IL1R8 Deficiency Drives Autoimmunity-Associated Lymphoma Development

Federica Riva1,2, Maurilio Ponzoni3, Domenico Supino2, Maria Teresa Sabrina Bertilaccio4, Nadia Polentarutti2, Matteo Massara2, Fabio Pasqualini2, Roberta Carriero2, Anna Innocenti3, Achille Anselmo2, Tania Veliz-Rodriguez4, Giorgia Simonetti4, Hans-Joachim Anders5, Federico Caligaris-Cappio4, Alberto Mantovani2,6, Marta Muzio4, and Cecilia Garlanda2,6

Abstract

Chronic inflammation, including that driven by autoimmunity, is associated with the development of B-cell lymphomas. IL1R8 is a regulatory receptor belonging to the IL1R family, which negatively regulates NF-kB activation following stimulation of IL1R or Toll-like receptor family members. IL1R8 deficiency is associated with the development of severe autoimmune lupus-like disease in Ipr mice. We herein investigated whether concomitant exacerbated inflammation and autoimmunity caused by the deficiency of IL1R8 could recapitulate autoimmune-associated lymphomagenesis. We thus monitored B-cell lymphoma development during the aging of IL1R8-deficient Ipr mice, observing an increased lymphoid cell expansion that evolved to diffuse large B-cell lymphoma (DLBCL). Molecular and gene-expression analyses showed that the NF-kB pathway was constitutively activated in I1r8−/−/Ipr B splenocytes. In human DLBCL, IL1R8 had reduced expression compared with normal B cells, and higher IL1R8 expression was associated with a better outcome. Thus, IL1R8 silencing is associated with increased lymphoproliferation and transformation in the pathogenesis of B-cell lymphomas associated with autoimmunity.

Introduction

The association between chronic inflammation and promotion of malignancy was first described in the nineteenth century (1) and is supported by epidemiologic and mechanistic data (2, 3). In particular, patients suffering from certain autoimmune or inflammatory conditions, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, and Sjogren syndrome, are prone to develop lymphomas, namely, B-cell non-Hodgkin lymphomas (B-NHL; refs. 4–7). The mechanisms triggering the transition from benign B-cell proliferation to malignancy are still only partially defined. Chronic inflammation, antigen stimulation, and B-cell receptor signaling, associated with the inherent genetic instability of lymphocytes, are known to play a central role in lymphoma development (8, 9). More specifically, gain-of-function mutations of MYD88 and constitutive activation of NF-kB have emerged among the most frequently recurring mutations in B-cell lymphoproliferative diseases (10).

Mice homozygous for the lymphoproliferation spontaneous inactivating mutation (Fas<sup>−/−</sup>) show systemic autoimmunity, massive lymphadenopathy associated with proliferation of aberrant T cells, arthritis, and immune complex glomerulonephrosis (11). In humans, germline mutations in the FAS gene have been associated with autoimmune lymphoproliferative syndrome (ALPS; ref. 12), and somatic FAS mutations have been found in multiple myeloma and B-NHL (4).

IL1R8 (also known as TIR8 or single Ig IL1-related receptor, SIGIRR) is a member of the interleukin-1 receptor (IL1R) family acting as a negative regulatory receptor (13). IL1R8 inhibits NF-kB and JNK activation following stimulation of IL1R or TLR family members by interfering with the recruitment of TIR domain-containing adaptor molecules (14–17). In combination with IL1RIRz, IL1R8 also serves as one of the receptor chains for the anti-inflammatory cytokine IL37, thereby activating anti-inflammatory responses (18).

IL1R8 deficiency leads to uncontrolled activation of IL1R or TLR family members and is associated with exacerbated inflammatory responses...
IL1R8 deficiency in \( lpr \) mice is associated with severe lymphoproliferation and autoimmune lupus-like disease (16), due to increased dendritic cell (DC) activation and B-cell proliferation in response to TLR7- and TLR9-activating autoantigens or nucleosomes (29, 30).

The involvement of IL1R8 in autoimmunity, and the critical role of constitutive activation of MyD88-dependent NF-κB activation in B-cell transformation, raised the hypothesis that IL1R8 might be involved in the autoimmune-associatied risk of developing lymphoma. Here, we show that IL1R8 deficiency was associated with significantly earlier death and increased susceptibility to lymphoproliferation, which evolved in transplantable diffuse large B-cell lymphoma (DLBCL). Analysis of clonality showed that multiple independent transformation events occurred in the same host. In humans, IL1R8 was poorly expressed in DLBCL cell lines and primary lesions when compared with peripheral blood or germinal center B cells and was associated with better outcome in terms of overall survival, suggesting that IL-1R8 downregulation is a driver of lymphomagenesis.

Materials and Methods

Animals and samples

IL1R8-deficient (\( Il1r8^{-/-} \)) mice were generated as described (14) and backcrossed to the C57Bl/6 background (Charles River Laboratories) up to the F11 generation. \( Il1r8^{-/-} \) and \( B6lpr/lpr \) (Charles River Laboratories) were crossed to generate \( Il1r8^{-/-}/lpr \) mice. Mice were housed in the SPF animal facility of Humanitas Research Hospital in individually ventilated cages. Mice were sacrificed at 12 to 18 months of age, unless they reached the established endpoints and organs were collected for histologic and molecular analysis. Procedures involving animals have been conducted in accordance with, and with the approval of the Institutional Animal Care and Use Committee of Humanitas Research Hospital and Italian Health Ministry (authorizations 43/2012-B released on August 2, 2012 and 828/2015-PR released on July 8, 2015), in compliance with national (D.L. n.116, G.U., suppl. 40, February 18, 1992; D.L. n.26, March 4, 2014) and international law and policies (EEC Council Directive 86/609, OJ L 358,1,12-12-1987; EEC Council Directive 2010/63/UE; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 2011). All efforts were made to minimize the number of animals used and their suffering.

Histopathology and IHC

Five-micrometer-thick sections of formalin-fixed, paraffin-embedded mouse tissues were stained with H&E. Based on lymphoid follicle morphology, a pathologic score was attributed to the spleen and lymph nodes of each 10–12-month-old mouse analyzed (normal = 0; reactive = 1; reactive > atypical = 2; atypical > reactive = 3; atypical = 4; atypical > lymphomatous = 5; lymphomatous > atypical = 6; lymphomatous = 7). Slides were analyzed by a certified hematopathologist (M. Ponzoni) and two investigators who were blinded to the experimental group. The following antibodies were used: anti-B220 (RA3-6B2, Serotec), anti-Ki67 (SP6, Neo Markers), anti-CD3 (1F4, Bio-Rad), anti-BCL6 (Rabbit polyclonal, Santa Cruz Biotechnology), anti-BCL2 (C21, Santa Cruz Biotechnology), and anti-Multiple Myeloma 1/Interferon Regulatory Factor 4 protein (MLM1/IRF4; 3E4, BioLegend; ref. 31).

Tumor transplantation

A total of 103± cells (5 x 106 splenocytes plus 5 x 106 lymph node cells) from 10- to 12-month-old \( Il1r8^{-/-}/lpr \) \( n = 8 \) or \( Il1r8^{-/-}/lpr \) \( n = 7 \) mice were injected i.p., s.c., or i.v. into C57Bl/6, nude or SCID mice \( n = 17 \) recipients of \( Il1r8^{-/-}/lpr \) cells; \( n = 16 \) recipients of \( Il1r8^{-/-}/lpr \) cells. Recipient animals were sacrificed when clinical signs (enlargement of mandibular lymph nodes or abdomen) were evident or 12 to 20 months after transplantation, and organs were collected for histologic and molecular analysis. The genotype of several tissues of all recipient mice was analyzed for \( lpr \) and \( Il1r8 \) mutations by PCR (14).

Western blot analysis

Western blot analysis of purified B-cell lysates (30 μg total proteins) was performed with the following antibodies: rabbit anti-p100/p52 (CS4882, 1:1,000, overnight at 4°C), mouse anti-Phospho-p65 (CS3036, 1:1,000, overnight at 4°C), rabbit anti-p65 (CS8242, 1:1,000, 2 hours RT; Cell Signaling Technology); anti-beta-actin-HRP (SIGMA A3852, 1:10,000, 2 hours room temperature), followed by anti-rabbit-HRP (Sigma A0545, 1:5,000) or anti-mouse-HRP (Sigma A3682, 1:5,000), using 10% or 4%-12% gradient precast gels (GenScript).

Real-time PCR and real-time PCR array

Total RNA from mouse spleen-purified B cells, DLBCL cell lines, and B cells from healthy donor buffy coats was isolated with a column-based kit followed by DNase treatment (Promega; for PCR array) or TRI reagent (Sigma-Aldrich; for PCR). RNA was retrotranscribed and cDNA used for gene-expression analysis by real-time PCR and real-time PCR array (Bio-Rad Prime PCR ARRAY code:10034381).

Real-time PCR was performed in QuantStudio 7 Flex (Applied Biosystems, Thermo Fisher) or 7900 Sequence Detection System (Applied Biosystems), in duplicate using Power Sybr Green PCR Master Mix (Applied Biosystems) and primers (300 nmol/L) in MicroAmp optical 96-well plates (25 μL). The following primer pairs were purchased from Invitrogen: Nfkbiz for 5’-CCGGCTTCCTGTGTGCTCC-3’; Nfkbiz Rev 5’-AGACTGCG-GATTCTCTC-3’; GAPDH for 5’-GCCAAGTGGAATTGTTGCACAT-3’; GAPDH Rev 5’-CTTGTG GCTCGCCATTGTTGA-3’; human IL1R8: For 5’-GGAGCTTATTGGAGATACT-3’; Universal actin For 5’-CCAAAGGCAACCCCGAGAGGT-3’; Universal actin Rev 5’-GTCCCGGCCGCGACGATCAG-3’. Experiments were repeated at least twice. The expression of the target gene was normalized using GAPDH or β-actin cDNA expression of the same sample and run, and reported as 2^(-ΔΔC_T).

For real-time PCR array, the analysis of 84 NF-kB signaling target genes was performed as described (32). Data were reported as 2^(-ΔΔC_T) relative to the average of six housekeeping genes. Of note, the specific assay for Fas mRNA expression is designed within exons 1 and 2, and it recognizes both wild-type and mutant Fas (33).
IgH gene rearrangement analysis

IgH gene rearrangement was investigated by Southern blot analysis of genomic DNA from different organs of one 20-month-old wild-type mouse transplanted s.c. with total lymph node, and spleen cells collected from one 11-month-old Il1r8−/-/lpr mouse. DNA (5 μg) was extracted from spleen, lymph nodes, and solid lesions (100 mg each), digested with EcoRI or Stul and subsequently hybridized with a 32P-labeled DNA probe PJ3 representing the JH4 region of the IgH locus, as described (34).

Cell culture

B cells were isolated from 100 × 10^6 splenocytes using a B-cell isolation kit (Miltenyi Biotech), plated in 48-well plate at 1 × 10^6/mL and cultured overnight.

Human DLBCL cell lines SU-DHL-2 (ATCC CRL-2956), SU-DHL-4 (ATCC CRL-2957), SU-DHL-6 (ATCC CRL-2959), SU-DHL-8 (ATCC CRL-2961), RC-K8 (DSMZ ACC-561), and RIVA (RI-1, DSMZ ACC-585) were received in 2013, expanded and frozen, and then thawed and grown for 10 days in RPMI or IMDM (RIVA, RC-K8) medium supplemented with 1%–20% FCS, 2 mmol/L L-glutamine, and streptomycin (100 U/mL) before the experiments. DLBCL cell lines were not authenticated, were routinely tested for Mycoplasma contamination, and only Mycoplasma-free cells were used for flow cytometry and molecular analysis.

Flow-cytometric analysis

Mouse spleen B cells overnight cultured in medium or in the presence of LPS (100 ng/mL, Sigma-Aldrich) were incubated with anti-mouse CD86 (GL1, eBioscience) and anti-mouse CD19 (1D3, BD Biosciences) for 30 minutes at 4°C for surface staining and analyzed by FACScanto II (Becton Dickinson).

IL1R8 cell-surface staining on human cells was performed with biotinylated goat anti-human IL1R8/SIGIRR (R&D Systems), followed by Alexa 647-conjugated streptavidin (Molecular Probes, Invitrogen), and analyzed with FACScanto I flow cytometer (BD Biosciences). Results are reported as mean fluorescence intensity (MFI) normalized on fluorescence minus one. Diva software (BD Pharmingen) and FlowJo (Tree Star) were used for data acquisition and analysis, respectively.

Analysis of IL1R8 and IL37 expression in human DLBCL

Public gene-expression data of DLBCL were retrieved from GEO. In the first study (GSE43677) samples of naive B cells (n = 8), germinal center (GC) B cells (n = 13), post-GC B cells (n = 9), tonsils (n = 10), and DLBCL (n = 12) were analyzed. In the second study (GSE32018), gene-expression profiling was conducted in a series of B-cell non-Hodgkin lymphoma patients [17 CLL, 22 DLBCL, 23 follicular lymphoma (FL), 24 mantle cell lymphoma (MCL), 15 marginal zone lymphoma (MZL), 13 nodal marginal zone lymphoma (NMZL)], and 7 freshly frozen lymph nodes. Differential expression analysis was performed using limma (version 3.26.8; ref. 35). For prognosis evaluation, expression and clinical data of 98 DLBCL cases selected from 220 lymphoma samples (GSE4475) were used. DLBCL patients treated with radiotherapy were excluded.

The Gene Set Enrichment Analysis (GSEA) software was used to perform the overrepresentation analysis with gene sets coming from the Molecular Signature Database (36). The entire data matrix containing normalized gene-expression values (log scale) was used, and the expression profile of the IL1R8 gene was tested as continuous phenotype label. The analysis was performed choosing the Pearson correlation as the metric to investigate gene sets enriched by genes correlated with the expression profile of IL1R8.

The Reactome database, belonging to the C2 collection (c2.cp.reactome.v6.1), was used. Resulting gene sets were considered significantly enriched according to the false discovery rate (FDR) threshold of 5%.

Statistical analysis

Statistical differences in mouse mortality and lymphoma incidence rates were analyzed with the Mantel–Cox test and the Fisher test, respectively. The Mann–Whitney test or Student t test with Welch correction was performed as specified. Survival analysis of human DLBCL was performed using the Kaplan–Meier and Mantel–Cox tests. The median expression value was used to classify patients into IL1R8low and IL1R8high or IL37low and IL37high gene-expression groups. A P < 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism software (GraphPad Software).

Results

IL1R8 deficiency is associated with severe lymphadenopathy and lymphoma in lpr mice

We previously observed that 6-month-old Il1r8−/-/lpr mice were affected by enhanced lymphoproliferation and lymph follicle hyperplasia compared with Il1r8+/+/-/lpr mice (16). In order to address whether this benign lymphoproliferation eventually evolved to malignancy, we analyzed survival and followed the evolution of lymphoid organs in older animals. As shown by survival curves reported in Fig. 1A, Il1r8−/-/lpr mice reached the endpoints earlier than Il1r8+/+/-/lpr mice, and mortality was 100% (23/23) at 15 months of age in Il1r8−/-/lpr mice compared with 22% (6/27) in Il1r8+/+/-/lpr mice (P = 0.0001, Mantel–Cox test). Splenomegaly and lymphadenomegaly were more pronounced in 10- to 12-month-old Il1r8−/-/lpr mice compared with Il1r8+/+/-/lpr mice of the same age (Fig. 1B). The spleen weight was significantly increased in both groups compared with wild-type or Il1r8−/-/mice, and in Il1r8+/+/-/lpr mice it was significantly greater (4-fold) than in Il1r8+/+/-/lpr mice (Fig. 1C).

Histopathologic analysis of the spleens of 12- to 14-month-old Il1r8−/-/lpr mice showed an enlargement of the white pulp and a complete loss of the normal architecture of the organ in most animals (Fig. 2A). In the spleens of 12- to 14-month-old Il1r8−/-/lpr mice, we observed a moderate enlargement of the white pulp, but the architecture of the organ remained recognizable despite the presence of enlarged germinal centers (Fig. 2A). Similarly, most (62.5%; 20/32) lymph node from 10- to 12-month-old Il1r8−/-/lpr mice presented abnormal histologic architecture, without any evident follicle (Fig. 2B). In contrast, lymph nodes from 10- to 12-month-old Il1r8+/+/-/lpr mice were enlarged but generally retained a preserved normal morphology of the follicles (Fig. 2B). As shown in Fig. 2C and D, the pathologic score of lymphoid follicles (based on the presence of normal, reactive, atypical, or lymphomatous follicles) was significantly higher in 10- to 12-month-old Il1r8−/-/lpr mice compared with 12- to 14-month-old Il1r8−/-/lpr mice (P = 0.0001 in spleen and P = 0.0022 in lymph nodes), and the diagnosis of lymphoma was mostly limited to Il1r8−/-/lpr mice. Splenic and lymph nodal plasmacytosis occurred in the spleen and lymph nodes of both Il1r8+/+/-/lpr and Il1r8−/-/lpr mice, in agreement with the role of TLR ligands
and autoantigens in inducing cellular differentiation into mature plasma cells and plasma cell expansion (37, 38).

Development of DLBCL in Il1r8−/−/lpr mice

Histopathologic analysis showed that a diffuse organ replacement by large cells in spleen and lymph nodes consistent with a diagnosis of lymphoma occurred in 13 of 26 Il1r8−/−/lpr mice and 3 of 23 Il1r8−/−/+/+ mice, and pathologic examination of lymph nodes and bone marrow of one recipient wild-type mouse transplanted with splenocytes and lymph node cells of one recipient wild-type mouse transplanted with spleenocytes and lymph node cells of one Il1r8−/−/lpr donor. The observation revealed bands of IgH rearrangements in DLBCL developed in different organs of one recipient wild-type mouse transplanted with spleenocytes and lymph node cells of one Il1r8−/−/lpr donor. The observation revealed bands of IgH rearrangement of different sizes in the spleen, lymph nodes, and other organs, indicating that multiple B-cell clones were transformed in the donor mouse (Fig. 4C and D).

These results indicate that lymphomas of Il1r8−/−/+/+ mice can be transplanted in wild-type recipient mice, giving rise to lymphoma.

Constitutive activation of the NF-κB pathway in Il1r8−/−/lpr B cells

Hyperactivation of the NF-κB pathway and overexpression of NFκBIZ are hallmarks of a subtype of DLBCL in humans (39–41). IL1R8 dampens NF-κB activation induced by TLR and IL1R family members (42), and Fas mutations affect B-cell activation (16). In addition, we previously showed that IL1R8 deficiency significantly increased B-cell proliferation upon exposure to RNA and DNA immune complexes and other TLR agonists (16). To further investigate the NFκB pathway in B cells of Il1r8−/−/+/+ mice, we observed activation of the canonical and noncanonical NFκB activation pathway in both Il1r8−/−/+/+ and Il1r8−/−/+/+ mice, whereas the canonical pathway appeared mostly activated in the Il1r8−/−/+/+ mice (Fig. 5A–C). We did not observe any significant difference in...
As indicated by fold change, 13 genes were upregulated, and only one was downregulated as shown in Supplementary Table S2). Of the 14 genes dysregulated in at least one group, 13 genes were upregulated, and only one was downregulated as compared with wild-type animals, again suggesting constitutive hyperactivation of this pathway in Il1r8−/− mice (fold difference = 1.08 and P = 0.8 as shown in Supplementary Table S2).

Next, we used a real-time PCR array to analyze 84 genes known to be targets of the NF-κB signaling pathway (Supplementary Table S1). We compared the results obtained from nonstimulated B cells collected from 4 wild-type mice, 4 Il1r8−/−/lpr mice, and 5 Il1r8+/−/lpr mice (Fig. 5D; Supplementary Table S2). Of the 14 genes dysregulated in at least one group, 13 genes were upregulated, and only one was downregulated as compared with wild-type animals, again suggesting constitutive hyperactivation of this pathway in Il1r8−/−/lpr and Il1r8−/−/lpr B cells from aged mice. Most of the NF-κB targets were upregulated in both Il1r8+/−/lpr and Il1r8−/−/lpr mice, including proinflammatory genes (e.g., Il1b, Ifng, Csf1, Stat1, Il2b) and genes associated with proliferation or antiapoptosis (e.g., Ccnd1; Supplementary Table S2). The Bcl2a1a gene coding for an antiapoptotic protein necessary for cell transformation and growth in anaplastic lymphoma (43) was downregulated in lpr mice (fold difference = 0.47 and P = 0.04 as shown in Supplementary Table S2), and although IL1R8 deficiency did not influence its expression (fold difference = 0.93; P = 0.8), expression was restored in Il1r8−/−/lpr mice (fold difference = 1.08 and P = 0.8 as shown in Supplementary Table S2).

We then analyzed a secondary response gene prototypically induced by TLRs and regulated by NF-κB, namely, Nfkbiz. In basal conditions, we observed low expression of Nfkbiz mRNA in B cells isolated from wild-type mice; however, Nfkbiz was significantly induced in Il1r8−/− mice, and this induction was sustained in Il1r8−/−/lpr mice (Fig. 5E), suggesting that a TLR-dependent NF-κB secondary response is constitutively activated in Il1r8−/− and Il1r8−/−/lpr mice.

In agreement with dysregulated activation of the NF-κB pathway, flow cytometry analysis showed that overnight-cultured, spleen-purified Il1r8−/−/lpr B cells had increased expression of CD86, an activation marker downstream of TLR activation (44, 45), compared with wild-type, Il1r8−/−, and Il1r8−/−/lpr B cells, both in basal conditions and after LPS stimulation (Fig. 5F).

Taken together, these results show that both Fas and IL1R8 deficiencies contribute to constitutive dysregulated NF-κB

Figure 2.

IL1R8 deficiency is associated with increased susceptibility to lymphoma development in lpr mice. A and B, Histopathologic analysis of the spleen (A) and lymph nodes (B) of 10- to 12-month-old Il1r8−/−, Il1r8+/−, and Il1r8+/−/lpr, and Il1r8−/−/lpr mice stained with H&E (400×; Axioskop 40 microscope equipped with AxioCam MRc camera and AxioVision Rel. 4.8 acquisition software; Zeiss). C and D, Pathologic score of the spleen (C) and lymph nodes (D) of 10- to 12-month-old Il1r8−/− (n = 2), Il1r8+/− (n = 2), Il1r8+/−/lpr (n = 20), Il1r8−/−/lpr (n = 26) mice (unpaired Student t test; mean and SD are indicated). E, Incidence of DLBCL in Il1r8−/−/lpr (3/23) and Il1r8−/−/lpr (15/26) mice (Fisher test).
signaling and increased B-cell activation with few differences associated with IL1R8 deficiency (e.g., induction of Nfkbiz). The double mutation rendered the cells highly responsive to TLR activation, as measured by CD86 expression.

IL1R8 expression is downmodulated in human DLBCL cells and correlates with prognosis

To assess the relevance of these results to human disease, we first studied the expression of IL1R8 in human lymphoma cell lines compared with normal circulating mature B cells. As shown in Fig. 6A and B, all DLBCL cell lines analyzed expressed lower IL1R8 mRNA and protein, respectively, compared with peripheral blood B cells.

Next, we studied IL1R8 expression in public gene-expression data of DLBCL retrieved from GEO, comparing different normal resting and activated B-cell populations and lymphomas. In the first study analyzed (GSE43677), the expression of IL1R8 was significantly downregulated in DLBCL samples versus naive B cells (logFC = −0.43, adj. P = 1.08E−04), GC B cells (logFC = −0.21, adj. P = 1.70E−02), post-GC B cells (logFC = −0.9, adj. P = 1.12E−09), and tonsils (logFC = −0.62, adj. P = 3.64E−08; Fig. 6C). The second study (GSE32018) showed significant downregulation of IL1R8 expression in DLBCL versus lymph node control samples (logFC = −1.34, adj. P = 1.65E−02), but also versus FL, an indolent form that may transform into DLBCL (logFC = 0.66, adj. P = 3.45E−02; Fig. 6D).

In a third study (GSE4475), the expression of IL1R8 was analyzed together with clinical data to evaluate a correlation with prognosis. DLBCL patients were divided into IL1R8 low and IL1R8 high based on the median gene expression. The resulting Kaplan–Meier curve showed that patients with IL1R8 expression above the median value had significantly prolonged overall survival (hazard ratio (HR) = 2.2; 95% CI, 1.2–3.8; P = 0.006; Fig. 6E), compared with patients below the median. In addition, the GSEA analysis retrieved a total of 60 pathways significantly enriched by genes positively correlated with IL1R8 gene-expression profile (Supplementary Table S3). Among these, the apoptotic process and the DNA damage response were two of the most enriched pathways (NES = 2.02, FDR q = 0.005 for the apoptosis process; NES =...
1.85, FDR q = 0.01 for the P53-dependent G1 DNA damage response) with a total of 70 and 29 genes, respectively, belonging to the core enrichment (Supplementary Tables S4 and S5). These results show a positive coregulation of the apoptotic process and DNA damage response genes with our gene of interest, suggesting a putative activation of the apoptotic process and DNA damage response in proliferative files with high IL1R8 expression compared with those with low expression.

Because IL1R8 is required for the anti-inflammatory activity of IL37 in inflammatory conditions triggered by TLR ligands (18, 46), we finally investigated whether IL1R8 and IL37 were coregulated in DLBCL. In the GSE43677 and GSE32018 studies, the expression of IL37 was significantly downregulated in DLBCL samples compared with normal B cells (logFC = −0.18, adj. P = 3.73E−02 for naïve B cells, logFC = −0.25, adj. P = 1.65E−03 for GC B cells) or FL cells (logFC = −0.37, adj. P = 4.30E−03), respectively, similarly to IL1R8 (Fig. 6F, G). However, in contrast to what was observed for IL1R8, the overall survival was not affected by IL37 expression in the GSE4475 study (HR, 0.6; 95% CI, 0.4–1.1; P = 0.1), indicating that the regulatory role of IL1R8 affects additional pathways.

These results indicate that IL1R8 is poorly expressed in DLBCL compared with healthy GC B cells and other B-cell lymphomas, and that lower IL1R8 expression is associated with shorter overall survival.

Discussion

IL1R8 is known to act as a negative regulator of NF-κB and JNK activation following stimulation of IL1R family members or TLRs. We herein showed that the increased susceptibility to lymphoproliferation observed in lpr mice deficient of IL1R8 is also associated with frequent development of DLBCL. The aggressive lymphomas that developed in Il1r8−/−lpr mice were transplantable and oligoclonal, possibly originating from multiple B-cell clones. In addition, we showed that IL1R8 expression is downregulated in human DLBCL cells in comparison with peripheral blood, GC B cells, and other lymphomas. Expression also correlated with overall survival, suggesting that IL1R8 silencing in DLBCL might contribute to dysregulated NF-κB activation, a frequent occurrence observed in DLBCL, lymphoproliferation, and transformation.

FAS-deficient lpr mice are a model of ALPS and SLE. FAS is a proapoptotic TNF receptor superfamily member, highly expressed on GC B cells. Mutations in the genes encoding FAS or its ligand cause massive accumulation of autoreactive B and T cells,
resulting in ALPS in humans (12). In addition, FAS mutation has been found associated with perforin deficiency in one case of ALPS and lymphoma (47), whereas in mice, increased lymphoma development was observed in lpr mice deficient of SPARC, the gene coding for osteonectin (48). In a previous study, we demonstrated that IL1R8 deficiency was associated with a more severe lymphadenopathy at 6 months of age in FAS-deficient lpr mice (16). This phenotype was mainly due to overactivation of DCs, B cells, and CD4+ T cells upon stimulation with lupus autoantigens, possibly through TLR7 engagement (16). Indeed, chromatin antigens in immune complexes can potently engage both the BCR and TLRs in B cells, leading to overstimulation and defective apoptosis of B cells, as well as to secondary inflammation (5). FAS mutations have also been observed in human lymphomas, indicating that longer lymphocyte survival may allow accumulation of additional oncogenic events (4).

In addition to these autoimmunity-dependent mechanisms, genetic alterations affecting components of the NF-κB signaling pathways have been shown to occur frequently in DLBCL. Constitutive NF-κB pathway activity is observed in almost all activated B-cell–like (ABC) types of DLBCL and in a large fraction of germinal center B-cell (GCB)–DLBCLs and is associated with the proliferation, differentiation, and survival of malignant lymphoid cells (49, 50). Among mutations of the NF-κB signaling pathway in B-cell lymphomas, MYD88 mutations have emerged as one of the most frequently recurring (40). MyD88 is an adaptor protein that mediates TLR and IL1R signaling. Gain-of-function mutations of MYD88 confer a cell survival advantage during the evolution of DLBCL by promoting NF-κB and JAK/STAT3 signaling (40). IL1R8 tunes TLR and IL1R-dependent signaling by interfering with the recruitment of TIR-containing adaptor molecules (51) and IL1R8 deficiency in mice is associated with uncontrolled inflammatory responses both in infectious and sterile conditions (42). Furthermore, genetic inactivation of IL1R8 was observed to cause earlier, more disseminated, and aggressive leukemia in the Em-TCL1 mouse model of CLL (26). In this model, the neoplastic transformation of B cells has an incidence of 100% and is mediated by the overexpression of the TCL1 oncogene; the absence of IL1R8 exacerbates CLL progression, but its impact on the B-cell transformation has not been

---

**Figure 5.** Dysregulated NF-κB activation in Il1r8⁺/⁺ and Il1r8⁻/-/lpr mice. A, Western blot of spleen B cells with the indicated antibodies. β-Actin expression was analyzed as internal control. B and C, Densitometric signal ratios of p52/p100 and phospho-p65/p65 shown in A (Mann–Whitney test). n = 4 Il1r8⁺⁺ , n = 5 Il1r8⁻⁻ , n = 4 Il1r8⁺⁺ /lpr, n = 6 Il1r8⁻⁻ /lpr mice. D, Real-time PCR array of NF-κB signaling target genes. Expression data are shown only for the genes for which a fold difference (FD) > 2 was observed in at least one comparison between two groups of mice (see Supplementary Table S2 for individual data). In the graph, a two-color scale formatting scheme was used to format cells: red is the maximum expression; blue, minimum. Each column represents one sample (from one mouse). E, Nfkbiz mRNA expression in purified B cells (unpaired Student t test with Welch correction). A–E, One experiment performed. F, FACS analysis of CD86 expression in overnight-cultured purified B cells in basal condition and after LPS stimulation. Top, representative histograms. Bottom, results are reported as MFI normalized on fluorescence minus one. One representative experiment with B cells collected from 3 to 6 mice (1 or 2 replicates per mouse) out of 2 performed is shown (unpaired Student t test with Welch correction). B, C, E, F, mean and SD are shown.
investigated (26). Finally, IL1R8 in association with IL18R serves a receptor chain for IL37, a cytokine that provides an anti-inflammatory environment in the aging bone marrow, preventing oncogenic transformation of B-cell progenitors (52). Our results show that Il1r8+/−/lpr mice spontaneously developed DLBCL at a very low frequency and at late age (12–18 months). IL1R8 deficiency increased the frequency and the severity of the disease, as well as accelerated the onset of disease to 8 to 12 months of age, indicating that IL1R8 has also a role in the neoplastic transformation of B cells, and not only in the progression of established B-cell leukemia or lymphoma.

The pathogenesis of lymphoma seen in patients with autoimmune diseases is complex and involves different factors contributing to lymphomagenesis, including both disease activity and
immunosuppression, as well as disease-specific mechanisms and mechanisms unique to lymphoma subtype (8). In the current study, we show that the Il1r8−/−flp mouse model recapitulated autoimmune-associated lymphomagenesis, suggesting that the absence of a negative regulator of the ILR- or TLR-MylD88 axis in an autoimmune-prone background is sufficient for the neoplastic transformation of B cells. These results are in line with data in humans, showing that aggressive B-cell lymphomas (particularly DLBCL) are more frequently associated with autoimmune conditions than more indolent lymphomas (particularly FL; ref. 4). It should be noted that IL1R deficiency in this model was not restricted to B cells and might also impact on antitumor immunity, as shown in other models (27, 28). Therefore, our results may underestimate the effect of selective IL1R8 deficiency in tumor B cells.

Western blot analysis of p52 and phospho-p65 demonstrated that both canonical and noncanonical NF-kB pathways are constitutively activated in Il1r8−/−flp mice. Moreover, the TLR-induced NF-kB–regulated Nfkbia gene was constitutively activated in Il1r8−/− mice. A combination of these pathways may contribute to the activation of distinct NF-kB target genes and B-cell activation observed in lymphoma prone Il1r8−/−flp mice. We previously observed that Il1r8−/−flp B cells had an increased proliferation rate after stimulations with autoantigens acting through TLR7, TLR9, and other TLR ligands, compared with flp B cells (16). In the present article, we described a high mitotic rate and diffusely elevated Ki67 immunoactivity in Il1r8−/−flp spleen, indicating an increased proliferation rate associated with immunoreactivity for Bcl-2, suggesting activation of the ant apoptotic machinery. Thus, the results presented here are in line with the view that the lack of a tuner of TLR and ILIR signaling–dependent NF-kB activation could impinge upon B-cell transformation in the context of lymphoproliferative syndrome. Indeed, other oncogenic events circumventing negative feedback mechanisms that attenuate NF-kB signaling, such as inactivation of the deubiquitinate A20, are associated with autoimmunity and lymphoma development (40, 53).

DLBCLs developed in Il1r8−/−flp mice were characterized by the presence of a monomorphic population of large B cells in lymphoid tissues. Neoplastic B cells displayed high proliferation rates and showed widespread involvement of distant organs including gut, liver, lung, and kidney. Histopathologic analysis and immunostaining for CD3, B220, Bcl-2, and Ki67 of spleen and lymph node specimens of Il1r8−/−flp mice documented sharply separated masses constituted by DLBCL arising within a background characterized by atypical lymphoproliferative disorder. Excessive lymphoproliferation associated with activation of ant apoptotic mechanisms were potentially responsible for multiple independent transformation events resulting in the polyclonal (or oligoclonal) development of different primary foci of DLBCL, as suggested by the detection of different bands of IgH rearrangement in the same mouse or in the same organ. Our results suggest that FAS deficiency was responsible for polyclonal B-cell expansion and very rarely lymphoma transformation, and the addition of the deficiency of IL1R8, causing hyperactivation of the MyD88–NF-kB axis, in response to autoantigens, resulted in tumors that resemble human ABC-DLBCL in Il1r8−/−flp mice, these tumors emerged sporadically and thus were likely to have acquired the additional oncogenic hits necessary to give rise to DLBCL.

In the present study, we show that the expression of IL1R8 in human DLBCL was downmodulated compared with peripheral blood or GC B cells from healthy donors and correlated with overall survival. The molecular mechanisms underlying IL1R8 downmodulation in human DLBCL are still undefined, and could include promoter methylation, as observed in human gastric carcinomas (54), alternative splicing leading to aberrant protein expression, as described in colorectal cancer (55), or promoter hypoacetylation as suggested by the analysis of hematologic cancer cell lines from publicly available data sets (Ensembl, UCSC Genome Browser). In addition, it was reported that genomic methylation affects IL1R8 expression, as azacytidine treatment of CLL cell lines restored IL1R8 mRNA expression (56). Very rarely, nonsense and somatic nonsynonymous mutations have been observed in the IL1R8 coding sequence as shown by Whole-Exome Sequencing data from The Cancer Genome Atlas and in sequenced samples within DLBCL patients (Dall'Aversa R, personal communication), but the functional consequences of these mutations or polymorphisms need to be investigated. The apoptotic process and DNA damage response were among the pathways significantly enriched by genes positively correlated with IL1R8 gene expression. This suggests that higher IL1R8 expression might be associated with increased apoptotic activity and better control of DNA damage, and as a consequence, with a less aggressive phenotype of lymphoma cells, thus leading to better prognosis.

Patients affected by DLBCL show different clinical courses, making prediction of prognosis and successful therapy difficult, leading to only 50% of patients being effectively treated (57). Our results demonstrate that IL1R8 activity limits B-cell activation and malignant transformation induced by autoimmune stimulation and contribute to the identification of genes and molecular pathways that could represent targets for novel therapeutic approaches in DLBCL treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C. Garlanda, F. Riva, M.T.S. Bertilaccio, A. Mantovanu, M. Muzio
Development of methodology: F. Riva, D. Supino, N. Polentarutti, A. Innocenzi, A. Anselmo, M. Muzio
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Riva, D. Supino, M.T.S. Bertilaccio, M. Massara, F. Pasqualini, A. Innocenzi, A. Anselmo, F. Caligaris-Cappio, M. Muzio
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Garlanda, F. Riva, M. Ponzoni, M.T.S. Bertilaccio, M. Massara, R. Carriere, M. Muzio
Writing, review, and/or revision of the manuscript: C. Garlanda, F. Riva, F. Caligaris-Cappio, M. Muzio
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Polentarutti, F. Pasqualini, H.-J. Anders, M. Muzio
Other (helped with experimental work): G. Simonetti, T. Veluz-Rodriguez

Acknowledgments
The study was supported by the European Commission (ERC project PHII-669415, FP7 project TIMER HEALTH-F4-2011-281608), Ministero dell’Istruzione dell’Università e della Ricerca (MIUR; project PRIN 2015SYKPNN, project FIRB RBAP11H29P), Associazione Italiana Ricerca sul Cancro (AIRC 19014 to A. Mantovanu and AIRC 5 × 1000 9962 to A. Mantovanu and C. Garlanda; AIRC IG 16777 and 13042 to M. Muzio; AIRC 5 × 1000 9965 to M. Muzio and F. Caligaris-Cappio), CARIPLO (project 2010-0795 to...
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Published OnlineFirst April 24, 2019; DOI: 10.1158/2326-6066.CIR-18-0698

Received October 6, 2018; revised January 28, 2019; accepted April 17, 2019; published first April 24, 2019.

References


IL1R8 Deficiency Drives Autoimmunity-Associated Lymphoma Development

Federica Riva, Maurilio Ponzoni, Domenico Supino, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-18-0698

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2019/04/24/2326-6066.CIR-18-0698.DC1

Cited articles
This article cites 57 articles, 21 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/7/6/874.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerimmunolres.aacrjournals.org/content/7/6/874.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.