cytokine in plasma cell survival. APRIL signalling occurs via TRAF mediators and STATs have not been implicated in signalling downstream of APRIL [37, 38]. Thus we hypothesise that the important role in negative regulation of cytokine signalling in plasma cells played by Lyn is restricted to STAT phosphorylation. It remains to be determined if Lyn also operates in the regulation of CXCL12, CXCL9 and CD44L signalling.

Our results reveal for the first time the molecular role for Lyn in plasma cell signalling and provide a possible explanation for the plasmacytosis of Lyn-deficient mice. We reveal also a previously unknown regulatory mechanism operating in plasma cells to regulate the magnitude of pro-survival signals in plasma cells. We show that loss of Lyn leads to hyper-responsive IL-6 and IL-3 signalling, suggesting that Lyn ordinarily activates an inhibitory mechanism downstream of these cytokines to limit the establishment of the long-lived PC population. In the absence of Lyn, fewer stimuli may be required to achieve signalling intensities sufficient to induce *Mcl1* expression and ensure plasma cell survival [5]. The identification of an inhibitory function for Lyn in PC signalling is an important step in understanding what controls plasma cell persistence and limits the size of the plasma cell population.

Materials and Methods

Mice

Lyn-deficient mice [13], Blimp-1^{gfp} reporter mice {Kovalchuk, 2002, 15492122} and B-cell deficient mice carrying the μMT allele of Igh6 [39] were previously described. Compound Lyn-deficient mice carrying the Blimp-1^{gfp} reporter or the μMT null mutation were generated by interbreeding and genotyping. Reconstitution experiments used donor bone marrow from B cell-deficient (μMT$^{-/-}$)[39] or *Lyn*$^{-/-}$ μMT$^{-/-}$ double deficient mice, mixed in the indicated ratios with Blimp-1^{gfp} or Lyn-deficient Blimp-1^{gfp} bone marrow. Recipient mice were γ-irradiated with two doses of 5.5Gy 2 hours apart. Reconstitution proceeded for 15 weeks prior to analysis. Four groups of mice were created: 1) 20 % WT (Blimp-1^{GFP}) cells + 80% WT μMT cells; 2) 20% *Lyn*$^{-/-}$ (Blimp-1^{GFP}) cells + 80% WT μMT cells; 3) 20% WT (Blimp-1^{GFP}) cells + 80% μMT *Lyn*$^{-/-}$ cells and 4) 20% *Lyn*$^{-/-}$ (Blimp-1^{GFP}) cells + 80% μMT *Lyn*$^{-/-}$ cells. All donor cells in these experiments were transferred into WT recipient animals. All mice were on a C57BL/6 background, bred and maintained at the Walter and Eliza Hall Institute of Medical Research (WEHI) or the Monash Animal Research Platform (MARP) Clayton and experimentation was conducted in accordance with National Health and Medical Research Council guidelines and protocols approved by the Animal Ethics Committee of the WEHI.

Antibodies and cell sorting

Single cell suspensions from bone marrow (BM) or spleen were treated with an ammonium chloride solution to lyse red blood cells and subsequently stained with antibody conjugates to detect mouse antigens by flow cytometry, including αB220 (clone RA3-6B2, maintained in-house), αCD138 (clone 281; Synd-1, BD Pharmingen), αCD123 (clone 5B11, BD Pharmingen), αCXCR4 (clone 2B11; CD184, eBiosciences), αCXCR3 (clone 220803, R&D), αCD44 (clone IM7, BD Pharmingen), αFcγRIIB (clone 2.4G2, maintained in-house), αMHC class II (IA/IE clone M5/114.15.2, eBiosciences), αFas (clone Jo2; CD95, BD Pharmingen), αCD84 (mCD84.7, BioLegend), αCD98 (RL388, eBiosciences). Plasma cells were identified by FACS as B220$^{-/int}$ CD138^{+} and Blimp-1^{gfp+}, basophils were identified as FcεRIa^{+} CD49b^{+} CD45^{low}), B cells were identified as B220 or CD19 positive. Live cells were identified by propidium iodide (PI) exclusion on a FACSDiVa (BD Instruments) or MoFlo (DaKo Cytomation, Colorado, USA). MFI was determined using geometric median fluorescence on gated B220^{int}CD138^{+}GFP$^{high/int}$ on cells from each mouse.

BrdU incorporation and JAK inhibition studies

To assess the turnover of cell populations, mice were injected intraperitoneally with a 100µL bolus of BrdU (10mg/mL in PBS) and then fed BrdU in their drinking water (0.5mg/mL BrdU, 2% glucose) for the indicated times. Cell suspensions from BM or spleen were then stained for surface markers, fixed in 2% PFA and permeabilized in PBS with 0.1% Tween20 (PBST) for 72h. Fixed cells were washed with PBST and incubated with 100µg/mL DNaseI (Sigma Aldrich) for 2hr at 37ºC, then stained with an anti-BrdU antibody conjugated to AlexaFluor647 (Molecular Probes) before analysis on an LSRII (BD Instruments). AZD1480 (AstraZeneca) compound[26] was administered to Lyn-deficient mice by oral gavage on four consecutive days at a dose of 60mg/kg to block JAK1/2 signalling. AZD1480 was made up in 0.5% hydroxypropyl methylcellulose (Dow; #009004-65-3)/0.1% Tween-80 (Fisher; #BP338-500). Control animals were treated with vehicle alone.

Cell culture

For in vitro viability assays, plasma cells were pre-enriched by MACS using αCD138 beads (Miltenyi Biotec) according to the manufacturer's instructions then sorted by FACS based on $B220^{-/int}$ CD138 and $Blimp\text{-}1^{gfp}$ or $B220^{-/int}$ expression. Purity was typically >95%. Plasma cells ($1\text{-}3\times10^3$) were cultured for the indicted times and viability measured based on PI exclusion or ELISpot. B cell in vitro cultures were performed using MACS purified splenic B cells. B cells were cultured in fresh RPMI 1640 supplemented with 10% FCS and 100µM β-mercaptoethanol. Approximately 100,000 cells were plated in 96-well plates, and CD40L (generated in-house), IL-4 (100U, Peprotech) and IL-5 (5ng/mL, Peprotech), and IL-6 where indicated (50ng/mL, generated in-house) were added. Plasma cell numbers (PI^- $CD138^+$) in B cell cultures were calculated by adding a known number of beads to each well prior to harvesting and measuring cell numbers by FACS.

ELISpot assay for plasma cell enumeration

ELISpot assays for the different immunoglobulin isotypes were conducted exactly as described [40]. Goat anti-mouse IgM/G/A (H+L) (Millipore) was used to coat MultiScreen-HA filter plates (Millipore). Cells were incubated for overnight at 37°C on pre-coated 96-well filter plates. Cells were plated at 500 cells/well. Goat anti-mouse Ig (H+L)- HRP (Southern Biotech) was used to detect bound antibody by addition of the substrate, 3-amino-9-ethyl carbazole (A-5754; Sigma-Aldrich). Spots were counted using the AID ELISpot Reader

System. Plasma cell viability was measured by ELISpot at the time points indicated in the relevant figures. Plasma cell frequency was determined in the starting population and then at the indicated times in cultures containing the cytokines being tested, including IL-3 (10ng/mL, eBioscience); αIL-3 (MP2-8F8, generated in house); IL-6 (10-50ng/mL, generated in-house); IL-21 (50ng/mL, R&D); APRIL (200ng/mL, Peprotech); BAFF (500ng/mL, generated in house) and Flt3L (125ng/mL, a kind gift from S. Nutt WEHI).

Quantification of gene expression

Lyn expression was quantified in FACS-sorted plasma cell populations using a Quantitect qPCR kit (Qiagen). Quantitative PCR (qPCR) was performed on a CFX-382 Real Time System machine (Bio-Rad). Relative expression of *Lyn* compared to 18S rRNA was used to determine the fold change in *Lyn* between B cells and plasma cells, using the formula $2^{-\Delta\Delta}Ct$.

Flow cytometric assay for STAT phosphorylation

Spleen cells were stimulated with IL-6 (50ng/mL) for up to 2h and then fixed with Lyse/Fix buffer for ten minutes at 37ºC (BD Pharmingen), permeabilized with cooled Perm II buffer (BD Pharmingen) on ice for 30 minutes, washed twice with PBS + 0.1% BSA then stained with αB220-FITC, αCD138-PE and α-pSTAT3(Y705)-AlexaFluor647 (BD Pharmingen) before analysis on a FACSCalibur (BD Instruments). STAT5 activation was assessed following IL-3 stimulation (50ng/mL) in an analogous manner except that cells were stained with α-pSTAT5(Y694)-PE (BD Pharmingen). In the indicated experiment, the Src family kinase inhibitor PP2 was added to isolated wild type plasma cells at a final concentration of 1 μM, 15 min prior to addition of IL-6. STAT3 phosphorylation was determined as in other experiments.

Western blots

Plasma cells (15,000 cells Fig. 5b, 95,000 cells Fig. 5c) were stimulated with IL-3 (50ng/ml) or IL-6 (50ng/ml), and lysed in EMBO buffer (20mM Tris-HCl pH 7.6, 137 mM NaCl, 10% Glycerol, 2mM EDTA, 1% Np40, Pervanadate 1mM) in the presence of protease inhibitor cocktail (Roche). Entire lysate was run in a single well of a NuPage 4-12% Bis-Tris Gel (Invitrogen). αLyn (Cell Signaling; 2732), αpSrc(Y416) (Cell Signaling) and rat αmouse IRF4 (3E4) (Dr. L. Corcoran, WEHI) were detected using appropriate secondary antibodies.

Statistical analyses

Unpaired, two-tailed student's t-tests were performed using Microsoft Excel software. Differences were deemed significant were *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$.

Supplementary Materials

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Figure legends

Figure 1 – Long-lived plasma cells accumulate in the absence of Lyn

The proportion of plasma cells populating (A) in the BM and (B) spleen of WT and Lyn-deficient mice at the indicated ages was measured by FACS, using Blimp-1^{gfp} and CD138 to identify plasma cells. Each point represents one mouse. (C) Representative FACS profile of Blimp-1 expression, as measured by GFP, in plasma cells (gated as $CD138^{+}$) from spleens of five week and 20 week old *Lyn*$^{-/-}$ mice, showing the shift from a mixture of Blimp-1^{hi} and Blimp-1^{int} to predominantly Blimp-1^{hi}. (D) Turnover of plasma cells in the spleen and BM was measured by continuously feeding mice with BrdU for seven or 14 days. Bars show the mean plus SEM for n=3 mice and data are representative of two separate experiments.

Figure 2 – Loss of Lyn causes B cell-intrinsic plasmacytosis.

(A) Representative FACS plots of spleens 15 weeks post-reconstitution revealing plasma cell frequency, identified as Blimp-1^{gfp+} $CD138^{+}$. Genotypes of BM donors in the mixed chimeras is indicated above the FACS plots with B-lineage cells derived obligatorily from the 20% donor while 80% of all other lineages derives from the 80% donor. (B) Plasma cell number in spleens of the recipient groups are shown as mean ± SEM (n=4), revealing plasmacytosis whenever B cells are Lyn-deficient, in a representative experiment. (C) Representation of plasma cells as a fraction of B cells within recipient groups showing the preponderance of plasma cells arising from Lyn-deficient B cells. Mean ± SEM (n=4) for a representative experiment is shown. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Figure 3 – Lyn regulates expression of plasma cell surface markers

Expression levels of the indicated cell surface proteins, determined by FACS and presented as mean fluorescence intensity (MFI), were measured on B cells (gated as total $B220^{+}$), $CD138^{+}$Blimp-1^{int} or $CD138^{+}$Blimp-1^{hi} plasma cells from the BM and spleen of WT and Lyn$^{-/-}$ mice. Means are shown as a horizontal bar in each case with n>4 mice analysed per genotype. Significant differences are indicated; * P ≤ 0.05, *** P ≤ 0.001. Filled squares, WT; open squares, *Lyn*$^{-/-}$.

Figure 4 – Lyn regulates survival and signalling in plasma cells

(A) Plasma cells were sorted from spleens and cultured in triplicate for 3 days with the indicated soluble factors. ELISpot assays at the end of the culture period were used to determine the number

of surviving antibody secreting cells in each condition and this was calculated as a proportion of the starting number. Bars, WT open, *Lyn*$^{-/-}$ grey, show mean and SEM for triplicate cultures, and data are representative of at least two separate experiments. (B) Example of ELISpot wells from (A) at the end of the culture period. (C) Blimp-1^{hi} plasma cells were sorted from spleens and cultured in triplicate for 5d with media or IL-3 or IL-3+αIL-3. ELISpot assays at the end of the culture period were used to determine the number of surviving antibody secreting cells as per (A). Graph as per (A) and data are representative of two separate experiments. (D) Example of ELISpot wells from (C) at the end of the culture period. (E) Spleen cells were cultured for the indicated times with IL-6 (50ng/mL), then fixed and stained for B220 and CD138 expression as well as intracellular pSTAT3 before analysis by FACS. Phospho-STAT3 intensity was measured as MFI (gated on B220$^{-/int}$CD138^{+} cells), which is plotted against time. Responses of cells from at least two individual mice are shown, and this experiment was repeated. (F) Equal induction of pSTAT3 with IL-6 in in vitro derived WT and Lyn$^{-/-}$ plasmablasts. B cells were cultured with CD40L, IL4 and IL5 for 3 days, stimulated for the indicated times with IL-6, fixed and stained as in (E). Plasmablasts were gated as (B220+ CD138+) and intracellular pSTAT3 MFI determined. (G). Sorted plasma cells from spleen were stimulated with IL-3 (50ng/mL) then prepared as in (E) for FACS determination of intracellular pSTAT5. N=3 mice. **P ≤ 0.01, ***P ≤ 0.001.

Figure 5 – Lyn is expressed and active in plasma cells

(A) Quantitative RT-PCR of Lyn expression in FACS-sorted splenic Blimp-1^{int} and Blimp-1^{hi} plasma cells compared with splenic B cells. Mean and SEM is shown from triplicate measurements, and this experiment was repeated with independent samples. (B) Western blot depicting Lyn expression in purified WT splenic plasma cells, with IRF4 used as a loading control. Plasma cells from Lyn$^{-/-}$ spleen are present as control. This experiment was repeated. (C) FACS-sorted spleen plasma cells were stimulated with 50 ng/mL IL-6 or 50 ng/mL IL-3 for the indicated times. An antibody directed against phosphorylated Src(Y416) was used to detect activated SFK in the lysates of stimulated WT and *Lyn*$^{-/-}$ plasma cells. The absence of a signal from this antibody in the *Lyn*$^{-/-}$ lysates indicates that Lyn is the predominant SFK in plasma cells. The increase in pSrc(Y416) signal in WT plasma cells after exposure to IL-3 indicates induced activation of Lyn. IRF4 was used as a loading control. This experiment was repeated twice with n > 6 mice per experiment.