Type I IFN sets the stringency of B cell repertoire selection in the bone marrow

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Abstract

Locally produced type I interferon (IFN-I) enhances the sensitivity of bone marrow B cell to IgM receptor ligation. The establishment of B cell repertoires, on the other hand, seems to involve selective processes that are critically dependent on B cell receptor (BCR) ligation. In order to assess the importance of BCR triggering thresholds on the selection of polyclonal unmanipulated B cell populations, we compared VH gene expression and reactivity repertoires in various B cell compartments of wild-type and IFN-I receptor-deficient mice (IFN-I-R⁻⁻). These analyses demonstrate that increased B cell sensitivity to BCR ligation mediated by IFN-I in the bone marrow (BM) has consequences on the stringency of B cell repertoire selection. Thus, the normal counter-selection of both VH 7183 gene family expression and multireactivity was impaired among immature BM B cells from mutant mice. Furthermore, as a result of reduced efficiency of BCR ligation-dependent inhibition of terminal differentiation, IFN-I-R⁻⁻ animals produce, in BM and thymus, higher numbers of plasma cells secreting antibodies that are more multireactive than wild-type animals. Finally, mutant serum IgM natural antibodies display a more reactive repertoire than controls, a likely reflection of the BM resident plasma cell repertoire. The present observations demonstrate, therefore, that local modulation of BCR triggering thresholds leads to important modifications in the generation and/or selection of normal B cell populations.

Introduction

The bone marrow (BM) constitutes a highly specific environment that allows the continuous development of the B lymphocyte lineage throughout adult life. Precursors differentiate and proliferate locally before reaching peripheral organs as mature lymphocytes. Later stages of differentiation characterized by cellular activation and followed by Ig secretion have been often neglected in developmental analysis although occurring in the absence of any immunization. This natural activation leads to the production of natural antibodies that display specific features such as multireactivity and connectivity (1).

Given that variable region diversity is randomly generated at the lymphocyte precursor stages, repertoire selection has to occur in a coordinated fashion with B cell development, such as to ensure the generation of competent lymphocytes, to preserve diversity and to prevent harmful levels of self-reactivity. Current views on repertoire selection favor the notion that B cells are 'screened' for specificity by receptor ligation at each developmental stage (2,3). This selection process seems to be initiated in the BM where newly generated cells first encounter antigens. Evidence for early selection at the pre-B cell stage via the Vpre-B receptor have been presented (4). This process is continued at the immature B cell stage, where a complete surface IgM is expressed. Formal evidence for such a cellular selection has been provided by analyses of transgenic mice expressing a B cell receptor (BCR) of known specificity (2,5,6). In such models, deletion and inactivation processes have been described, and are believed to ensure the absence of pathogenic levels of self-reactivity in the periphery. In addition, analyses of unmanipulated, polyclonal B cell populations in normal mice revealed physiological negative selection of B cell repertoires, leading to a decrease in BCR multireactivity along B cell maturation (7). Similarly, B cell development is associated with modifications in the usage of VH gene families. Thus, B cell precursors use a restricted set of VH segments, while mature B cell populations express a wide diversity (8–10). In mouse, the more D-proximal genes, e.g. VH 7183 and VH Q52 gene families, are preferentially expressed early in cellular
development, both by fetal liver and adult BM precursors. This preferential usage decreases drastically from pro-B to pre-B cells and from immature to mature B lymphocytes, thus indicating a counter-selection of cells expressing these genes. Antigen interaction with BCR initiates a cascade of signals that may lead to cell survival, activation or death (11). It has long been established that immature BM B cells are more sensitive than mature peripheral lymphocytes to BCR ligation (reviewed in 12). Our previous work, however, has demonstrated that differential sensitivity to BCR ligation is not an intrinsic property of B cell sub-sets, but is modulated by type I IFN (IFN-I) locally produced in the BM (13). Using IFN-I receptor (IFN-I-R) mutant mice, we could investigate, therefore, the consequences of raising the threshold of BCR triggering in a normal, polyclonal B cell population.

The development and selection of B cell repertoire were now compared in wild-type and IFN-I-R−/− mice. These studies demonstrate both the physiological process of B cell repertoire selection and the respective role of IFN-I. Thus, local modulation of B cell sensitivity to BCR ligation alters repertoire selection of BM immature B cells and IgM-secreting plasma cell populations, resulting in significant changes of serum IgM reactivity.

Methods

Mice

IFN-I-R-deficient mice (14) and 129sv control mice were bred and kept in our animal facilities. All analyses were performed on 8- to 12-week-old animals.

Cell purification and cultures

Splenocytes and thymocytes were obtained by grinding the organ between frosted ends of glass slides. BM cells were obtained by flushing the bone with medium using a 23 gauge needle. Purification of immature and mature BM cells was done on the basis of the expression of the surface marker CD23. Positive and negative selections were performed using a biotinylated anti-CD23 antibody (PharMingen, San Diego, CA) by MiniMACS magnetic cell sorting according to the manufacturer (Miltenyi Biotec, BergischGladbach, Germany). The positive fraction provided the mature B cells while the negative fraction was submitted to a second step of positive purification using an anti-B220 biotinylated antibody (Pharmingen). FACS analysis indicated that the enrichment was >90%. Resting and activated spleen cells were separated according to their densities on a Percoll gradient, and enrichment confirmed by forward scatter analysis. Thymus B cells were enriched by complement-mediated killing of the Thy-1-expressing cells. Briefly, total thymocytes were incubated at 37°C for 40 min with the hybridoma J7/7 supernatant produced in the laboratory and rabbit complement (Sigma) diluted 1/15 in RPMI. All cell cultures were performed in RPMI 1640 medium containing Glutamax (Gibco/BRL, Gaithersburg, MD) supplemented with 10% FCS (Boehringer Mannheim, Meylan, Germany), 50 μM β2-mercaptoethanol, antibiotics (50 IU/ml penicillin and 50 μg/ml streptomycin), 10 mM HEPES buffer and 1 mM sodium pyruvate (all from Difco/BRL). The same media containing only 2% FCS was used to perform an ELISA spot assay (ESA). B cell activation was induced by lipopolysaccharide (LPS, Salmonella typhymurium, Sigma) at 25 μg/ml, anti-CD40 (gift from J. F. Kearney) at 2 μg/ml and IL-4 supernatant from 3T3IL-4 diluted 1/10. IFN-αβ (mouse fibroblast; Sigma) pretreatment was performed with 400 U/ml during 24 h. Inhibition of LPS stimulation assay is described in (15) and was done using mAb against IgM produced in the laboratory from the 331 hybridoma.

In situ hybridization

In situ hybridization of LPS-activated cells was performed according to Haase et al. (16), following modifications performed and extensively described in (10). The cDNA probes specific for Vµ,7183, VµJ558 and VµQ52 gene families are described in (10). The control Cµ probe is the Pst fragment in exon 2. Probes were labeled by random priming with [35S]dCTP (500 Ci/mmol; ICN) and specific hybridization revealed by autoradiography in Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY). The number of positive cells was scored by counting between 30 and 100 microscopic fields (~2000–30,000 cells scored), as well as the mean number of total cells per field, from which the frequency of positive cells was calculated. A cell was scored positive if it contained at least 20 silver grains, but in general positive cells were heavily labeled with uncountable number of silver grains.

ELISA and ESA

Sera were obtained by retro-orbital plexus bleeding and kept at −20°C until used. Total IgM and IgG concentrations were determined by ELISA as described in detail elsewhere (17,18). Total or antigen-specific IgM and IgG plasma cell numbers in ex vivo experiments or after LPS stimulation were evaluated by a modification of the ESA (7,19). Briefly, ELISA plates (Luxlon M29 LSE; CEB) were coated with anti-IgM (Southern Biotechnology, Birmingham, AL), DNA, myosin, thyroglobulin, actin, ribonuclease, β-galactosidase or hen egg lysozyme (all from Sigma). The plates were blocked with gelatin, and the cells were seeded according to a serial dilution and incubated at 37°C for 4–5 h. After washing out the cells, the spots, generated by the specific binding of secreted Ig, were revealed with alkaline phosphatase-conjugated anti-mouse IgM or IgG (Southern Biotechnology Associates, Birmingham, AL) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The number of plasma cells per seeded cell was determined by counting the number of spots in at least three different dilutions of cells. When evaluating the number of naturally occurring plasma cells in various organs, the number of spots was further readjusted to the number of B cells as determined by FACS analysis.

Analysis of IgM and IgG sera repertoire by quantitative immunoblot assay

Mouse tissues and bacteria extracts were obtained and used in an improved immunoblot assay for analysis of natural antibody repertoires as previously described (20,21). Immunoblot was performed using the sera samples adjusted to 10 μg/ml of IgM. Quantitation of immunoreactivities was performed by densitometry in reflective mode. Blotted total...
proteins were then stained using colloidal gold, and subjected to a second densitometric analysis to score the protein profile and to quantitate transferred proteins. The scanned images were digitalized, and data processed and analyzed using the software Igor (Wavemetrics, Lake Oswego, OR) with special macros written to allow comparison between immunoreactivity profiles after adjustment of irregularities of protein profiles due to electrophoretic migration distortions and also to perform multiparametric statistical analysis (22,23).

Results

IFN-I does not regulate the steady state numbers of B lymphocytes

Several lines of evidence suggest that (pre-)BCR signaling is necessary for cell survival and progression along the differentiation pathway to mature B cells (24–27). Given the IFN-I-dependent regulation of cellular sensitivity to BCR ligation, as well as its recent implication in the control of pre-B cell proliferation (28), we investigated B cell development in IFN-I-R–/– (14) animals. A detailed quantitative FACS analysis compared the composition of B lymphocyte subpopulations in adult mutant and wild-type mice. Absolute numbers of BM pro-B (B220+, IgM+, CD43+), pre-B (B220+, IgM+, CD43+), immature B (B220+, IgM−, CD23−, IgD+, CD22−) and mature B cells (B200+, IgM+, CD23+, IgD−, CD22+) were determined, as well as those of naive and activated B cells in the spleen and lymph nodes (forward scatter, IgM density, CD23 and syndecan expression). These analyses revealed that B lineage compartments of either BM or peripheral organs are equally represented in adult IFN-I-R–/– and in control mice (data not shown), indicating that constitutively produced IFN-I in the BM does not modify the steady-state composition of early, intermediate or mature stages of B cell development.

Altered V\textsubscript{H} gene family expression of BM immature B cell populations in the IFN-I-R deficient mice

Modifications in the representation of the V\textsubscript{H} gene segments used in the composition of BCR repertoires is another hallmark of B cell development that is associated with BCR-dependent signaling and selection. Thus, numerous studies demonstrated major differences in V\textsubscript{H} gene usage between various BM compartments and peripheral organs (8–10). We therefore compared the genetic repertoires of B cell subpopulations in IFN-I-R–/– and genetically identical control mice (129/sv). Using \textit{in situ} RNA hybridization, we scored the relative representation of V\textsubscript{H} gene families in purified B cells after \textit{in vitro} polyclonal activation by LPS. BM mature and immature B cells were isolated according to differential expression of CD23, as described previously (13), while resting and activated splenocytes were separated by density gradient centrifugation. For simplicity, only the relative usage of the V\textsubscript{H}7183, V\textsubscript{H}J558 and V\textsubscript{H}Q52 gene families in the different subpopulations are represented. As shown in Fig. 1, left panel, analyses of the expression of these three V\textsubscript{H} families in control wild-type mice confirmed that B cell maturation is accompanied by a decreased utilization of the most D-proximal (V\textsubscript{H}7183 and V\textsubscript{H}Q52) genes in the periphery (10). Moreover, these results show that the shift in V\textsubscript{H} gene family representation takes place already at the transition from CD23+ to CD23− B cells, since the latter population is comparable to small spleen cells in this respect. Thus, in line with previous observations on V\textsubscript{H}7183.1 expression (9), we conclude that a major selective shift in V\textsubscript{H} gene repertoire composition occurs just prior to B cell emigration to the periphery (29).

Genetic repertoire analyses of the IFN-I-R–/– mice revealed a major difference as compared to control mice in the compartment of BM immature B cells. As can be seen in Fig. 1, right panel, there is no shift in V\textsubscript{H} gene repertoires between CD23+ and CD23− BM cells. Thus, the representation of V\textsubscript{H}7183 genes in immature and mature B cell populations is comparable (average 6.5%) and similar to that scored in wild-type mature B cells (6%). A slightly decreased representation of the V\textsubscript{H}J558 family in the immature (CD23+) population is inversely affected (28% in the mutant versus 14% in the control). Interestingly, the representation of the V\textsubscript{H}Q52 family is not affected by the absence of IFN-I-R.

These results demonstrate that IFN-I is involved in the selective processes accompanying BM B cell differentiation and are compatible with the notion that IFN-I sets the stringency of BCR signaling operating in the counter-selection of V\textsubscript{H}7183-expressing cells. It looks as though IFN-I plays a critical role on repertoire selection at the immature/mature BM cell transition and that, in its absence, other mechanisms operate to initiate counter-selection of V\textsubscript{H}7183 expression at a stage of development prior to that defined by the IgM+ CD23− phenotype. As a result, IFN-I-R–/– and wild-type mature B cell compartments display similar V\textsubscript{H} gene family representation.

IFN-I modulates the reactive repertoire selection of newly generated B cells

Previous evidence for selection against antibody (BCR) multireactivity throughout B cell maturation (7,30) strongly suggests that such selective processes are driven by BCR V-region-mediated signaling. In order to assess the role of IFN-I in this process, we tested the reactivity of various B cells populations on a panel of self and non-self antigens. Purified cells were activated to antibody secretion by LPS treatment \textit{in vitro} and
Table 1. Frequencies of antigen-specific IgM-secreting cells in adult IFN-I-R–/– and control mice

<table>
<thead>
<tr>
<th></th>
<th>BM CD23–</th>
<th>BM CD23–</th>
<th>Spleen (small)</th>
<th>Spleen (large)</th>
<th>Spleen (plasma cells) (IgM+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>KO</td>
<td>Wild-type</td>
<td>KO</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Anti-µ2</td>
<td>17,600</td>
<td>25,024</td>
<td>107,160</td>
<td>119,524</td>
<td>190,269</td>
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<tr>
<td>TNP-BSA–</td>
<td>26.0</td>
<td>33.0</td>
<td>8.8</td>
<td>7.7</td>
<td>8.0</td>
</tr>
<tr>
<td>DNA</td>
<td>9.5</td>
<td>26.0</td>
<td>2.4</td>
<td>6.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Myosin</td>
<td>3.6</td>
<td>4.4</td>
<td>1.8</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>3.1</td>
<td>4.8</td>
<td>1.5</td>
<td>3.1</td>
<td>0.7</td>
</tr>
<tr>
<td>HEL</td>
<td>1.7</td>
<td>1.8</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>43.9</td>
<td>70.0</td>
<td>15.0</td>
<td>19.6</td>
<td>10.2</td>
</tr>
</tbody>
</table>

aLPS-treated purified population.  
bUntreated population.  
cAnti-µ reveals the total number of IgM-secreting cells analyzed in the assay.  
dResults expressed as percentage of antigen-specific IgM-secreting cells out of total number of IgM-secreting cells.  
Results of one representative experiment out of three independent measures.

Fig. 2. Reactivity repertoire alteration in IFN-I-R–/– mice is restricted to BM immature B cells. For each B cell subpopulation, the frequencies of antigen-specific IgM-secreting cells detailed in Table 1 are summed up and presented as a histogram. Wild-type, control mice; KO, IFN-I-R–/– animals. SD established on three different experiments for each point was <2.5% of the mean.

then assayed by ESA. This strategy has been shown to reveal an unbiased repertoire sampling of the corresponding B cell population (31–33), where the total reactivity scored is directly correlated to the antibody multireactivity (frequency of multireactive cells and degree of individual V-region degeneracy) of the cell population (7).

Analyses of the wild-type mice confirmed previous data demonstrating that the frequencies of B cells expressing IgM reactive to the antigenic panel are highest amongst BM B cell populations and lowest among naturally activated splenic plasma cells (Table 1). Furthermore, we show here that BM immature CD23− B cells constitute the most multireactive cell pool, since up to 43.9% of all cells scored on this very limited panel of antigens. In contrast, the CD23+ population displays a frequency of reactive clones similar to those of peripheral cells (15 versus 10.2 and 11.8%) (Fig. 2), indicating again that a major step in B cell repertoire selection takes place at the transition between CD23− and CD23+ cells.

In the present experiments, peripheral resting and activated B cells are indistinguishable, whereas the frequency of reactive cells in the pool of natural plasma cells is drastically decreased (2.9%). Since such high frequency of cells recognizing only five antigens must correspond to antibody multireactivity (7), our results indicate that selection against multireactivity in normal B cell repertoires occurs in two major steps: (i) at the developmental transition from immature to mature stages in BM, and (ii) at the final phase of differentiation upon activation to terminal differentiation and antibody secretion.

Analyses of B cell reactivity repertoires in IFN-I-R–/– animals reveal a similar loss of multireactivity in the peripheral repertoire (Fig. 2B). In contrast, the frequency of multireactive clones in the total BM population is increased when compared to control, the difference being due to the immature CD23− population (70 versus 43.9%), where the frequency of anti-single-strand DNA reactivities is markedly increased (Table 1). Increased multireactivity is also detected, but to a lesser extent, in the mature BM cell population (19.6 versus 15%). Strikingly, IFN-I-R–/– and control mice are again comparable in the reactivities of splenic resting and activated B cells. Thus, the alterations in repertoire selection in BM are compensated by alternative mechanisms along progression to the peripheral compartments.

Taken together, our results support the notion that IFN-I sets the stringency of BCR signaling in BM and thus participates in the physiological process responsible for the counter-selection of multireactivity that occurs at immature B cell stages.

IFN-I-R–/– mice produce increased numbers of BM and thymic plasma cells

Production of natural IgM plasma cells is a highly controlled process both as to the number and specificity of cells in this compartment (30,34). Although often neglected, the BM natural IgM plasma cell population contributes to quite a significant fraction of all serum IgM in unimmunized animals (35). Our original observations on IFN-I-dependent regulation of BCR-signaling thresholds had precisely concerned inhibition of plasma cell generation by BCR ligation (13). It seemed appropriate, therefore, to evaluate the physiological impact of local IFN-I synthesis on the production of natural IgM plasma cells, particularly because the present experimental system offers an inbuilt control, i.e. the periphery, where
constitutive production of IFN-I is absent. The numbers of IgM- and IgG-secreting cells in each lymphoid compartment of both mutant and control mice were determined, and are displayed in Fig. 3. Strikingly, BM from IFN-I-R−/− mice contains 3- to 4-fold increase in the numbers of IgM-secreting plasma cells, as well as those of IgM- and IgG-secreting plasma cells in the peripheral lymphoid organs are comparable in both types of animals (as shown for spleen in Fig. 3, central panel).

Thymus is another site of natural IgM plasma cell production and the constitutive expression of the IFN-β gene in this organ has been recently described (36). Although neither the number nor the representation of thymic B cells differ significantly in control and mutant mice (not shown), a 3- to 4-fold increase in the numbers of IgM-secreting plasma cells was scored in IFN-I-R−/− animals (Fig. 3, right panel).

These results indicate that locally produced IFN-I inhibits natural IgM plasma cell formation in BM and thymus, in line with our previous in vitro findings (13).

IFN-I-mediated inhibition of terminal differentiation in BM is BCR ligation related and results in decreased multireactivity of the secreted IgM antibodies

The physiologic effects of IFN-I on the generation of BM and thymic natural IgM plasma cells seem to operate independently of cell activation, since the numbers of activated B cells (as scored by forward scatter) are indistinguishable in control or mutant BM and spleen (not shown). Regulation of terminal differentiation, however, could result from BCR-related or -unrelated mechanisms. To distinguish between those alternatives we performed in vitro assays to score the effects of IFN-I on BCR-independent terminal differentiation of B cells. Upon activation by LPS, CD40 or CD40 together with IL-4, splenic B cells from wild-type pre-incubated or not with IFN-αβ developed comparable proliferative responses (Fig. 4A) and similar numbers of IgM-secreting plasma cells (as exemplified for LPS in Fig. 4B). Similar data were obtained when monitoring proliferation induced by those stimuli on wild-type and mutant BM B cells (not shown). These results demonstrate that IFN-I does neither interfere with B cell activation nor with BCR-independent terminal maturation of activated B cells. As also shown in Fig. 4B, however, functional exposure of BCR-ligated B cells to IFN-I, results in marked inhibition of terminal differentiation for critical levels of ligation. As before (13), we take this as evidence that IFN-I enhances the sensitivity of activated B cells to BCR-dependent inhibition of terminal differentiation. We therefore interpret the increases of BM and thymus natural IgM plasma cells in mutant mice as resulting from the absence of such a physiological role of IFN-I. This hypothesis implies that a fraction of in vivo activated B cells is not secretory because of BCR-dependent inhibition of terminal differentiation. In support of this notion, preliminary results have shown that a significant fraction of naturally activated splenic B cells, if washed and diluted in serum-free culture medium, ‘spontaneously’ develop into IgM-secreting cells within 48 h, in the absence of any proliferation (results not shown). Taken together, these observations indicate that IFN-I restricts the number of activated B cells entering terminal differentiation by lowering the threshold of sensitivity to BCR ligation. Again, the effects are restricted to BM and thymus, where local production of IFN-I takes place. Our analysis therefore strongly supports the idea that the increased number of BM plasma cells in mutant mice results from the release of BCR-mediated inhibition of terminal differentiation associated to the lower sensitivity of the B cells.

Selection of B cells to engage in natural plasma cell differentiation is a self-antigen-dependent process, under very stringent controls by genetic and epigenetic factors (37,38). If IFN-I regulates the number of BM natural IgM plasma cell in a BCR ligation-dependent process, this should be reflected on the plasma cell repertoires of BM (but not of spleen). Analyses of IFN-I-R−/− animals (Table 2) demonstrate 3- to 4-fold increases in total BM IgM plasma cell (multi)reactivity, when compared to the mutant splenic plasma cells or to either of the wild-type populations.

Taken together, these results therefore indicate that a fraction of naturally activated BM B cells is physiologically inhibited of secreting multireactive antibodies by locally produced IFN-I, via the control of sensitivity to BCR ligation.
IFN-I-dependent antibody repertoire selection

Fig. 4. BCR-independent activation of B cell is not affected by IFN-I. Percoll-purified resting B cells from wild-type spleen preincubated with media alone or IFN-αβ were monitored for proliferation (A) and terminal differentiation (B) induced by standard polyclonal stimuli. Thymidine incorporation was scored at day 3 for LPS, CD40 and CD40IL-4 stimuli. ESA were performed 4 days after LPS, LPS + 10 ng anti-µ and LPS + 100 ng anti-µ treatments.

Table 2. Frequencies of antigen-specific natural IgM-secreting cells

<table>
<thead>
<tr>
<th></th>
<th>BM wild-typea</th>
<th>BM KOa</th>
<th>Spleen wild-typea</th>
<th>Spleen KOa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-µb</td>
<td>596</td>
<td>1417</td>
<td>5248</td>
<td>4350</td>
</tr>
<tr>
<td>TNP-BSAc</td>
<td>0.9</td>
<td>7.0</td>
<td>2.4</td>
<td>1.9</td>
</tr>
<tr>
<td>DNA</td>
<td>0.2</td>
<td>0.6</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Histone</td>
<td>1.2</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Myosin</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Total</td>
<td>2.4</td>
<td>9.5</td>
<td>2.9</td>
<td>2.6</td>
</tr>
</tbody>
</table>

aUntreated populations.
bNumber of total IgM+ natural plasma cells/10^6 mononuclear cells.
cResults expressed as percentage of antigen-specific IgM-secreting cells out of total number of IgM-secreting cells.

The reactive repertoire of serum natural IgM antibodies is indirectly regulated by IFN-I produced in the BM

As previously demonstrated, the contribution of BM plasma cells to the pool of serum natural IgM antibodies is considerable and it exceeds that of spleen from 3 months of age onwards (35). Given the above results on the apparent increased multireactivity of BM IgM-secreting cells in IFN-I-R−/− animals, we would expect that natural IgM antibody repertoires in these mice differ from controls.

Using a semi-quantitative immunoblot technique, we could compare global natural antibody repertoires from many individuals against a very large panel of antigens (20,21). Such a comparative analysis of sera originating from the wild-type and the IFN-I-R-deficient mice is illustrated in Fig. 5. Strikingly, although serum IgM levels were comparable in both groups of mice (not shown), natural IgM antibody produced by the mutant mice consistently displayed a more reactive pattern when tested against homologous tissues (Fig. 5A) or, to a lesser extent, bacterial extracts (Fig. 5B). Appropriate multiparametric statistics, such as principal component analysis, clusters all animals from each group in different areas of the two-dimensional space defined by the first two factors (Fig. 5C), confirming that mutant mice display distinct serum IgM reactivity profiles, when compared to wild-type. No differences were scored when the natural IgG antibody repertoire was analyzed (data not shown).

These results indicate that the restriction in multireactive antibody secretion mediated by IFN-I on the BM IgM plasma cell repertoire has systemic consequences for the serum natural antibody composition. Incidentally, these results also establish, for the first time, that BM IgM plasma cells functionally contribute to the constitution of the natural antibody pool. Surprisingly, despite the increased number of IgM plasma cells in the mutant BM, the serum concentration of IgM is equal in mutant and wild-type mice, thus indicating that regulatory mechanisms other than the control of production operate in the strict maintenance of serum IgM levels in unimmunized animals.

Discussion

The present work establishes that physiological modulation of BCR signaling thresholds by IFN-I constitutively produced in the BM has local consequences for B-lineage cell composition and results in systemic alterations of natural antibody repertoires. We show here that mice lacking the receptor for type-I IFN display alterations in the repertoire of the immature BM B cell population, and in the numbers and reactivity of IgM-secreting BM plasma cells. As a consequence, serum IgM natural antibodies from the IFN-I-R−/− mice display higher levels of (multi)reactivity.

The present experiments also show that immature B cells are the main target of repertoire selection in BM, while at the periphery, the crucial selective step seems to pertain to the process of ‘natural’ activation to IgM antibody secretion. A
Fig. 5. Increased reactivities of serum IgM from IFN-I-R−/− mice. Immunoreactivities of serum IgM from five IFN-I-R−/− (dashed line) and five control mice (solid line) were scored by immunoblot and the densitometric profile of each individual mouse is shown (see Methods). For each sample, IgM concentration was readjusted to 10 µg/ml and the reactivity tested against mouse liver (A) and Escherichia coli extracts (B). The values related to immunoreactivities intensities (peak value) were submitted to principal component analysis. The two first principal component analysis factors are shown in a two-dimensional plot (C).
continuous selection of $V_{H}$ gene usage along B cell development, starting at the pre-B cell stage and extending to plasma cell formation, has been well documented by numerous studies. A major switch in $V_{H}$ gene utilization occurs between BM and periphery (8–10), corresponding on functional terms to a major drop in multireactivity between BM and the peripheral B cells (7). In the present study we subdivided further the BM population in immature and mature B cells, and could show that mature B cells in BM and the periphery are essentially indistinguishable. These results are in total agreement with the accumulating evidence indicating that mature B cells (B220$^{hi}$, CD23$^+$, IgD$^+$) in BM may essentially correspond to recirculating lymphocytes re-entering BM trough the blood vessels while newly generated mature B cells are rapidly released into blood circulation (29, 39). The $V_{H}7183$ gene family has been shown to be over-represented in BM precursor B cells and counter-selected together with the expression of a surface receptor. Conversely, the $V_{H}558$ gene family is characteristically dominant in peripheral populations (8–10). Our result of a lower representation of cells expressing the $V_{H}7183$ gene family, associated to an increased representation of $V_{H}558$ expression, in the immature BM population of mutant mice indicates that, in the absence of IFN-I signaling, $V_{H}7183$ gene expression is counter-selected at a stage of development prior to that defined by the phenotype B220$^+$, IgM$^+$, CD23$^-$. Analyses of precursor repertoires and of IFN-I effects on pre-BCR ligation may elucidate this point. Alternatively, enhanced representation of $V_{H}558$-expressing cells may correspond to their expansion by a positive selection process that requires low levels of BCR ligation sensitivity, as found in the periphery and in the BM of mutant mice. Strikingly, immature BM B cells in mutant mice also display increased levels of (multi)reactivity as compared to the corresponding wild-type population. Thus, assuming that selection against multireactivity results from the elimination of B cells that strongly interact with self ligands, this result is consistent with the escape of such B cells in the BM of mutant mice, due to the higher threshold of sensitivity to BCR triggering. Similar alterations of negative selection by modification of BCR-mediated signaling have been reported in self-reactive Ig transgenic models (40, 41). All together the present results support the idea that the composition of immature B cell populations in BM (B220$^+$, IgM$^+$, CD23$^-$) reflects a continuous process of previous and ongoing selection, operating prior to export from the BM. Moreover, according to the idea that the quantity of BCR triggering defines the fate of a B cell, including all ranges of stimuli and consequently of responses, modification in the threshold of sensitivity to BCR triggering must alter the repertoire equally for negatively and positively selected populations, thus leading to the complex picture we present. In addition, similarities in the peripheral mature B cell repertoire of mutant and control mice suggest that ‘normalization’ of repertoires occurs in the periphery, an environment that is devoid of IFN-I.

A marked effect of IFN-I on B cell development that was revealed by the present experiments concerns the generation and selection of natural IgM plasma cells. In the absence of IFN-I action, there are 3–5 times more IgM-secreting plasma cells in BM and thymus. This difference is restricted to the IgM isotype, in agreement with the notion that most IgM-secreting cells are locally produced in BM, while IgG-secreting cells are activated in the periphery and only then migrate to BM (42, 43). The processes that ‘naturally’ activate B cells and a fraction of these into antibody secretion in normal animals are still unknown. For the present discussion, it is worth noting, however, that in the absence of intentional immunization, the number of antibody-secreting cells (e.g. as scored by ESA) is only a minor fraction (1–10%) of all activated B cells that represent up to 10% of the total numbers of B cells in the organism. Lack of antibody secretion by activated B cells could be due to the inhibition of terminal differentiation by BCR ligation, as previously demonstrated in vitro (44). The physiological relevance of this mechanism, however, has remained entirely speculative. The present observations constitute the first indication for such a physiological role. Thus, we show here that lack of IFN-I signaling, resulting in higher thresholds of sensitivity to BCR-dependent inhibition of terminal differentiation, leads to an increased number of multireactive BM plasma cells. Since (i) the precursor populations are equally represented in both wild-type and mutant animals, (ii) IFN-I does not affect BCR-independent B cell activation, and (iii) plasma cell numbers only differ between IFN-I-deficient and competent mice wherever there is constitutive production of IFN-I (BM and thymus, but not in the spleen), we propose that antibody secretion by activated B cells is physiologically inhibited, in normal animals, by BCR ligation with self ligands. Such a mechanism provides yet another level to regulate the composition of natural antibody repertoires in physiology. Driven by autoreactivity, as demonstrated by the normal numbers of IgM-secreting cells in ‘antigen-free’ mice, selected by self ligands for a very conserved repertoire of reactivities in the species (1), natural antibody production seems to proceed within a range of self-reactivity that is high enough to activate the B cells, but low enough to escape BCR-dependent inhibition of terminal differentiation. This hypothesis is in full agreement with the data in Table 1 showing that peripheral B cell auto- or multireactivity is maximal in the compartment of ‘naturally’ activated but non-secretory B cells. Activated B cells that are inhibited from secreting by BCR ligation resemble the so-called ‘anergic B cells’ described in transgenic model systems: both are characterized as self-reactive, ‘large’ and non-secretory (45).

Given such increased numbers of multireactive IgM-secreting cells in BM (and thymus) of mutant mice, we would have expected to find the serum natural IgM antibody pool [antibodies present in the serum of healthy individuals in the absence of deliberate immunization (1)] accordingly increased in size and multireactivity. Thus, in adult mice, BM resident IgM-secreting cells account for a significant fraction of the total number of natural plasma cells (35,42 and our personal observations). Surprisingly, however, while the analysis of natural IgM antibody reactivity revealed that mutant mice do display higher levels of (multi)reactivity, the concentrations of serum IgM were not altered. These results indicate, therefore, that serum natural IgM antibody repertoire composition owes significantly to BM plasma cells and that mechanisms other than the control of production rates must regulate serum levels of natural IgM antibodies. These are likely to include complex formation and removal, particularly given...
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