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Signal-transducing adaptor protein-2 delays recovery of B-lineage lymphocytes during hematopoietic stress

Michiko Ichii,[1] Kenji Oritani,[2] Jun Toda,[1] Hideaki Saito,[1] Henyun Shi,[1] Hirohiko Shibayama,[1] Daisuke Motooka,[3] Yuichi Kitai,[4] Ryuta Muromoto,[4] Jun-ichi Kashiwakura,[4] Kodai Saitoh,[4] Daisuke Okuzaki,[3] Tadashi Matsuda,[4] and Yuzuru Kanakura[1,5]

[1]Department of Hematology and Oncology, Osaka University Graduate School of Medicine, Suita; [2]Department of Hematology, Graduate School of Medical Science, International University of Health and Welfare, Narita; [3]Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, Suita; [4]Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo and [5]Sumitomo Hospital, Osaka, Japan

Haematologica 2021
Volume 106(2):424-436

ABSTRACT

Signal-transducing adaptor protein-2 (STAP-2) was discovered as a C-FMS/M-CSFR interacting protein and subsequently found to function as an adaptor of signaling or transcription factors. These include STAT5, MyD88 and IκB kinase in macrophages, mast cells, and T cells. There is additional information about roles for STAP-2 in several types of malignant diseases including chronic myeloid leukemia; however, none have been reported concerning B-lineage lymphocytes. We have now exploited gene targeted and transgenic mice to address this lack of knowledge, and demonstrated that STAP-2 is not required under normal, steady-state conditions. However, recovery of B cells following transplantation was augmented in the absence of STAP-2. This appeared to be restricted to cells of B-cell lineage with myeloid rebound noted as unremarkable. Furthermore, all hematologic parameters were observed to be normal once recovery from transplantation was complete. In addition, overexpression of STAP-2, specifically in lymphoid cells, resulted in reduced numbers of late-stage B-cell progenitors within the bone marrow. While numbers of mature peripheral B and T cells were unaffected, recovery from sub-lethal irradiation or transplantation was dramatically reduced. Lipopolysaccharide (LPS) normally suppresses B precursor expansion in response to interleukin 7; however, STAP-2 deficiency made these cells more resistant. Preliminary RNA-sequencing analyses indicated multiple signaling pathways in B progenitors to be STAP-2-dependent. These findings suggest that STAP-2 modulates formation of B lymphocytes in demand conditions. Further study of this adapter protein could reveal ways to speed recovery of humoral immunity following chemotherapy or transplantation.

Correspondence:

MICHIKO ICHII
michii@bldon.med.osaka-u.ac.jp

Received: April 26, 2019.

Accepted: January 23, 2020.

Pre-published: January 23, 2020.

https://doi.org/10.3324/haematol.2019.225573

Introduction

Production of blood cells in bone marrow (BM) is highly regulated. Billions of blood cells are derived from multipotent hematopoietic stem cells (HSC). Indeed, a wide spectrum of hematologic lineages is produced on a daily basis over an individual's lifetime.[1,2] Hematopoiesis is flexible enough to respond to various types of stress, including chemotherapy, acute or chronic infections, and injuries.[3] In such situations, myeloid lineage cells often respond first to resolve inflammatory events, after which they need to be rapidly regenerated.[4] Recent studies have shown that HSC play an important role in driving this emergency myelopoiesis. For example, hematopoietic progenitors (HPC) and HSC in BM can respond to stimulation of toll-like receptors (TLR) that detect microbial products. This results in HSC expansion, increased myeloid differentiation and depletion of lymphoid progenitors via direct and indirect ways.[5-8] Besides this, proinflammatory cytokines such as interleukin (IL)-1, IL-6, tumor necrosis factor alpha (TNFα), interferons (IFN), and granulocyte-colony stimulating factor (G-CSF) impact the fate of multipotent

hematopoietic stem/progenitor cells (HSPC).[5,9,10] Many studies have focused on the pathophysiology of HSC, while few have investigated the role of lineage-committed progenitors, which have great capacity for proliferation.

Treatments for hematologic malignancies such as leukemia and lymphoma have been dramatically improved by recent advances in chemotherapy, immunotherapy and HSC transplantation (HSCT). However, compromising the immune system remains a frequent complication of various types of therapy, and induces the risk of non-relapse mortality. Especially in allogeneic HSCT settings, which is the only curative therapy for patients with refractory malignancies and severe BM failure diseases, regeneration of cellular and humoral immunity occurs over one year, while the recovery of innate immune cells, megakaryocytes and erythrocytes is usually observed within one month of HSCT.[11] Similar to clinical observations, murine HSCT experiments show relatively slow recovery of lymphocytes. Under regenerative conditions, HSC and myeloid-biased multipotent progenitors (MPP) enter cell-cycle, supporting early recovery of myeloid cells.[12,13] However, the mechanisms of lymphoid reconstitution is less well understood.

In 2003, we identified signal-transducing adaptor protein-2 (STAP-2) as a C-FMS/M-CSFR interacting protein.[14] STAP-2 contains an N-terminal pleckstrin homology domain, a proline-rich region and an YXXQ motif. Its central region is distantly related to the Src homology 2-like (SH2) domain. As the adaptor protein structure predicts, we and others identified roles in inflammatory reactions, cell survival, migration and cell adhesion in macrophages, T cells or mast cells.[15-18] Although interactions with inflammatory molecules such as STAT5, MyD88, and IκB kinase (IKK) have been shown in immune cells, the importance of STAP-2 to hematopoiesis in BM remains unknown. Therefore, we investigated STAP-2-mediated regulation of stress hematopoiesis using genetically modified mice.

Methods

Mice

STAP-2 knockout (KO) and transgenic (Tg) mice of the C57BL/6J strain were generated and maintained as described previously.[14] For the generation of STAP-2 Tg mice, a cDNA fragment including the full coding region of the human *STAP-2* gene was subcloned into a p1026X vector, which consisted of the murine Lck proximal promoter, the Ig intronic H chain enhancer E , and a human growth hormone (hGH) cassette, as previously described.[19] Eight to 16-week old mice were used in all experiments except for those involving aged mice. Some mice were administered BrdU intraperitoneally (100 mg/kg of body weight) 12 hours prior to BM collection for cell cycle analyses. To examine age-related hematopoiesis, 12-22-month old mice were used. All experimental procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee of Osaka University.

Bone marrow transplantation

Six thousand lineage$^-$ Sca1$^+$ cKit^{high} (LSK) cells sorted from C57BL/6-Ly5.2 (CD45.2) mice were mixed with 6x10^5 unfractionated adult BM cells obtained from wild-type (WT) C57BL/6-Ly5.1 (CD45.1) mice. The mixture of cells was transplanted into C57BL/6-Ly5.1 mice lethally irradiated at a dose of 8.5 Gy.

Flow cytometry

Flow cytometric analysis and sorting were performed using a FACS Aria IIu or FACSCanto (BD Biosciences). The antibodies used for flow cytometric sorting and analysis are listed in *Online Supplementary Table S1*. Antibodies to CD19, CD45R/B220, CD11b/Mac1, Gr1, Ter119, CD3, and CD8 were used as lineage markers. FITC-Annexin V apoptosis Detection Kit (BD Biosciences) for apoptosis detection, and BrdU Flow Kit (BD Biosciences) for cell proliferation were also used according to the manufacturer's protocol. FlowJo software (Tree Star) was used for data analysis.

Cultures

The details of culture methods are provided in the *Online Supplementary Methods*.

RNA-sequencing

Sequencing was performed using an Illumina HiSeq 2500 platform in a 75-base single-end mode. The raw data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (GSE127939). The normalized values compared to control were defined to show signal ratios with greater than 2-fold increases or decreases. Bioinformatic analyses were conducted with Ingenuity Pathway Analysis software (Ingenuity Systems).

Real-time quantitative polymerase chain reaction analyses of gene expression

Semi-quantitative and real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to assess mRNA expression. Reactions were quantified using fluorescent TaqMan technology.

Statistical analysis

Statistical analysis was carried out using unpaired two-tailed Mann-Whitney tests. The error bars used throughout indicate standard deviation (SD) of the mean.

Results

Loss of STAP-2 accelerates B-cell recovery in bone marrow transplantation

It has been reported that STAP-2 affects immune cell responses by directly binding to a variety of proinflammatory molecules, including MyD88, IKKα/β, C-FMS, STAT3, and STAT5.[14-18] In tumorigenesis, STAP-2 regulates cell migration, proliferation and therapy resistance for tyrosine kinase inhibitors.[20-23] Hematopoiesis under steady state appears normal in STAP-2 deficient mice;[24] we, therefore, hypothesized that STAP-2 may regulate stress hematopoiesis.

Here, we first conducted immunophenotypic characterization of STAP-2 deficient HSPC with flow cytometry, which were found to be comparable between the WT and knockout (KO) BM under homeostatic conditions (Figure 1A). Next, LSK derived from STAP-2 KO or WT mice (CD45.2$^+$) was intravenously injected into congenic recipients (CD45.1$^+$). One month after HSCT, we found that CD45.2$^+$ donor chimerism in peripheral B cells in KO HSPC transplanted mice was significantly higher than that in controls (WT donor, 51.8±1.0%; STAP-2 KO donor, 72.0±1.5%; P<0.001), while recovery of other lineages including Mac1$^+$ or Gr-1$^+$ myeloid and CD3$^+$ T cells was comparable (Figure 1B). The calculation of blood lin-

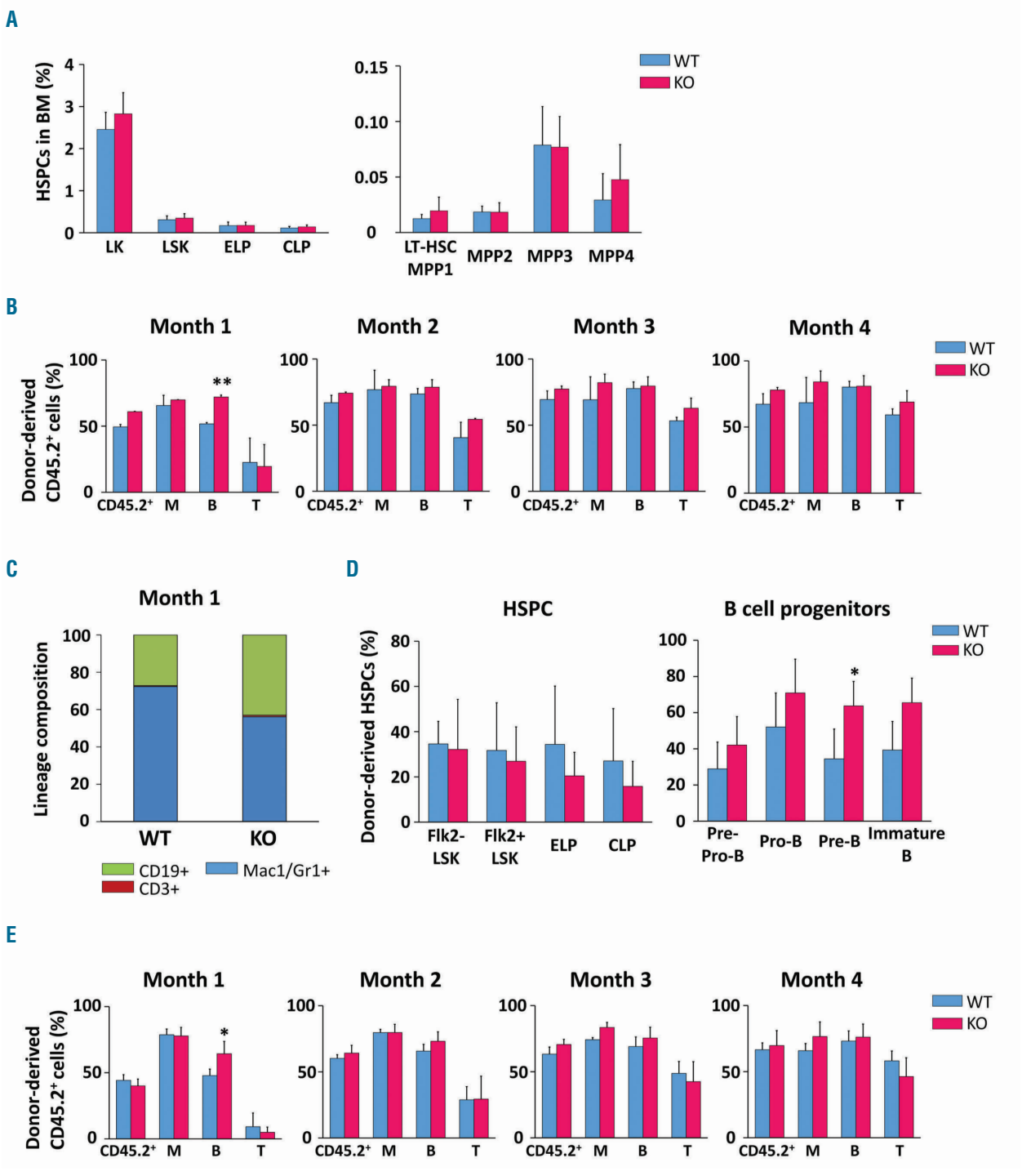


Figure 1. B-cell reconstitution following transplantation is improved in the absence of STAP-2. (A) The proportion of the indicated subsets (LK; Lineage$^-$ Sca1$^-$ cKit^{high}, LSK; Lineage$^-$ Sca1$^+$ cKit^{high}, early lymphoid progenitor (ELP); Flk2^{high} IL7Rα$^-$ LSK, common lymphoid progenitor (CLP); Lin$^-$ Sca1$^+$ cKit^{low} Flk2^{high} IL7Rα$^+$, LT-HSC and multipotent progenitor (MPP)1; Flk2$^-$ CD48$^-$ CD150$^+$ LSK, MPP2; Flk2$^-$ CD48$^+$ CD150$^+$ LSK, MPP3; Flk2$^-$ CD48$^+$ CD150- LSK, MPP4; Flk2- CD48- CD150- LSK) in bone marrow (BM) were analyzed with flow cytometry using wild-type (WT) and STAP-2 knockout (KO) mice (WT, n=5; STAP-2 KO, n=4). (B-E) Competitive transplantation experiments were conducted using KO mice. LSK cells (CD45.2$^+$) derived from KO or WT were injected intravenously with competitor BM cells (CD45.1$^+$). (B) Flow cytometry was used to evaluate hematopoietic recovery of Mac1$^+$ or Gr1$^+$ myeloid (M), CD19$^+$ B- and CD3$^+$ T-lineage cells in CD45.2$^+$ donor-derived peripheral blood at the indicated times after transplantation. (C) The lineage distribution in CD45.2$^+$ cells one month after transplantation. (D) BM cells were collected one month after transplantation, and donor chimerism (CD45.2$^+$) was evaluated in Flk2$^-$ or Flk2$^+$ LSK cells, ELP and CLP (left panel). The proportion of CD45.2$^+$ cells in the B lineage progenitor subsets (pre-pro-B; B220$^+$ CD43$^+$ CD19$^-$ IgM$^-$, pro-B; B220$^+$ CD43$^+$ CD19$^+$ IgM$^-$, pre-B; B220$^+$ CD43- CD19$^+$ IgM-, immature B; B220$^+$ CD43- CD19$^+$ IgM$^+$) was also analyzed (right panel). (E) The same experimental strategy was used to assess the effects of STAP-2 on aged hematopoiesis in 12-22-month old KO mice, using age-matched WT mice as a control. Similar results were obtained in three independent experiments. Six to ten mice were used for each experiment. The results are shown as means±standard deviation. Statistical significances relative to WT controls were determined by unpaired two-tailed Mann-Whitney tests: *P<0.05; **P<0.01.

eage distribution in donor-derived cells showed that the proportion of B cells was increased in KO donors, compared to that in WT donors (Figure 1C). Two months following transplantation, B-cell chimerism reached a plateau at which the engraftment and lineage distribution were indistinguishable between KO and WT donors (Figure 1B). Interestingly, we noticed that the proportion of KO donor-derived B cells one month after transplantation was similar to that at two months, indicating that B-cell reconstitution was completed by one month in KO LSK transplanted mice, while it took two months in WT control.

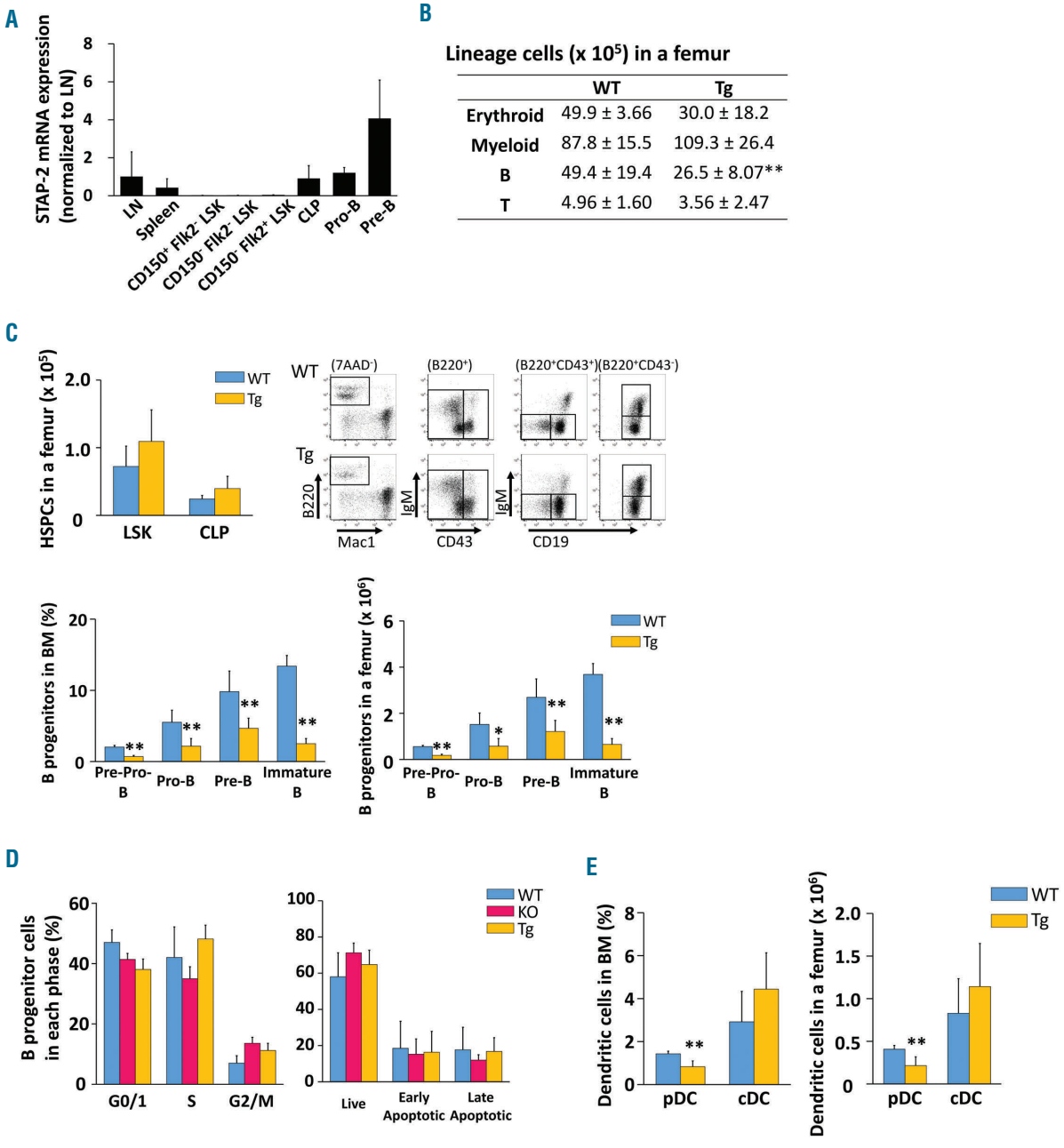


Figure 2. STAP-2 overexpression impairs early B lymphopoiesis in bone marrow (BM). BM cells from transgenic mice (Tg) that overexpress human STAP-2 under the control of an Eμ enhancer and Lck proximal promoter were used to evaluate roles of STAP-2 in early B lymphopoiesis. (A) The expression level of the inserted human STAP-2 was analyzed with real-time polymerase chain reaction (RT-PCR). The transcript levels in the indicated subsets (LSK; Lineage- Sca1$^+$ cKit^{high}, common lymphoid progenitor [CLP]; Lin$^-$Sca1$^+$ cKit^{low} Flk2^{high} IL7Rα$^+$, pro-B; B220$^+$ CD43$^+$ CD19$^+$ IgM$^-$, pre-B; B220$^+$ CD43- CD19$^+$ IgM) were normalized to the median of lymph node cell (LN) samples in Tg mice. (B) The percentages of Ter119$^+$ erythroid cells, Gr1$^+$ or Mac1$^+$ myeloid cells, CD19$^+$ B cells, or CD3$^+$ T cells, were determined by flow cytometry, and exact numbers were calculated. (C and E) Without initial separation, the samples were stained for lineage-associated surface markers for flow cytometric analysis. Representative gating for B-progenitor subsets is shown in (C) (top right panel). Proportion and number of cells in BM of B220$^+$ CD19$^-$CD11c^{low} Ly6C$^+$ plasmacytoid dendritic cells (pDC) and B220$^-$CD19$^-$ CD11c$^+$ conventional dendritic cells (cDC) were analyzed (E). (D) Ki67 and 7AAD staining was used for the analysis of the cell cycle status in B220$^+$ CD19$^+$ cells (left panel). Cell apoptosis was analyzed by Annexin V and 7AAD in B220$^+$cells using flow cytometry (right panel). Similar results were obtained in three independent experiments. Six to ten mice were used for each experiment. Results are presented as mean±standard deviation. Statistical significances relative to wild-type (WT) control were determined by unpaired two-tailed Mann-Whitney test: *P<0.05; **P<0.01. KO: knockout; CLP: common lymphoid progenitor; HSPC: hematopoietic stem/progenitor cells.

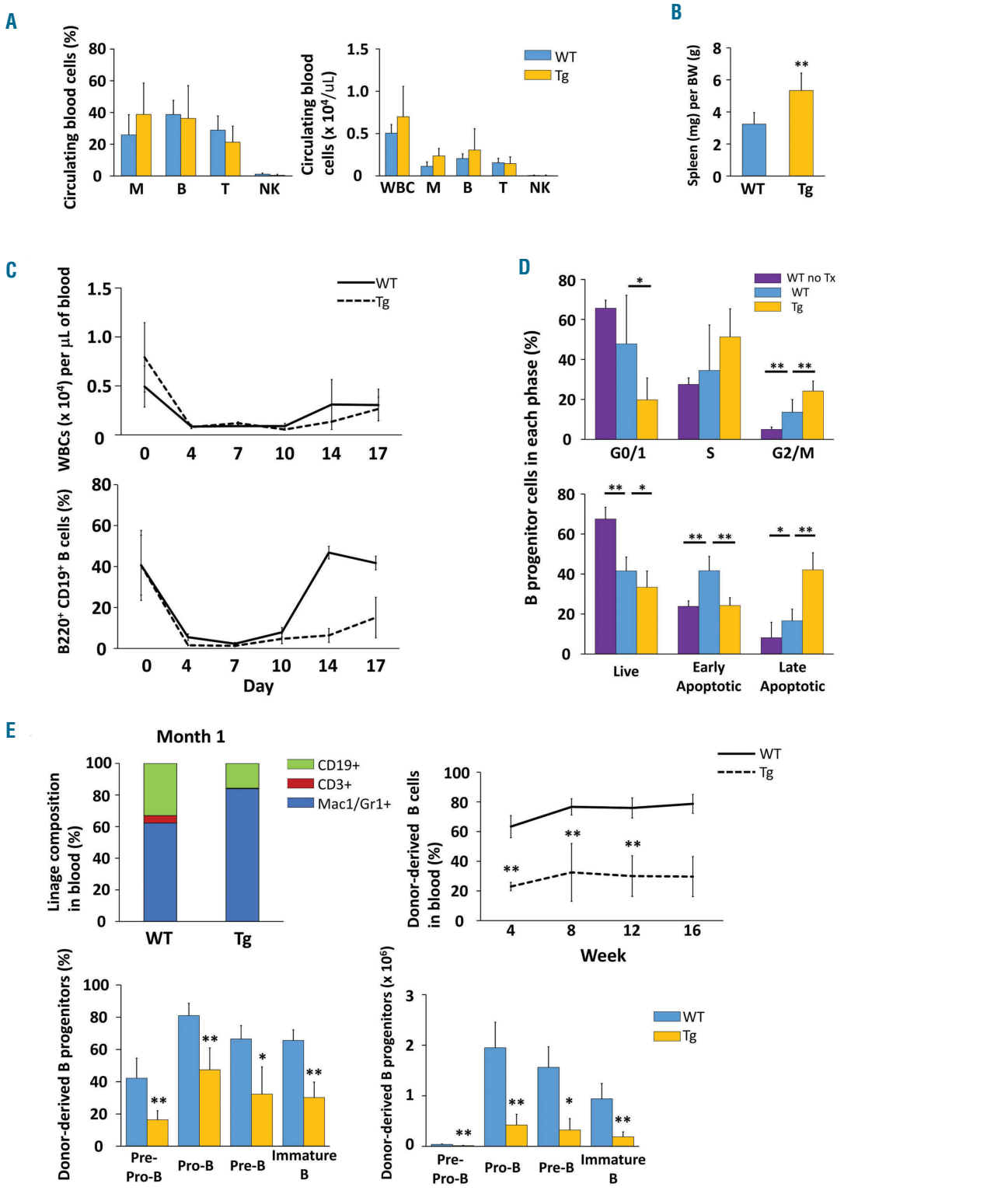


Figure 3. STAP-2 is essential for B-cell recovery during stress hematopoiesis. (A) Percentages of Mac1$^+$ or Gr1$^+$ myeloid (M), CD19$^+$ B, CD3$^+$ T-lineage cells and NK1.1$^+$ natural killer (NK) cells in peripheral blood of STAP-2 transgenic (Tg) and wild-type (WT) mice were analyzed via flow cytometry, and the number of white blood cells (WBC) and different lineages are shown (n=6 in each). (B) Weight of spleen was measured and the ratio to body weight (BW) was calculated (n=6 in each group). (C and D) WT or Tg mice were sub-lethally irradiated (3.5 Gy). (C) Peripheral blood was examined twice per week, and WBC counts were compared at each time point (top panel). Percentages of B220$^+$ CD19$^+$ B cells (bottom panel) were calculated using flow cytometry. Data represent three independent trials with similar results. (D) BrdU was administered 12 hours before analysis of the cell cycle status of B220$^+$ CD19$^+$ cells in bone marrow (BM) via flow cytometry (top panel). Cell apoptosis was analyzed by Annexin V and 7AAD in B220$^+$ cells in BM using flow cytometry (bottom panel). Data were pooled from two independent experiments (n=5 in each). (E) LSK cells (CD45.2$^+$) derived from Tg or WT were injected intravenously with competitor BM cells (CD45.1$^+$) (n=7 in each). Flow cytometry was used to evaluate hematopoietic recovery of myeloid, B- and T-lineage cells in CD45.2$^+$ donor-derived peripheral blood at the indicated time after transplantation (top panels). BM cells were collected one month after transplantation, and donor chimerism (CD45.2$^+$) was evaluated in B-lineage progenitor subsets (bottom panels). Similar results were obtained in two independent experiments. Results are shown as mean±standard deviation. Statistical significances relative to WT control were determined using unpaired two-tailed Mann-Whitney tests: *P<0.05; **P<0.01.

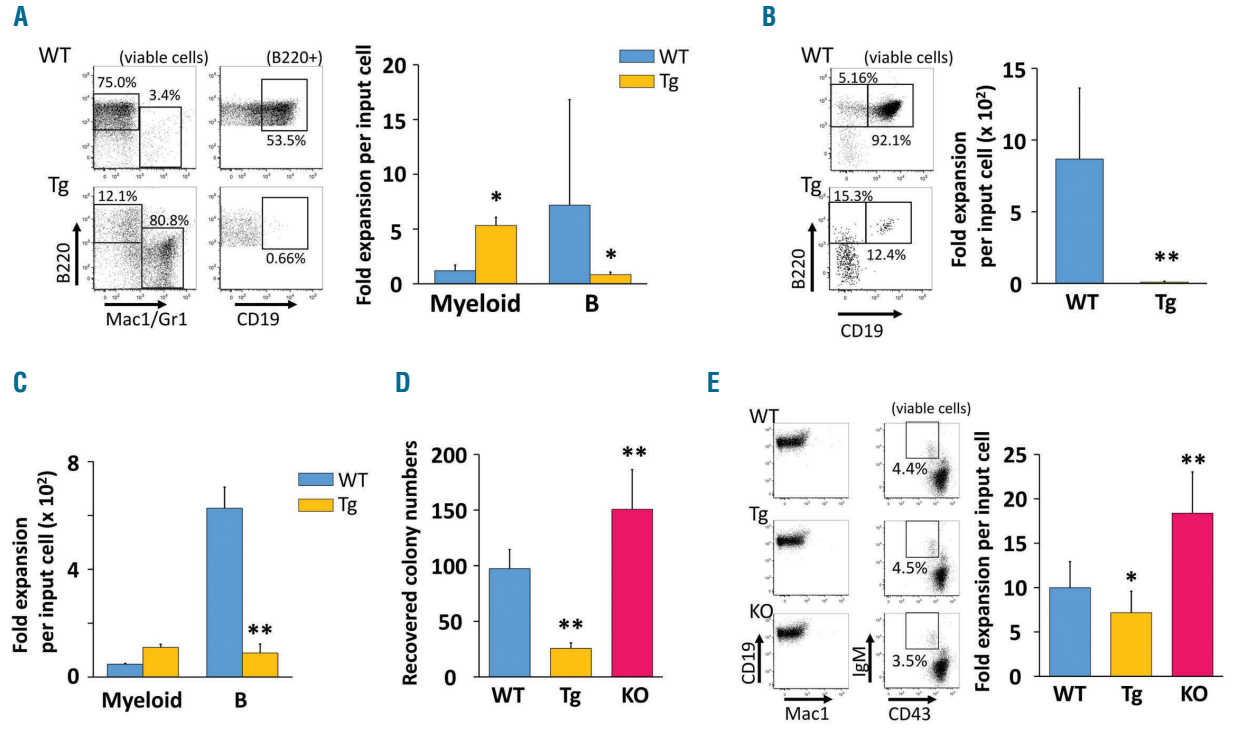


Figure 4. STAP-2 impairs B-cell differentiation and pre-B-cell proliferation. (A) LSK cells derived from STAP-2 transgenic (Tg) marrow were cultured under stromal-cell free, B-cell differentiation-supporting conditions. Flow cytometry was used to evaluate differentiation. (Left) Representative gating for Mac1$^+$ or Gr1$^+$ myeloid and B220$^+$ CD19$^+$ B cells. The number of myeloid and B cells recovered were used to calculate yields per input cell (fold expansion). (B and C) Lin$^-$ Sca1$^+$ cKit^{low} Flk2^{high} IL7Rα^+ CLP derived from Tg were cultured without (B) or with (C) OP9 stromal cells under B-cell conditions, and the production of B220$^+$ CD19$^+$ B cells was analyzed. (D) In colony-forming unit (CFU pre-B) assays, the ability to generate pre-B colonies was evaluated. (E) The stromal cell-free culture system was used to evaluate the proliferation of pre-B cells and differentiation to IgM$^+$ immature B cells. Similar results were obtained in three independent experiments. The results are shown as mean±standard deviation. Statistical significances relative to wild-type (WT) control were determined by unpaired two-tailed Mann-Whitney tests: *P<0.05; **P<0.01. KO: knockout.

We analyzed HSPC in BM one month after transplantation via flow cytometry and found that there were no differences in the number of each subset of multipotent HSPC and early lymphoid progenitors between WT and KO donors (Figure 1D). In contrast, STAP-2 deficiency significantly increased pre-B-cell subset. These results indicate that STAP-2 in B progenitors is involved with B-cell recovery under regenerative conditions.

It is known that self-renewal capacity and lineage commitment in HSPC change according to the type of hematopoietic stress to which they are exposed;[1,2] therefore, the effects of STAP-2 on aged hematopoiesis were also analyzed. In aged KO mice, no abnormalities were observed with respect to hematopoietic parameters in BM or peripheral blood (*Online Supplementary Figure S1*). In old KO LSK-transplanted mice, comparable long-term engraftment was observed. We found B-cell reconstitution from aged HSPC was also accelerated by STAP-2 deletion (Figure 1E).

Collectively, our findings indicate that STAP-2 suppresses B-cell recovery during stress hematopoiesis at the pre-B-cell stage in BM, while STAP-2 does not affect the functions of multipotent HSPC.

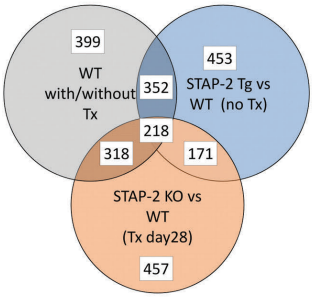
STAP-2 is critical for B-cell differentiation during stress hematopoiesis

To further investigate the role of STAP-2 in early B lymphopoiesis, we generated transgenic mice that overexpress STAP-2 under the control of an Eμ enhancer and Lck proximal promoter. This promoter could drive expression of the inserted cDNA in lymphoid lineage cells in BM, T-cell precursors in thymus, and peripheral mature lymphoid cells. The consistent upregulation from the common lymphoid progenitor (CLP) stage to mature lymphoid cells in lymph nodes was confirmed with RT-PCR (Figure 2A). Tg mice showed no apparent defects, and had normal life-spans.

We conducted a thorough analysis of marrow hematopoiesis in STAP-2 Tg mice that were 8-16 weeks old. No abnormalities were found with respect to marrow cellularity or proportions and numbers of HSPC and CLP (Figure 2B and C). In contrast, numbers of B220$^+$ B progenitors were significantly reduced (Figure 2B). By immunophenotyping B-cell development, the reduction of all BM B-lineage-committed progenitor stages (pre-pro-B, pro-B and pre-B) was confirmed (Figure 2C). Analyses of the proliferation and apoptosis status showed no statistical differences among WT, KO, and Tg mice under steady states (Figure 2D). Dendritic cells that can be generated from CLP, were also affected by STAP-2 overexpression (Figure 2E). These findings suggest that STAP-2 has a critical function in maintaining the integrity of the B-progenitor compartment.

Although overexpression of STAP-2 in B precursors seemed to increase the sensitivity to subtle stress under homeostasis, peripheral B-cell cellularity could be maintained through compensatory mechanisms, and the sizes of spleen were rather enlarged (Figure 3A and B). We therefore investigated whether B-cell recovery is compro-

A



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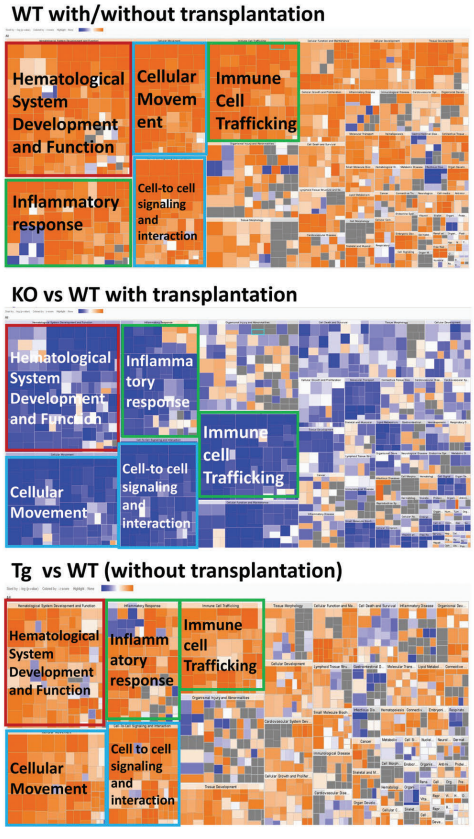


C

Differentially expressed pathways downstream of STAP-2

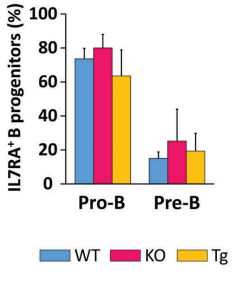
	Canonical Pathway	WT-Tx	Tg	KO-Tx
1	TREM1 Signaling	3.74	2.45	-1.90
2	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	3.00	2.83	-1.60
3	Complement System	2.33	0.82	-2.65
4	Colorectal Cancer Metastasis Signaling	1.71	1.89	-1.88
5	Acute Phase Response Signaling	1.50	2.11	-1.81
6	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.00	2.53	-1.89
7	Role of NFAT in Regulation of the Immune Response	1.51	1.50	-2.31
8	PPAR Signaling	-2.12	-1.41	1.67
9	IL-6 Signaling	1.60	1.90	-1.60
10	Eicosanoid Signaling	2.00	1.63	-1.34
11	VDR/RXR Activation	N/A	2.00	-2.24
12	Interferon Signaling	2.24	2.00	N/A
13	Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1.26	2.24	
14	NF-κB Signaling	1.21	1.60	-1.39
15	Dendritic Cell Maturation	1.28	1.50	-1.41
16	HMGB1 Signaling	0.90	2.11	-1.15
17	ILK Signaling	0.77	1.60	-1.70
18	Tec Kinase Signaling	1.07	1.89	-1.07
19	p38 MAPK Signaling	1.94	1.00	-1.07
20	Toll-like Receptor Signaling	1.13	1.34	-1.41

D

Differentially indicated upstream regulators of STAP-2

	Upstream regulators	WT-Tx	Tg	KO-Tx
1	lipopolysaccharide	7.11	6.72	-5.25
2	IFNG	7.08	6.22	-5.04
3	TNF	6.12	5.74	-5.14
4	IL1B	6.06	4.91	-4.90
5	poly rI:rC-RNA	5.75	4.69	-3.84
6	Interferon alpha	5.85	5.04	-3.07
7	tretinoin	4.51	5.53	-3.44
8	STAT1	4.97	5.22	-3.16
9	phorbol myristate acetate	4.38	4.18	-4.09
10	IRF7	5.77	4.97	
11	TGM2	5.05	3.79	-3.34
12	NFkB (complex)	4.91	2.85	-4.12
13	E. coli B4 lipopolysaccharide	4.84	3.56	-3.05
14	RELA	4.63	3.42	-3.40
15	TLR3	4.54	4.04	-2.77
16	MYD88	4.37	4.78	-2.00
17	ACKR2	-4.58	-4.24	2.33
18	IL10RA	-4.65	-3.60	2.78
19	IRF3	5.33	4.27	-1.39
20	CSF3	3.79	3.49	-3.47

E



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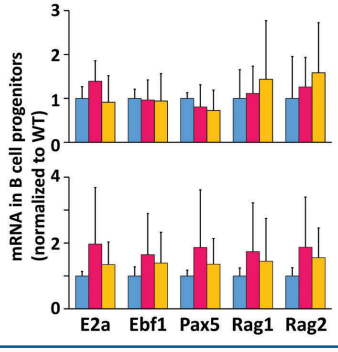

Figure 5. STAP-2-dependent inflammatory signals at the pre-B stage regulate B lymphopoiesis during hematologic stress. (A-D) RNA-Seq experiments with pre-B cells derived from wild-type (WT) and STAP-2 transgenic (Tg) bone marrow (BM) under steady-state as well as WT and STAP-2 knockout (KO) BM one month after transplantation (Tx) was conducted, and the genes were grouped according to a hierarchical clustering technique with Ingenuity Pathway Analysis based on patterns of expression. (A) Number of genes extracted. (B) A color-coded heatmap allows for the visualization of the differential gene expression data categorized by their functions in the Ingenuity Knowledge Base. Color bar indicates the z-score for each category: the strongest predicted increases (orange square) correspond to z-score > 2, the strongest predicted decreases (blue square) correspond to z-score < -2. Gray and white colors indicate categories with a -2 < z-score < 2 and without z-score, respectively. Size reflects the associated log of the calculated *P*-value with s significance threshold of *P*=0.05 (Fisher's exact test). Larger squares indicate more significant overlap among the affected genes contained in the dataset. (C and D) Datasets were compared, and a bioinformative approach identified shared pathways regulated under hematologic stress and by STAP-2 expression. (E) The proportion of IL7 receptor α^+ in the B-lineage progenitor subsets in WT, KO, and Tg BM (pro-B; B220$^+$ CD43$^+$ CD19$^+$ IgM$^-$, pre-B; B220$^+$ CD43$^-$ CD19$^+$IgM) was analyzed with flow cytometry (n=6 in each). (F) Indicated transcript levels in pro-B cells (top panel) and pre-B cells (bottom panel) were evaluated with real-time polymerase chain reaction and normalized to the median of WT samples (WT, n=9; KO, n=4; Tg, n=8).

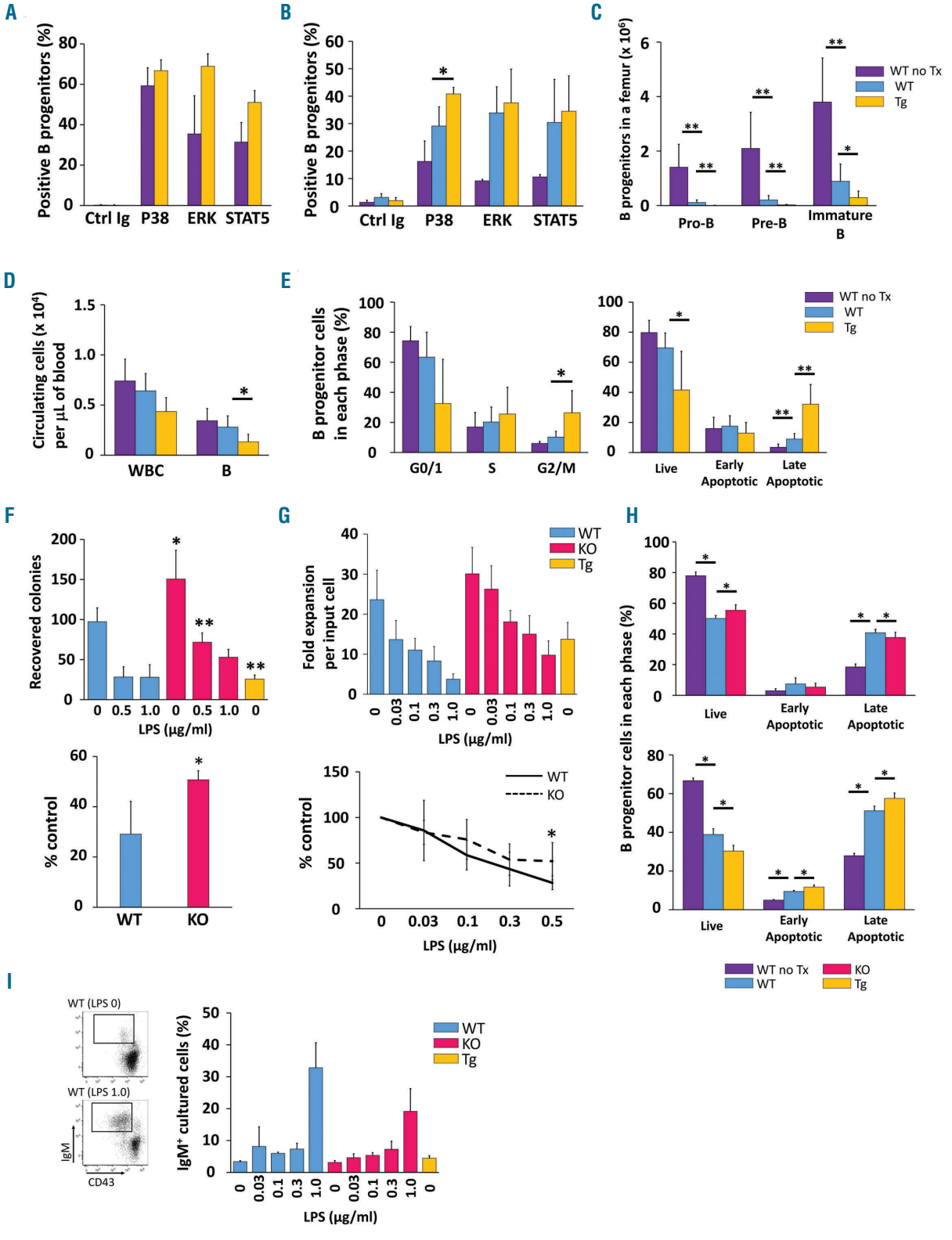

Figure 6. STAP-2 deficiency attenuates the inhibitory effects of lipopolysaccharide (LPS) in pre-B cells. (A and B) Phosphorylation status of P38/MAPK (pT180/pY182), ERK1/2 (pT202/pY204), and STAT5 (pY649) in B220⁺ cells from wild-type (WT) or STAP-2 transgenic (Tg) mice, under steady state (A) and 12 hours after sub-lethal irradiation (B), were analyzed by flow cytometry. (C-E) Three days after 1.0 mg /kg of LPS had been administered intraperitoneally, flow cytometric analysis was conducted. Number of B-lineage progenitor subsets (pro-B; B220⁺ CD43⁺ CD19⁺ IgM⁻, pre-B; B220⁺ CD43⁻ CD19⁺ IgM⁻, immature B; B220⁺ CD43⁻ CD19⁺ IgM⁺) in bone marrow (BM) (C), as well as white blood cells (WBC) and B220⁺ CD19⁺ B cells in peripheral blood (D) are shown. BrdU was administered 12 hours before analysis of the cell cycle status in B220⁺ CD19⁺ cells (E, left). Cell apoptosis was analyzed by Annexin V and 7AAD in B220⁺ cells using flow cytometry (E, right). Data represent three independent experiments (n=6 in each). (F-I) Indicated concentration of LPS was added into CFU pre-B (F) or B-progenitor suspension cultures (G-I) using cells derived from STAP-2 knockout (KO), Tg, or WT mice. Recovered colony (F) or cell (G) numbers were counted, and the proportion normalized to cells without LPS stimulation (control) were calculated. (H) Cell apoptosis was analyzed by Annexin V and 7AAD using flow cytometry after 4-day cultures with LPS (1.0 µg/mL). (I) Differentiation to IgM⁺ immature cells was evaluated via flow cytometry. Results are shown as mean±standard deviation. Statistical significances relative to WT were determined by unpaired two-tailed Mann-Whitney tests: *P<0.05 and **P<0.01.

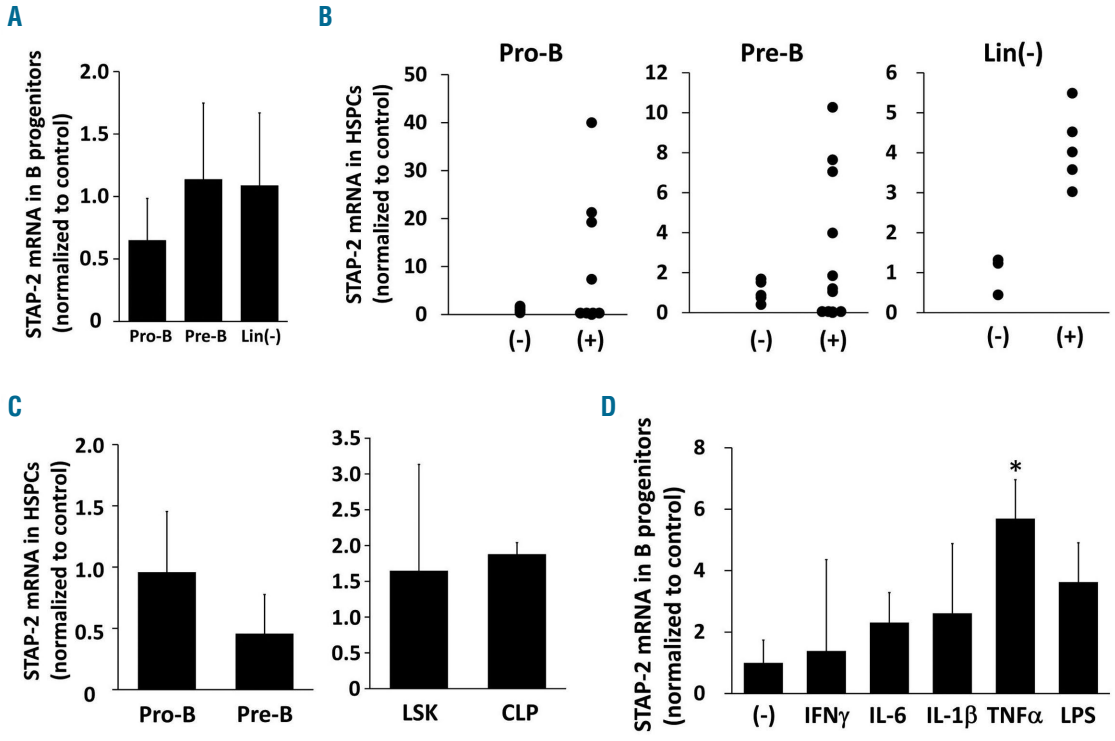

Figure 7. STAP-2 expression under hematologic stress. (A-C) The indicated subsets (pro-B; B220$^+$ CD43$^+$ CD19$^+$ IgM$^-$, pre-B; B220$^+$ CD43$^-$ CD19$^+$ IgM$^-$, Lin($^-$); lineage marker negative, LSK; Lineage$^-$ Sca1$^+$ cKit^{high}, common lymphoid progenitor [CLP]; Lin$^-$ Sca1$^+$ cKit^{low} Flk2^{high} IL7Rα$^+$) of hematopoietic cells derived from wild-type (WT) mice three days after LPS administration (A) (n=6 in each), or 12 hours (B) (control, n=6; with irradiation, n=13) or one month (C) (control, n=3; with irradiation, n=5) after sub-lethal irradiation (3.5 Gy) were analyzed for Stap-2 expression by real-time polymerase chain reaction (RT-PCR). Stap-2 transcript levels were normalized to the median of control samples without treatment. (D) Indicated cytokines (IFN-γ, 0.1 ng/mL; IL-6, 50 ng/mL; IL-1β, 5.0 ng/mL; TNFα, 50 ng/mL; LPS, 5.0 μg/mL) were added to B-progenitor cultures. Cells were collected after 24-hour stimulation, and RT-PCR was performed. Results are expressed as fold change relative to the controls, and are representative of results obtained in three independent experiments. Results are shown as mean ± standard deviation. Statistical significances relative to controls without stimulation were determined by unpaired two-tailed Mann-Whitney tests: *P<0.05.

mised by STAP-2. Sub-lethal irradiation eliminates most hematopoietic cells, leading to rapid proliferation and differentiation of HSPC, and STAP-2 requirement was assessed following 3.5 Gy sub-lethal irradiation (Figure 3C). Four days post irradiation, loss of myeloid, T, and B cells was observed in both WT and Tg mice, whose total white blood cell counts were indistinguishable before and after irradiation. However, while control mice regained normal levels of B cells two weeks after irradiation, almost no recovery was observed in STAP-2 Tg mice at that stage. Analyses of the proliferation and apoptosis status after irradiation revealed that B progenitors in Tg mice showed more progressive cell division, and rapid induction of apoptosis (Figure 3D). To test the effect of STAP-2 on undamaged B progenitors following direct stress, B-cell recovery was monitored after congenic BM transplantation. One month after transplantation to lethally irradiated recipients, CD45.2$^+$ donor chimerism in blood B cells of Tg LSK-transplanted recipients was significantly lower than in WT transplanted mice (WT donor, 63.3±7.5%; STAP-2 Tg donor, 23.0±2.8%; P<0.001). The percentage and cellularity of BM B progenitors one month after transplantation were significantly reduced in Tg LSK transplanted mice compared to control (Figure 3E). The suppression of B-cell recovery lasted for 4 months at the end of follow-up. Taken together, we concluded that STAP-2 governs B-cell reconstitution following hematologic stress.

STAP-2 impairs proliferation of B progenitor at the pre-B stage

To gain further insight into STAP-2 functions, we evaluated B-cell differentiation of *Stap-2* gene targeted mice *in vitro*. The capacity for B-cell lineage development was reduced, when HSPC derived from Tg were cultured under stromal-cell free, B-cell conditions in the presence of IL-7, FL and SCF (Figure 4A). The same was true in cultures of LSK cells as well as CLP with or without OP9 stromal cells (Figure 4B and C).

After rearrangement and expression of immunoglobulin (Ig) heavy chain loci at the pro-B stage, large pre-B cells enter cell-cycle and undergo several rounds of proliferation. The Ig light chain gene is then rearranged, and cells begin to express a functional B-cell receptor, progressing to an IgM$^+$ immature B-cell stage. Colony-forming unit (CFU) assays were used to study the effect on pre-B-cell proliferation, and we found that STAP-2 deficiency promoted the generation of pre-B colonies, while overexpression of STAP-2 inhibited colony recovery with statistical significance (recovered colony number; 90.0±9.5 in WT, 40.7±3.1 in Tg; P<0.001, and 200.0±5.3 in KO; P<0.001) (Figure 4D). Under stromal-cell free culture conditions of Mac1$^-$ B220$^+$ CD19$^+$ CD43$^+$ IgM$^-$ pro-B cells, STAP-2 regulated the expansion in the same way as was found in the CFU assay, while the differentiation into immature B cells was unaffected (Figure 4E).

These results indicate that STAP-2 is involved in the

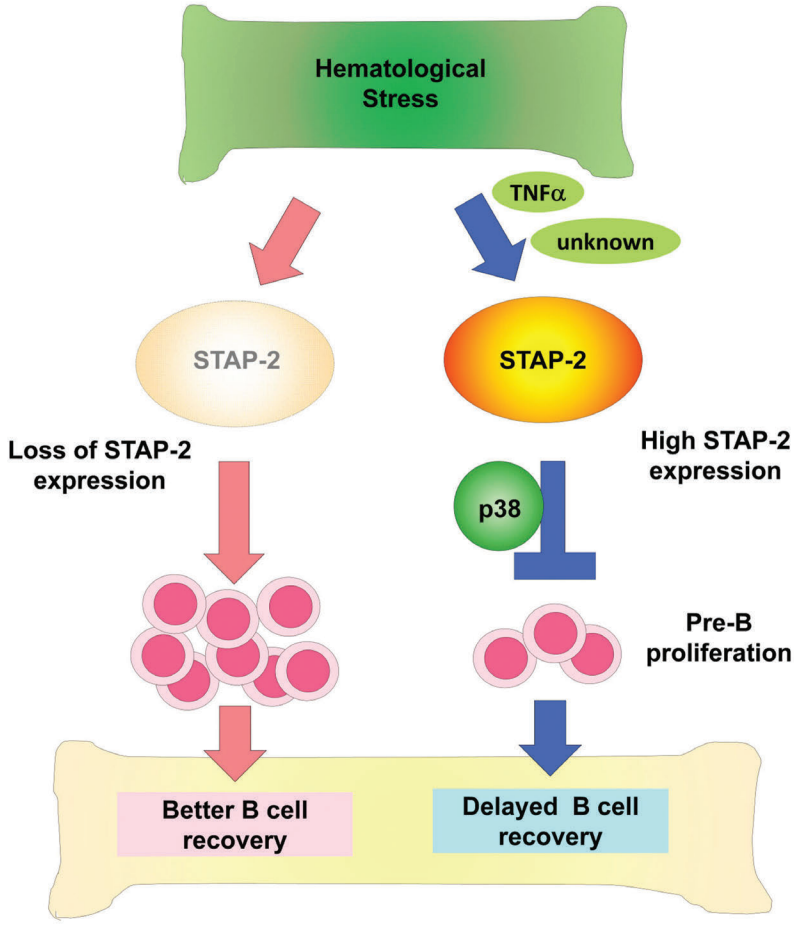

Figure 8. STAP-2 attenuates B-cell recovery under hematologic stress. A graphic depiction of hematologic stresses that variably regulate STAP-2 mRNA expression via the direct effects of several hematologic stress factors, such as TNFα, as well as indirect effects imposed by the affected microenvironment. STAP-2 impairs B-cell recovery under hematologic stress.

proliferation of pre-B cells leading to compromised B-cell recovery during hematologic stress.

Inflammatory signals upregulated following hematopoietic stem cell transplantation are exaggerated by STAP-2

To determine the molecular basis for the inefficient B lymphopoiesis in STAP-2 Tg BM, transcriptome analysis was used to identify the pathways regulated by STAP-2 under hematologic stress. We evaluated global gene expression profiles of pre-B cells derived from WT and Tg BM under steady-state as well as WT and KO BM one month post transplantation by RNA-sequencing (RNA-Seq) experiments (Figure 5). The number of significantly regulated transcripts is shown in Figure 5A.

First we compared pre-B cells isolated one month after transplantation with cells under homeostasis. It was expected that pathways related to hematologic development and genes with lymphoid signatures would be significantly regulated (Figure 5B). Furthermore, a bioinformatic approach with Ingenuity Pathway Analysis revealed that groups of genes involved in inflammatory responses were strongly upregulated during stress hematopoiesis. Differential display between WT and KO after transplantation showed that the deletion of STAP-2 prevented inflammatory changes induced by stress. STAP-2 overexpression activated these pathways even under steady-state conditions.

Next, we investigated which signals were commonly regulated among the following three pairs: WT with or without transplantation, WT or KO with transplantation, and WT or Tg under steady state (Figure 5C and D). As a result, pathways related to cytokines (IFN, TNF, IL-1β and IL-6), innate immune system (TLR and complement system), NFκB and p38 MAPK were extracted. Among them, the pathway activated to the greatest degree by STAP-2 and regenerative stress triggered receptor expression on myeloid cells type-1 (TREM-1) signaling (Figure 5C). This molecule has recently been shown to play an important role in controlling the intensity of innate immune responses.[25-27] Upregulation of TREM-1 was verified by quantitative PCR and flow cytometry (*Online Supplementary Figure S2A-C*). Similar to macrophages, the activation of TLR4 signaling in Baf-3 pre-B cells upregulated the expression of TREM-1 (*Online Supplementary Figure S2D*). There were no shared pathways involved in B-cell development, and protein expression of IL7R, and mRNA expression of *E2a, Ebf1, Pax5, Rag1* and *Rag2* were not regulated under these conditions (Figure 5E and F).

Inflammatory network regulates stress B lymphopoiesis in bone marrow

Our findings from RNA-Seq experiments indicate STAP-2 modulation with inflammatory signals delays B-cell recovery after hematologic stress. This was also confirmed with the evaluation of phosphorylated forms of the target proteins of inflammatory signaling pathways, such as JAK/STAT and MAPK in B progenitors *in vivo*.

When the levels of p38 MAPK, ERK1/2 and STAT5 phosphorylation in B progenitors were evaluated under steady state, there were no differences between WT and Tg (Figure 6A). After mice were irradiated, the phosphorylation was activated. We found that STAP-2 overexpression exaggerated p38 phosphorylation significantly, compared to WT B progenitors (Figure 6B).

While several inflammatory molecules including LPS are known to be activators of B cells at the maturation stage following export to the periphery, recent studies showed that the TLR4 signaling pathway inhibits proliferation of pro-B and pre-B cells, and promotes the differentiation to IgM^+ immature B cells in BM.[28,29] Consistent with previous studies, the suppression of B-cell differentiation in BM was observed 3 days following sub-lethal dosing of LPS treatment (1.0 mg/kg) (Figure 6C). When Tg mice were treated, the actual B-cell number in peripheral blood decreased and a significant reduction of B progenitors in BM was observed (Figure 6C and D). The proliferation and apoptosis analyses, including BrdU and Annexin V staining methods, revealed a similar pattern to those after sub-lethal irradiation *in vivo*, indicating that the same mechanism is employed under these hematologic stressors (Figure 6E). Treatment of LPS-induced septic Tg mice with the p38 MAPK inhibitor SB203580 increased the number of B progenitors in BM, but this increase was not statistically significant (*Online Supplementary Figure S3*).

To study the specific interaction between each signal and STAP-2, pre-B-culture systems were used. This made it possible to eliminate the influence of HSPC and other lineage cells, which are also targets of the same signals such as IFN, TNFα, IL-1β, MAPK, and TLRs.[5-10] When LPS was added to CFU pre-B cultures, we found that deletion of STAP-2 attenuated the inhibitory effect of LPS (Figure 6F). The cancelation, although incomplete, was statistically significant, and the same was true in stroma-free liquid cultures (Figure 6G). Similar to reports following LPS treatment *in vivo*, apoptosis was induced (Figure 6H); however, BrdU analysis revealed that differences were not observed with or without treatment (*data not shown*). Moreover, STAP-2 deficiency led to a statistically significant reduction in the effects (Figure 6H). Regarding effects on differentiation, the expression of IgM after one week of culture was identical between WT and KO pre-B-cell cultures (Figure 6I). The limited effects on TLR4 signaling indicates the existence of other pathways involved with STAP-2. The addition of IFNγ, TNFα, IL-1β and IL-6 into pre-B-cell cultures did not induce statistically significant differences between WT and KO cultures (*data not shown*).

Our previous studies showed that Stap-2 mRNA is induced in murine hepatocytes in response to stimulation by LPS and IL-6.[14] However, the mRNA level in pre-B or pro-B cells did not change after LPS treatment *in vivo*, indicating the sustained protein level is sufficient to serve as the suppressor of B-progenitor proliferation (Figure 7A). In contrast, we found the expression level varied after sub-lethal irradiation (Figure 7B). When 13 mice were irradiated, the expression in pre-B cells was upregulated in five mice (38.5%) while in the same number of mice that was completely lost. The same was observed in pro-B cells. Interestingly, *Stap-2* expression in lineage negative cells was increased approximately four times compared to controls. The changes recovered to normal levels one month after irradiation (Figure 7C). These results indicated that *Stap-2* mRNA expression may be regulated via the direct effects of several hematologic stress factors, as well as indirectly by the affected microenvironment.

Lastly, we sought to determine if the upregulated inflammation during hematologic stress could alter STAP-2 expression in pre-B cells. Several cytokines such as IFNγ, IL-6, IL-1β, TNFα, and LPS were added to pre-B-cell cultures, and the expression of *Stap-2* mRNA was analyzed after 24 hours of stimulation (Figure 7D). Interestingly, only TNF significantly increased Stap-2 expression. These results indicate that inflammatory signals during hematologic stress may use STAP-2 for negative regulation of B lymphopoiesis.

Discussion

Here we demonstrate that STAP-2 governs early B lymphopoiesis in BM during hematopoietic stress. While STAP-2 did not influence stemness of HSC or homeostatic hematopoiesis, short-term recovery of B cells was impaired by STAP-2 following transplantation. This effect was due to the inhibited proliferation of pre-B cells. RNA-Seq analyses showed that activation of several inflammatory signals occurs one month after transplantation in Tg mice. Culture experiments indicated developmental stage-specific inhibitory regulation of the TLR4-STAP-2 pathway as one of the mechanisms. Our study unveiled the critical role of STAP-2 in B-cell lineage progenitors in short-term responses to hematologic stress (Figure 8).

Previously, we and others reported that STAP-2 functions as an adaptor protein in a variety of signaling pathways, including immune responses and tumorigenesis. STAP-2 regulates cell growth, integrin-mediated adhesion, migration induced by CXCL12, and apoptosis in T cells.[17,30,31] The production of cytokines in macrophages is also affected by STAP-2. In tumorigenesis, STAP-2 binds to BCR-ABL in chronic myeloid leukemia cells, and also interacts with Brk, which mediates STAT5 activation in breast cancers.[21,22] Recent studies reported that STAP-2 has a significant impact on disease severity in mouse models of IgE allergy and inflammatory bowel disease.[18,24] In this study, we show a novel function of STAP-2 during stress hematopoiesis. Consistent with previous reports, STAP-2 modulates inflammatory signals such as TLR4 in pre-B cells.

Sustained hematopoiesis is regulated by various mechanisms, including cell-intrinsic and -extrinsic factors, and the role of HSC in hematologic stress has been intensively studied.[1-3,5,9] Unlike HSC, lineage-committed progenitors lose multipotency, yet retain the ability to proliferate during differentiation. In murine erythropoiesis, it is reported that robust expansion of erythroid progenitors in BM and spleen occurs to counteract anemic stress using signals different from those in homeostatic conditions.[32,33] In B lymphopoiesis, various factors such as infection, aging and G-CSF administration, are known suppressors of BM B lymphopoiesis.[34-38] However, some studies focused on the immune functions of B lymphocytes,[39] and the effects of G-CSF on B progenitors in BM are indirect.[36,37] How B progenitors in BM respond to stress is not well understood. In the current study, we show that STAP-2 impairs pre-B-stage proliferation and delays the recovery of B cells during hematologic stress, such as transplantation and irradiation. The proliferation of lineage-committed progenitors appears to be more important in hematologic regeneration than previously believed.

Interestingly, pre-B cells were strongly affected by various inflammatory signals at the early phase after hematopoietic stem cell transplantation (Figure 5). Among these signals, we focused on the TLR4 pathway. B-lineage cells express multiple pathogen recognition receptors, such as TLR, from the earliest stage.[7,40] As far as the impact on B progenitors in BM is concerned, two research groups previously showed that signaling through TLR4 inhibits pro-B and pre-B proliferation, and enhances differentiation to IgM^+ immature B cells, using different methods.[28,29] In our genetically modified mice, STAP-2 displayed synergistic effects on pre-B-cell proliferation with the TLR4 pathway. However, STAP-2 effects did not involve differentiation to IgM^+ immature B cells. In macrophages, STAP-2 binds directly to MyD88 and IKKα/β but not to TNFR-associated factor 6 or IL-1R-associated kinase 1.16 As in macrophages, STAP-2 may modulate specific TLR4 functions. We also found that TREM-1 expression is upregulated by STAP-2 overexpression and LPS stimulation. Although it is known that TREM-1 is expressed on B-lineage cells, its potential roles in B lymphopoiesis remain unknown.[25-27,41] When the antagonized antibody and neutralized Fc chimera was added into the pre-B-cell cultures, no effects were observed (*data not shown*). Thus, a gene-modified approach may be required to clarify the function.

Considering the nature of STAP-2 as an adaptor protein, the total protein expression as well as its phosphorylation is essential for the acting process. Previously, we showed that phosphorylated STAP-2 binds directly to several types of inflammatory proteins, including MyD88.[16,17,30,31] In this study, we evaluated the transcript under hematologic stress. As shown in Figure 7, the expression is sustained after LPS treatment. Alternatively, we observed expression changes according to the individual condition after sub-lethal irradiation, indicating that several direct and indirect factors affected by hematologic stress are associated with the expression of STAP-2.

Among several candidates, TNFα upregulates the expression of STAP-2 in pre-B cells (Figure 7D). In clinical settings, prolongation of hematologic suppression after chemotherapy is sometimes observed when patients suffer from severe complications such as infection. Our findings indicate the existence of a complex network induced by hematologic stress under inflammatory milieu. Interestingly, we found *Stap-2* expression in the lineage negative HSPC fraction was upregulated following irradiation (Figure 7B). Since the intensity of cKit decreases while that of Sca1 increases under hematologic stresses, such as irradiation and LPS administration, it is difficult to sort LSK or CLP cells via flow cytometry, and, therefore we were unable to identify in which population HSPC expression increased. Based on our *in vivo* and *in vitro* results, STAP-2 in CLP may affect B lymphopoiesis under hematologic stress.

In this study, we revealed a detailed mechanism underlying the impaired effects of STAP-2 on B-cell reconstitution under hematopoietic stress. The target of STAP-2 during stress hematopoiesis appears to be very specific to B progenitors in BM, and its expression is altered following exposure to hematologic stress, while the expression of STAP-2 is ubiquitous under steady state. Our findings provide insights that may aid in the development of new therapeutic approaches, with fewer adverse effects, or increased prognostic value, for patients given severe myeloablative therapy or allogeneic HSCT.

Disclosures

MI have received speakers bureau from Celgene, Kowa Pharmaceutical, Takeda Pharmaceutical and Novartis outside the submitted work. HS have received honoraria from Jansen, honoraria and research funding from Bristol-Meyer Squibb, Celgene, Fujimoto Pharmaceutical, Mundipharma, Novartis, Ono Pharmaceutical, and Takeda Pharmaceutical outside the submitted work. KO have received speakers bureau from Novartis outside the submitted work. YK have received consultancy, honoraria, and research funding from Alexion outside the submitted work. The remaining authors declare no competing financial interests.

Contributions

MI designed research, collected data, analyzed data, and wrote the paper. KO, HShib, and TM analyzed data, and wrote the paper. HShih, JT, HS, DM, and DO collected and analyzed data. YK, RM, JK and KS supplied materials. YK supervised the research and wrote the paper. All authors reviewed and approved the manuscript.

Acknowledgments

The authors would like to thank Dr. Kincade (Oklahoma Medical Research Foundation) for constructive suggestions. We are also grateful for Ms. Habuchi, and Mr. Takashima for technical support.

Funding

This work was supported in part by Japan Society for the Promotion of Science KAKENHI (grant no. 16K09872, 19H03364, 19K08815), Otsuka Pharmaceutical, and Kyowa Kirin Co., Ltd.

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