Autocrine Secretion of Interferon γ Negatively Regulates Homing of Immature B Cells

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Abstract

The mechanism by which immature B cells are sequestered from encountering foreign antigens present in lymph nodes or sites of inflammation, before their final maturation in the spleen, has not been elucidated. We show here that immature B cells fail to home to the lymph nodes. These cells can actively exclude themselves from antigen-enriched sites by downregulating their integrin-mediated adhesion to the extracellular matrix protein, fibronectin. This inhibition is mediated by interferon γ secretion. Perturbation of interferon γ activity in vivo leads to the homing of immature B cells to the lymph nodes. This is the first example of autocrine regulation of immune cell migration to sites of foreign antigen presentation.

Key words: lymph nodes • adhesion • migration • interferon γ • invariant chain$^{-/-}$ mice

Introduction

B cells develop in the bone marrow and, through a process that involves rearrangement and expression of Ig genes, produce an antigen-specific receptor, which is first manifested in the immature stage. Immature B cells emerge from the bone marrow to the periphery and migrate into the spleen for their final maturation step. This migration proceeds through the terminal branches of central arterioles to blood sinusoids of the marginal zone (1, 2). Newly made B cells then penetrate the marginal zone sinus to end up in the outer zone of the periarteriolar lymphocytic sheath (3). At this site in the spleen, B cells are still immature and can be distinguished from their mature counterparts (1, 2, 4, 5).

One of the most fundamental and important aspects of the immune system is its ability to recognize and respond to virtually any foreign antigen while maintaining strict unresponsiveness to self-antigens. For B cells, antigens encountered during the immature stage, at anatomic sites populated with these cells, are likely to be self-antigens, and therefore, reactive clones are eliminated. The selection process occurring within the immature B cell compartments is also extended to the periphery, allowing deletion of all B cells recognizing peripheral self-antigens outside of the bone marrow (1, 6, 7).

Immature B cells leave the bone marrow and migrate to the spleen where they can mature before antigen encounter. Like other naive lymphocytes, before their arrival to the spleen immature B cells might recirculate to nonsplenic secondary lymphoid organs, which are specialized tissues for collecting antigens (8), or to sites of infection and inflammation. In these secondary lymphoid organs, where differentiation to mature cells does not occur, antigen encounter would lead to the death of the immature B cells and elimination of effective clones due to the negative selection process. Although recently it was suggested that CD40 signaling can induce immature B cell responsiveness to multiple members of the γ chain–common cytokine family (9), physiological stimulation of immature cells is known to usually lead to an arrest of cell differentiation and clonal deletion (1, 6, 7). In this work, we postulated the existence of biological mechanisms to prevent immature B cells from reaching antigen-enriched sites before maturation.
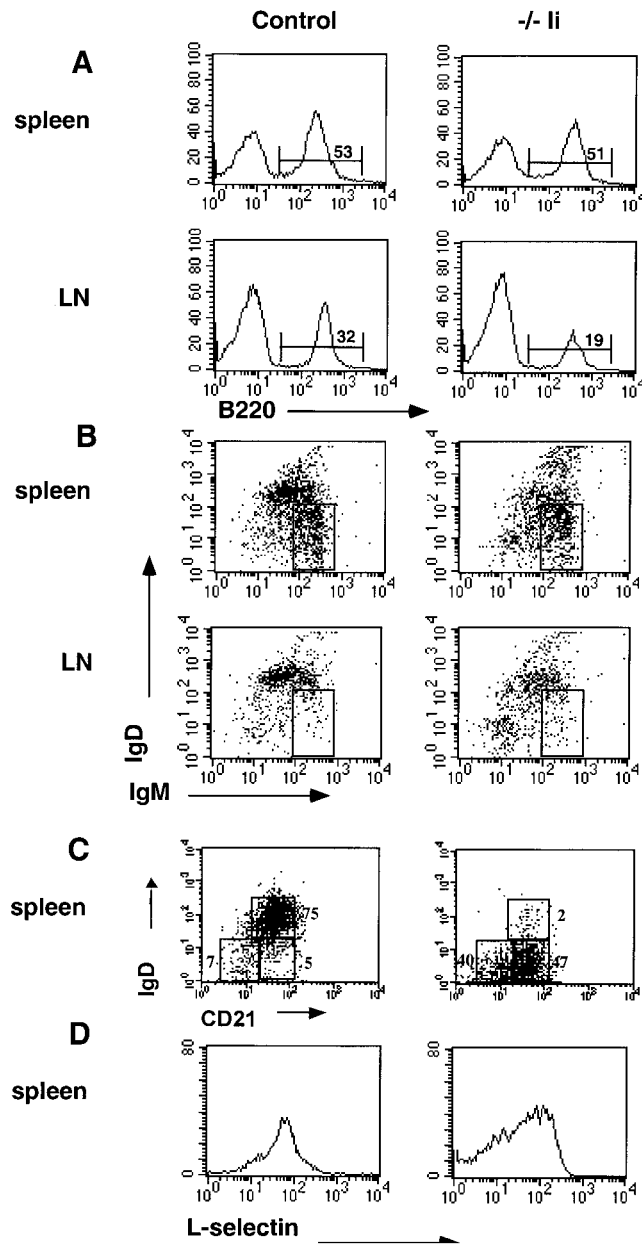
Herein we show that by autocrine secretion of IFN-γ, B cells developmentally regulate their recruitment to LNs.

Materials and Methods

Cells.   Cells were obtained from control C57BL/6, invariant chain (Ii)$^{-/-}$ (10), or IFN-γ–deficient mice on C57BL/6 background (The Jackson Laboratory) at 6–8 wk of age. Spleen and LN cells were purified as described previously (10).

Separation of B Cells. B cells were purified by treating the splenocyte suspension with anti–Thy 1.2, CD4, and CD8 (Southern Biotechnology Associates, Inc.), followed by low Tox-M complement (Cedarlane). To separate between the control IgD⁻ cells from the IgD⁺ population, we used the MACS system (Miltenyi Biotec). The IgD⁻ population was incubated with anti-CD45RA (B220) magnetic beads and reseparated using the MACS system. For comparison of populations, in those experiments in which the control immature B cell population was separated, the Ii⁻/⁻ B cells were purified by the MACS system using the CD45RA beads. T cells were obtained by collecting the B220⁻ population. To separate between the IgD⁻CD21⁺ and IgD⁻CD21⁻ populations, IgD⁻ cells were divided according to their CD21 expression using the MACS system. The IgD⁻CD21⁻ cells were incubated with anti-CD45RA magnetic beads and reseparated. The purity of each cell population was determined by flow cytometry.

Immunofluorescence Staining. The following antibodies used in the experiments were purchased from BD PharMingen: 14.8 anti-CD45RA, B3b4 anti-CD23, 7G6 anti-CD21/CD35, R6-60.2 anti-IgM, AMS 9.1 anti-IgD, 7G6 anti-CD21/CD35, MEL-14 anti–L-selectin, R1-2 anti–very late antigen (VLA)-4, 5H10-27 anti–VLA-5, 2D7 anti-LFA-1, and anti-α4.

Adhesion Assay. Adhesion assays were performed as described previously (11). 96-well plates were coated with fibronectin (100 μg/ml). ⁵¹Cr-labeled cells (2 × 10⁵/well) were suspended in RPMI supplemented with 0.1% FCS or conditioned medium collected from control or Ii⁻/⁻ cells. The cells were plated in each coated well, in the presence or absence of LPS (10 μg/ml), PMA (0.25 μg/ml), IL-2 (0.2 U/μl), stromal cell–derived factor (SDF)-1 (1 μg/ml), EDTA (5 mM), or LDV- and RGD-containing peptides (800 μg/ml). After 30 min, plates were washed. The adherent cells were lysed and the radioactivity was determined. Results were expressed as the mean percentage (± SD) of bound cells from quadruplicate wells. The percentage of cells that adhered was calculated as follows: (total counts of residual cells in the well)/(total counts of cells added to the well) × 100.

RNA Isolation and Reverse Transcription. Total RNA was isolated from cells using the TRI reagent kit (Molecular Research Center). Reverse transcription was carried out using Superscript II RT (GIBCO BRL). The primers and PCR conditions were as described previously (12).

Detection of IFN-γ. Cells were lysed as described previously (10). IFN-γ was immunoprecipitated by overnight incubation with the R4-6A2 monoclonal antibody to IFN-γ (BD PharMingen) followed by protein G–Sepharose beads (Amersham Phar-

Figure 1. Cytofluorometric analysis of B cells from spleen and LNs and their maturation markers. (A) Control and Ii⁻/⁻ splenocytes or pooled LN cells were stained with anti-B220. Histograms show the percentage of B cells in each population. (B) Splenocytes and LN cells were triple stained with anti-B220, anti-IgM, and anti-IgD. FACS® analysis demonstrates the expression of IgM and IgD on B220⁺-gated cells. Boxes mark the immature IgD^low population. (C) Dot plots show CD21 expression on B220⁺ cells. (D) L-selectin expression on B cells. Control and Ii⁻/⁻ total splenotyes were double stained with anti-B220 and anti–L-selectin. Histograms show the expression of L-selectin on B220⁺ cells. The differences between B and C in IgD expression pattern are not significant and result from normal experimental errors. The percentage of this population in both experiments falls in the standard deviation.

Table I. *Total Spleen Cell Number and the Percentage of the B Cell Population and the Mature (IgD⁺) and Immature (IgD⁻) Subpopulations in Control and Ii⁻/⁻ Mice*

	Control	Ii⁻/⁻
Spleen		
Total cells	9.3 ± 2 × 10⁷	6 ± 1.6 × 10⁷
Percent B cells	46.5 ± 8.2	47 ± 10.2
Percent IgD⁺	39.5	2.2
Percent IgD⁻	6.8	44.5
LNs		
Percent B cells	36 ± 3	15 ± 4
Percent IgD⁺	35	13.3
Percent IgD⁻	1	1.6

macia Biotech). Lysates or immunoprecipitated proteins were separated by 12% (wt/vol) SDS-PAGE. The proteins were transferred onto nitrocellulose and probed with the R4-6A2 anti–IFN-γ antibody (BD PharMingen), followed by horseradish peroxidase–conjugated goat anti–rat IgG (Jackson ImmunoResearch Laboratories).

Tracking of Cells In Vivo. Cells were incubated for 30 min with various treatments. The cells were then washed and labeled for 30 min with the fluorescent marker BCECF-AM (10 μg/ml; Molecular Probes; ~97% of cells were stained). 5×10^7 washed cells were injected intravenously into C57BL/6 mice in duplicates. After 3.5 h, spleen and LNs were removed, and the percentage of fluorescent cells in both organs was analyzed by FACS®. Inhibition of cell accumulation was calculated as: 100% − (the amount of cells present after treatment/the cells present in the control mice). The results presented are the average of three different experiments.

Laminar Flow Assay. Polystyrene plates were coated with fi-

bronectin (30 μg/ml; Sigma-Aldrich) in the presence or absence of SDF-1 (0.5 μg/ml). These plastic plates were assembled to form the lower wall in a parallel wall flow chamber as described previously (13). Cells were allowed to settle in the coated capillary tube for 2 min before being subjected to increasing laminar flow. Cell images were videotaped and manually quantitated by analysis of images directly from the monitor screen.

Cytokine Concentration. Cytokines (GIBCO BRL) were added to the adhesion assay at the following concentrations: IL-4 (10^3 U/ml), IL-5 (16 U/ml), IL-6 (16 U/ml), IL-12 (3.5 ng/ml), and IFN-γ (0.1 U/ml).

Results and Discussion

To determine whether immature B cells are indeed excluded from secondary lymphoid organs while recirculating through the body, we first compared the B cell distribution
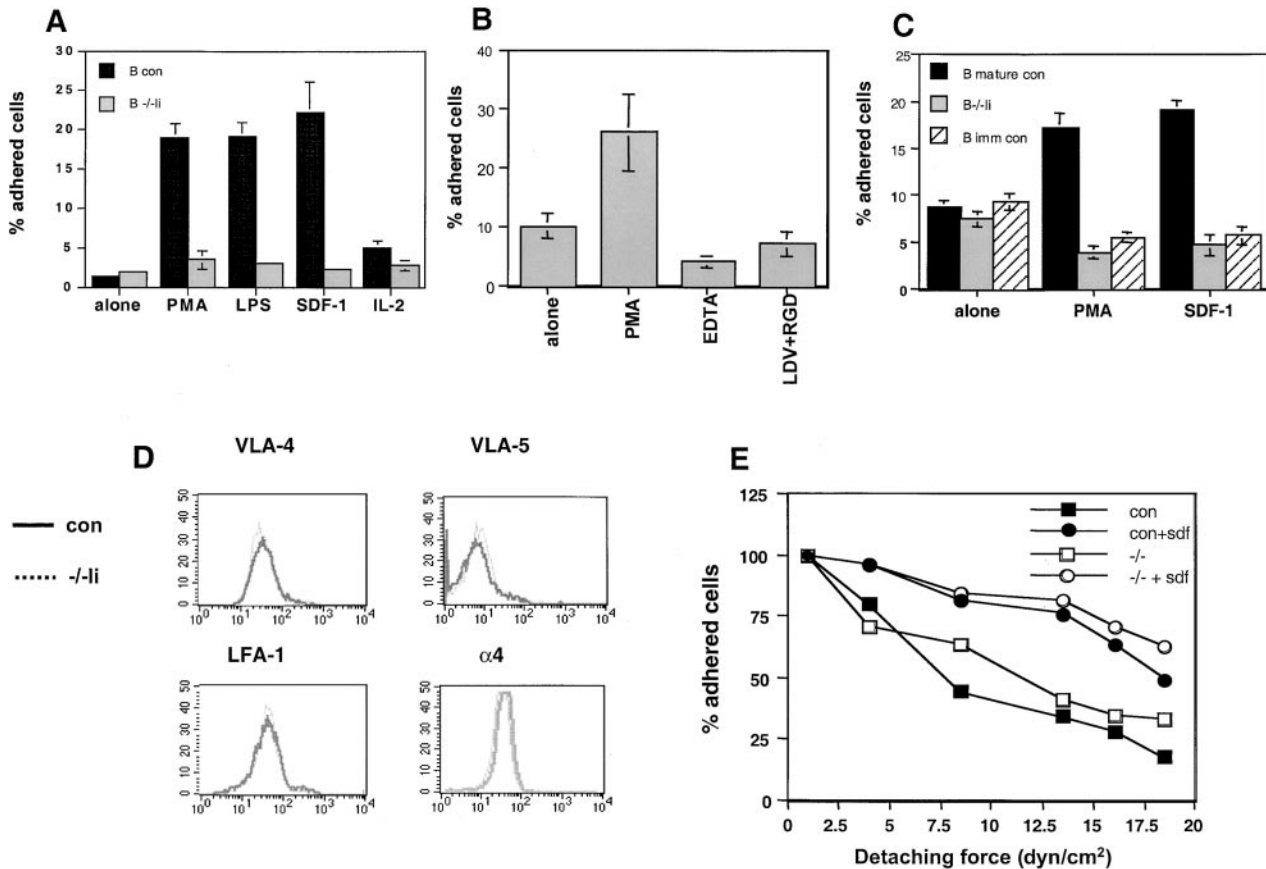

Figure 2. Immature B cells do not increase their long-term integrin-mediated adhesion response to stimulation, although they have a normal short-term response. (A) Wild-type mature B cells (B con) or immature B cells from Ii$^{-/-}$ mice (B $-/-$Ii) were labeled with ^{51}Cr, washed, resuspended in adhesion medium, and added to fibronectin-coated wells in the presence of PMA, LPS, SDF-1, or IL-2 stimulation. The adherent cells were lysed and radioactivity was determined. One experiment representative of five is depicted. (B) Control B cells were ^{51}Cr-labeled and tested for adhesion in the presence or absence of PMA, EDTA (5 mM), LDV, or RGD (800 μg/ml). (C) Mature IgD$^+$ B cells from control mice (B mature con), immature Ii$^{-/-}$ B cells (B $-/-$Ii), and immature IgD$^-$ B cells from control mice (B imm con) were ^{51}Cr-labeled and tested for adhesion. One experiment representative of three is depicted. (D) Cytofluorometric analysis of integrin expression on immature and mature B cells. Control and Ii$^{-/-}$ splenocytes were double stained with anti-B220 and anti–VLA-4, VLA-5, LFA-1, or α4β7. Histograms show an overlay of the expression levels of the different integrins on control (light) and Ii$^{-/-}$ (dark) B cells. (E) Resistance to shear stress by mature and immature B cells on fibronectin in a short-term adhesion assay. Control (con) and Ii$^{-/-}$ ($-/-$) B cells were allowed to attach to plates coated with fibronectin, alone or coimmobilized with SDF-1 (sdf). After attachment, flow was initiated and increased in 2–2.5-fold increments every 10 s. The number of cells remaining bound at each interval was determined and is expressed as the percentage of input cells remaining bound. The data depict one experiment representative of three.

in spleen and LNs in control and in Ii–deficient ($Ii^{-/-}$) mice. B cells from $Ii^{-/-}$ mice are arrested at an immature stage and have an immature phenotype expressing low levels of IgD and CD23 and partial CD21 expression (14–16; Fig. 1, B and C, and Table I). Moreover, these $Ii^{-/-}$ B cells proliferate in response to LPS stimulation, but anti-IgM causes their death (data not shown). However, control and $Ii^{-/-}$–derived B cells found in the LNs were enriched for cells expressing a more mature phenotype, higher levels of IgD. Moreover, as was described previously, in $Ii^{-/-}$ mice there was a striking decrease in the proportion of B cells populating the LNs (15–17; Fig. 1 and Table I). These observations confirm that immature B cells are depleted from the LNs, although the $Ii^{-/-}$ population expresses significant levels of L-selectin on their cell surface (Fig. 1 D).

To migrate to the LNs, leukocytes, including B cells, must interact with adhesive components of the extracellular matrix (ECM; 18). We therefore compared the adhesion response of mature (control) and immature ($Ii^{-/-}$) B splenocytes by studying their ability to adhere, upon activation, to fibronectin, a major ECM-based cell adhesive integrin ligand. ^{51}Cr-labeled B cells were plated for 30 min on fibronectin–coated microtiter wells in the presence of PMA, a potent agonist of integrin-mediated adhesion (19, 20), LPS, a B cell mitogen, or SDF-1, a potent B cell chemoattractant (21). After stimulation, control B cells dramatically increased their adhesion to fibronectin. In contrast, the adhesion of $Ii^{-/-}$ immature B cells remained unchanged after

activation (Fig. 2 A). As expected, B cells did not respond to stimulation with IL-2. The adhesion of B lymphocyte to fibronectin in response to stimulation was abrogated in the presence of the integrin inhibitor, EDTA, as well as by the LDV and RGD peptides, selective blockers of VLA-4 and VLA-5 (Fig. 2 B). Thus, the adhesion response observed was integrin mediated.

The inability of $Ii^{-/-}$ B cells to increase their adhesion to fibronectin could be ascribed to the developmental stage of the cells, or to Ii deficiency. Therefore, we compared the adherence of purified control immature IgD^- and $Ii^{-/-}$ B cells in response to SDF-1 or PMA stimulation. Like immature B cells derived from $Ii^{-/-}$ mice, IgD^- immature B cells from control mice were unable to respond to stimulation and did not increase their adhesion to fibronectin (Fig. 2 C). Thus, the stage of maturity, and not the lack of Ii, determined the response. Similar cell surface expression levels of the integrins VLA-4, VLA-5, LFA-1, and $\alpha 4$, the major fibronectin receptors, were detected on both $Ii^{-/-}$ and control B cells (Fig. 2 D). Thus, immature B cells possess the adhesive molecules required for stimulation and adherence to the ECM. To verify that intrinsic integrin function is not impaired, we tested both control and $Ii^{-/-}$ populations in a short-term adhesion assay on fibronectin-coated surfaces assembled in a flow chamber apparatus. In the absence of stimulation, $Ii^{-/-}$ B cells exhibited a slightly stronger spontaneous adhesion to fibronectin than that of the control cells. Surprisingly, rapid adhesion stimulation to fi-
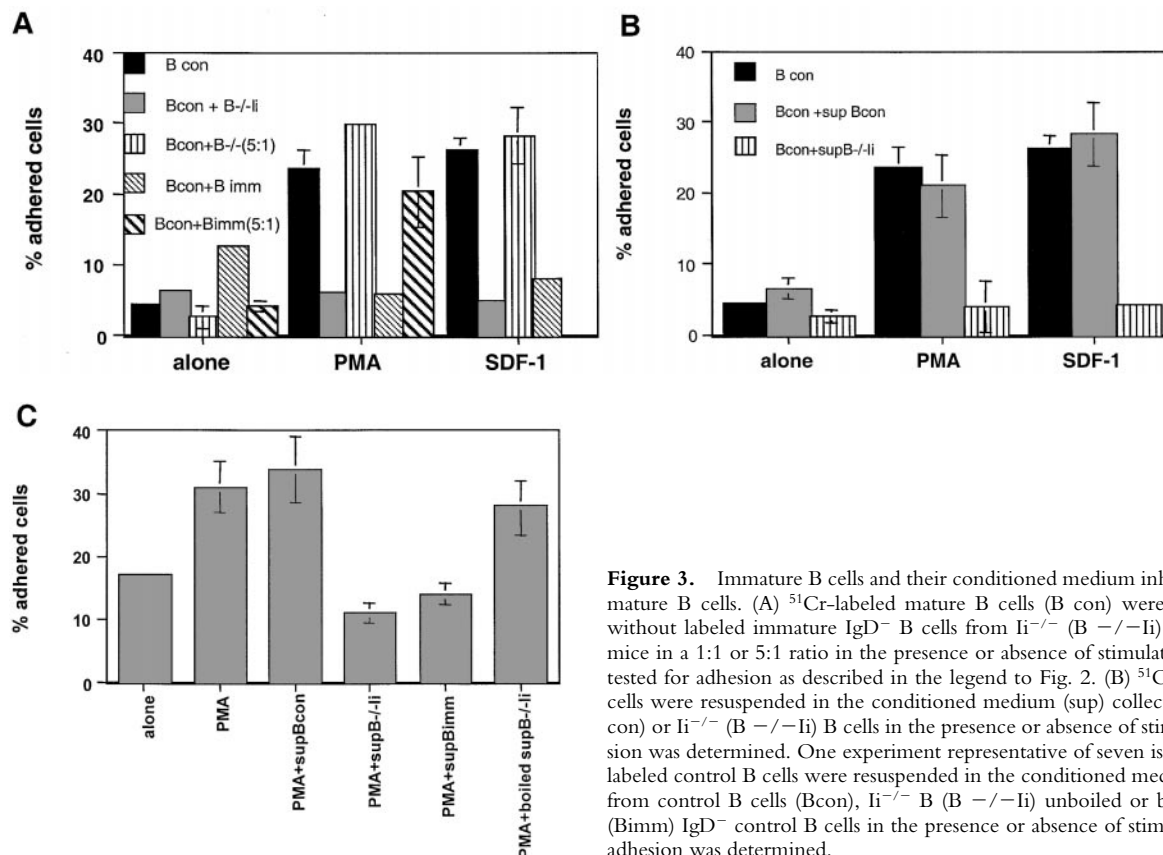
Figure 3. Immature B cells and their conditioned medium inhibit the adhesion of mature B cells. (A) ^{51}Cr-labeled mature B cells (B con) were incubated with or without labeled immature IgD^- B cells from $Ii^{-/-}$ (B $-/-$Ii) or control (Bimm) mice in a 1:1 or 5:1 ratio in the presence or absence of stimulation. The cells were tested for adhesion as described in the legend to Fig. 2. (B) ^{51}Cr-labeled control B cells were resuspended in the conditioned medium (sup) collected from control (B con) or $Ii^{-/-}$ (B $-/-$Ii) B cells in the presence or absence of stimulation, and adhesion was determined. One experiment representative of seven is depicted. (C) ^{51}Cr-labeled control B cells were resuspended in the conditioned medium (sup) collected from control B cells (Bcon), $Ii^{-/-}$ B (B $-/-$Ii) unboiled or boiled, or immature (Bimm) IgD^- control B cells in the presence or absence of stimulation (PMA), and adhesion was determined.

bronectin was induced in both cell types by 2-min treatment with SDF-1 (Fig. 2 E) or PMA (not shown). These results indicate that both the expression of integrin receptors for fibronectin and their ability to respond to rapid stimulation are normal in immature B cells. Thus, the lack of adhesion by immature cells during the 30-min adhesion assay appeared to result from inhibitory events subsequent to normal initial integrin-mediated adhesion.

To test whether the inhibition of adhesion may be transferred to other cells, we determined the ability of purified wild-type IgD$^-$ immature or Ii$^{-/-}$ B cells to inhibit the adhesion of purified mature IgD$^+$ B cells when comixed. Surprisingly, both the control IgD$^-$ and Ii$^{-/-}$ immature B cells had an inhibitory effect on the adhesion of mature B lymphocytes to fibronectin (Fig. 3 A); the inhibition could be abrogated by fivefold dilution of the immature cells within the mature population.

The inhibitory effect mediated by immature B cells could be explained by direct cell–cell interaction, or by secretion of a soluble inhibitory factor. To determine

whether a factor secreted from the cells could affect B cell adhesion, we analyzed the activity of conditioned medium collected from the cells. Wild-type or Ii$^{-/-}$ immature B cells were plated on fibronectin-coated microwells and 30 min later their conditioned medium was collected. The ability of stimulated control B cells to adhere to fibronectin in the presence of conditioned medium derived from control or Ii$^{-/-}$ immature B cells was studied. Whereas conditioned medium collected from control B cells did not affect cell adhesion, cells incubated with conditioned medium collected from Ii$^{-/-}$ B cells (Fig 3, B and C) or control IgD$^-$ immature B cells (Fig. 3 C) maintained a low adhesion response after stimulation. Thus, the conditioned medium derived from immature B cells contained a factor(s) that inhibited the adhesion of stimulated B cells. The factor was heat sensitive (Fig. 3 C).

To determine the identity of this factor, various cytokines were screened for their ability to suppress agonist-stimulated B cell adhesion to fibronectin. Of the chemokines tested, only IFN-γ could mimic the effect of the condi-
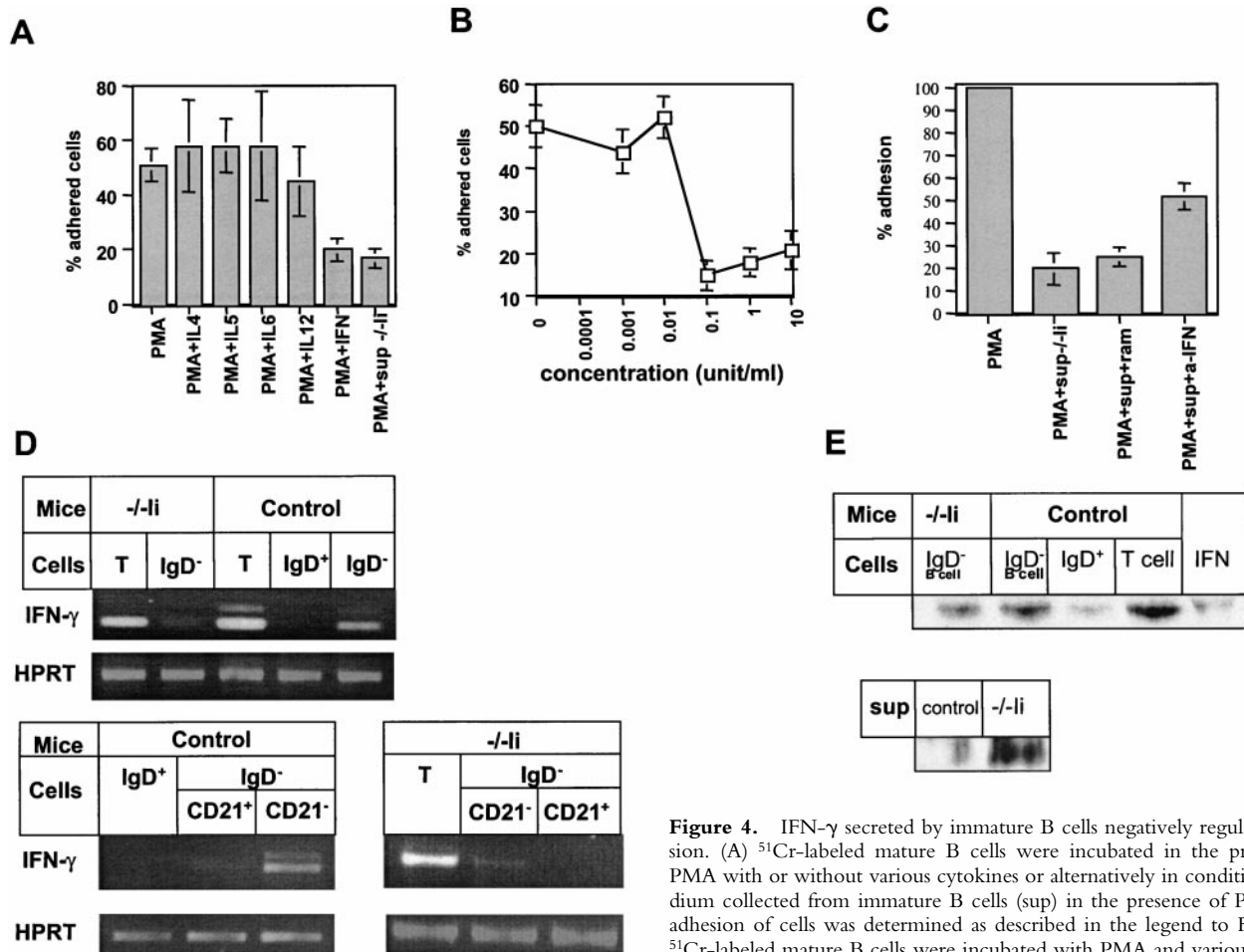
Figure 4. IFN-γ secreted by immature B cells negatively regulates adhesion. (A) ^{51}Cr-labeled mature B cells were incubated in the presence of PMA with or without various cytokines or alternatively in conditioned medium collected from immature B cells (sup) in the presence of PMA. The adhesion of cells was determined as described in the legend to Fig. 2. (B) ^{51}Cr-labeled mature B cells were incubated with PMA and various concentrations of recombinant IFN-γ and their adhesion was determined. (C) ^{51}Cr-labeled mature B cells were incubated with PMA. The adhesion response of the cells was designated as 100%. The adhesion response of these cells incubated in conditioned medium derived from Ii$^{-/-}$ immature B cells (sup) itself or conditioned medium treated overnight with rat anti–mouse IgG (ram) or rat anti–IFN-γ (a-IFN) antibodies was calculated as a percentage of the PMA response. (D) Reverse transcription PCR was performed as described in Materials and Methods. (E) Steady-state levels of IFN-γ in total cell lysates of IgD$^-$ B cells from control or Ii$^{-/-}$ mice, mature IgD$^+$ B and T cells from control mice, or IFN-γ immunoprecipitated from conditioned medium (sup) of control and Ii$^{-/-}$ B cells. The band representing IFN-γ is indicated.

tioned medium derived from immature B cells (Fig. 4 A), and acted in a concentration-dependent manner (Fig. 4 B). Antibodies against IFN-γ partially blocked (∼50%) the inhibitory effect of immature B cell–conditioned medium. The partial nature of the antibody blocking may indicate involvement of an additional factor(s) in this process (Fig. 4 C). Reverse transcription PCR analysis revealed that IFN-γ expression by immature B cells is developmentally regulated at the transcriptional level. Although mRNA was detected in both control and Ii$^{-/-}$IgD$^-$ immature B cells (15–17; Fig. 1), this transcript was not found in IgD$^+$ B cells from control mice (Fig. 4 D). Furthermore, the low partially mature IgD$^+$ B cell population, which is present in Ii$^{-/-}$ mice, downregulated its IFN-γ mRNA levels as well (data not shown). Newly emigrant immature B cells can be separated from the marginal zone cells by the expression of the CD21 marker. To determine which population secretes IFN-γ, we analyzed IFN-γ message in the CD21$^+$ and CD21$^-$ IgD$^-$ populations. As can be seen in Fig. 4 D, the IFN-γ mRNA can be detected in the IgD$^-$CD21$^-$ cells from both control and Ii$^{-/-}$ mice. After acquisition of CD21, the cells downregulate their IFN-γ expression. Thus, IFN-γ is transcribed mostly in newly arrived immature B cells. The Western blot analysis of IFN-γ expression in B cells during maturation revealed maturation-dependent regulation of IFN-γ production and secretion. Whereas immature (IgD$^-$) B cells from control or Ii$^{-/-}$ mice express high levels of IFN-γ, mature (IgD$^+$) cells produce lower IFN-γ levels. Importantly, conditioned medium derived from Ii$^{-/-}$ cells contained significant levels of IFN-γ, indicating that the cytokine is both produced and secreted by immature B cells (Fig. 4 E).

We further studied the effect of the conditioned medium and IFN-γ on the entry into LNs in vivo (Fig. 5 A). Control or Ii$^{-/-}$ total splenocytes were preincubated in the presence of fresh medium (Fig. 5 A, bars 1 and 2), IFN-γ (Fig. 5 A, bar 6), conditioned medium collected from control B cells (Fig. 5 A, bar 3) or from Ii$^{-/-}$ B cells (Fig. 5 A,

bar 4), or conditioned medium collected from Ii$^{-/-}$ immature B cells treated with anti–IFN-γ (Fig. 5 A, bar 5). The cells were then washed, labeled with the fluorescent dye BCECF-AM, and an equal number of live cells was injected intravenously into control mice. The proportion of labeled cells recovered in the spleen and LNs was determined 3.5 h after injection. The extent of labeled cell accumulation in the spleen was unaffected by the various pretreatments. In contrast, migration of Ii$^{-/-}$ and of control cells incubated with Ii$^{-/-}$ conditioned medium or IFN-γ to the LNs was significantly decreased, whereas cells incubated with conditioned medium derived from control B cells or control medium exhibited a degree of migration similar to untreated cells. When control cells were incubated in Ii$^{-/-}$ conditioned medium treated with antibodies against IFN-γ, the ability of this conditioned medium to inhibit homing of the cells to the LNs was partially blocked (∼50%). In agreement with previous studies, our results suggest that the entry of lymphocytes from the blood into the spleen does not depend on integrin–fibronectin interactions, whereas migration to peripheral LNs is critically regulated by these adhesive interactions (22).

To determine the biological significance of our studies, we analyzed the B cell profile in spleen and LNs of genetically IFN-γ–deficient mice (IFN-γ$^{-/-}$). Although spleens derived from IFN-γ$^{-/-}$ exhibited wild-type levels of immature and mature B cells, there was a striking difference in the profile of the B cell population in the LNs. LNs of IFN-γ$^{-/-}$ were populated with significantly greater levels of immature B cells compared with their control counterparts (Fig. 5 B). These results demonstrate the physiological role of IFN-γ in regulating the arrival of immature B cells to the LNs.

En route to the spleen, for their final maturation, immature B cells recycle through the blood and can be recruited either to LNs or to sites of inflammation. Immature B cells prevent premature encounter with antigen by downregulating their own migration into the LNs. This inhibition is
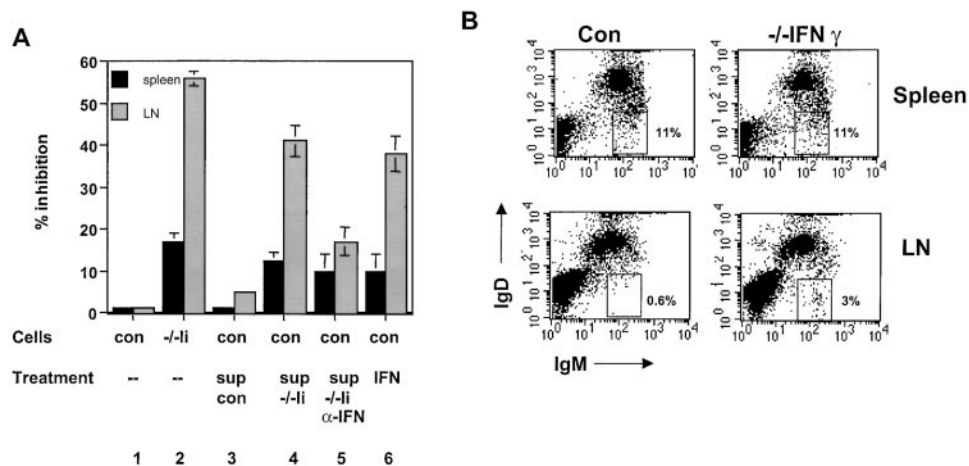
Figure 5. IFN-γ regulates the migration and arrival of immature B cells to the LNs in vivo. (A) Inhibition in the arrival of labeled cells to spleen or LNs. The proportion of control cells which homed to control spleen or LNs was designated 100%. The arrival of Ii$^{-/-}$ cells or control cells treated with conditioned medium derived from control (sup con) or Ii$^{-/-}$ (sup −/−Ii) B cells or Ii$^{-/-}$-conditioned media treated with anti–IFN-γ (sup −/−Ii α-IFN) or in the presence of IFN-γ (1 U/ml) was calculated as the percentage of control homing. Inhibition was defined as: 100% − the percentage of cells arrived in each treatment. The data shown represent the average of three experiments.

(B) Analysis of spleen and LNs derived from control and IFN-γ–deficient mice. Dot plots show the expression of the markers IgD and IgM on the total spleen and LN cells. The percentage of the immature population out of the 100% of B cells is calculated. The data depict one mouse out of four analyzed.

mediated by IFN-γ, which is transcribed and secreted by immature B cells but dramatically downregulated in mature B cells. Our in vivo results directly demonstrate the physiological significance of IFN-γ secretion in preventing immature B cell arrival to sites of foreign antigen presentation. In addition, low levels of IFN-γ have the ability to regulate the adhesion of mature B cells (23; Fig. 4). As immature B cells normally migrate to specific microenvironments, and the proportion of immature B cells in the circulation is low, it is more likely that immature B cells use IFN-γ to locally influence their integrin function rather than to exert global effects on mature B cells in vivo. The powerful inhibitory effect of IFN-γ on adhesion of B lymphocytes both in vitro and in vivo suggests that this protein if properly targeted may have therapeutic clinical application as a B cell–specific antiinflammatory agent in addition to its many other functions.

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