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Hepatocyte growth factor regulated tyrosine kinase substrate in the peripheral development and function of B-cells

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ABSTRACT

Hepatocyte growth factor (HGF)-regulated tyrosine kinase substrate (Hrs) is a vesicular sorting protein that functions as one of the endosomal-sorting proteins required for transport (ESCRT). Hrs, which binds to ubiquitinated proteins through its ubiquitin-interacting motif (UIM), contributes to the lysosomal transport and degradation of ubiquitinated membrane proteins. However, little is known about the relationship between B-cell functions and ESCRT proteins *in vivo*. Here we examined the immunological roles of Hrs in B-cell development and functions using B-cell-specific Hrs-deficient ($Hrs^{flox/flox}$;$mb1^{cre/+}$:Hrs-cKO) mice, which were generated using a cre-*LoxP* recombination system. Hrs deficiency in B-cells significantly reduced T-cell-dependent antibody production *in vivo* and impaired the proliferation of B-cells treated *in vitro* with an anti-IgM monoclonal antibody but not with LPS. Although early development of B-cells in the bone marrow was normal in Hrs-cKO mice, there was a significant decrease in the number of the peripheral transitional B-cells and marginal zone B-cells in the spleen of Hrs-cKO mice. These results indicate that Hrs plays important roles during peripheral development and physiological functions of B lymphocytes.

1. Introduction

Cell surface proteins are internalized and degraded to regulate their functions [1,2]. Ubiquitination of these proteins plays an important role in controlling membrane transport by facilitating internalization and degradation [3]. Ubiquitinated membrane proteins such as epidermal growth factor (EGF) receptors are initially recognised by the endosomal-sorting complex required for transport (ESCRT)-0 complex and then sequentially engage ESCRT-I, -II and -III [1]. ESCRT proteins form multimolecular complexes that control multivesicular body formation and transport ubiquitinated membrane proteins to the endosomes.

Hepatocyte growth factor (HGF)-regulated tyrosine kinase substrate (Hrs) was originally identified as a molecule that is phosphorylated in cells treated with HGF [4] or interleukin (IL)-2 [5]. Hrs is a vesicular sorting protein belonging to the class of ESCRT-0 and contributes to the control of multivesicular body formation, lysosomal transport and degradation of ubiquitinated membrane proteins [6–8]. Hrs contains two ubiquitin-binding sites, the Vps27/Hrs/STAM (VHS) domain and ubiquitin interacting motif (UIM) [9], through which Hrs binds to ubiquitinated proteins. We previously reported that the lysosomal degradation of the EGF receptor and gp130, a cytokine receptor, is severely impaired in mouse embryonic fibroblasts (MEFs) isolated from an Hrs-deficient mouse, suggesting an important role for Hrs in the endosomal-sorting machinery [10–12]. Furthermore, the impairment of Hrs-mediated degradation of gp130 leads to a prolonged and amplified IL-6 signal in Hrs-deficient MEF cells [12]. Therefore, we postulated that Hrs contributes to the control of B-cell development and function through protein turnover and degradation of immune receptors.

B-cell development, selection, activation and tolerance are critically dependent on the surface expression of the B-cell receptor (BCR) and BCR-mediated signal transduction [13]. The BCR is composed of a membrane-bound immunoglobulin and an immunoreceptor tyrosine-based activation motif-containing αβ heterodimer [14]. BCR ubiquitination, which induces the internalization of BCR, controls BCR-mediated antigen (Ag) processing and presentation [15]. The ubiquitination process mediated by E3 ligases plays important roles in B-cell development and activation [16]. However, little is known about the contribution of ESCRT proteins to the functions of B-cells.

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B-cells undergo a highly ordered and defined developmental program [17]. The earliest bone marrow pro-B-cells differentiate to the pre-B-cell stage [18]. Immature bone marrow B-cells are negatively selected based on their avidity for self-Ag and enter the spleen from the bone marrow. Newly formed transitional type 1 (T1) B-cells traffic to the spleen where they encounter selection signals for continued selection/maturation. T1 B-cells subsequently advance into the follicle and are then called transitional type 2 (T2) B-cells. These T2 cells are further selected and either die by apoptosis or develop into marginal zone (MZ) or follicular mature B-cells [19].

The strength of signalling through the BCR complex is important for determining the fate and activities of a developing B-cell [20]. Mature B-cells present peptide-major histocompatibility complex (MHC) class II complexes to $CD4^+$ T-cells to elicit humeral immune responses, including antibody (Ab)-class switching and proliferation. Efficient presentation of Ag by B-cells depends upon initial BCR-mediated Ag binding and internalization [21]. BCR can be ubiquitinated [15] and is constitutively internalized through clathrin-coated pits [22]. Therefore, the surface expression of BCR may be controlled by ESCRT proteins, including Hrs through BCR internalization.

Although several studies using Hrs mutants or on mammalian cells and mice with deletions of *Hrs* demonstrate that Hrs contributes to morphogenesis, development and survival of neurons [23,24]; however, whether Hrs functions in B-cells is unknown. To understand the physiological function of Hrs in B-cells, using a cre-*LoxP* system, we generated a mouse model in which Hrs expression is specifically deficient in B-cells. Here we show that Hrs deficiency caused a significant reduction in the numbers of transitional B-cells and MZ B-cells in the spleen. Furthermore, Hrs deficiency led to decreased surface expression of BCR, which was accompanied with hyporesponsiveness to Ag stimulation. The present study demonstrates that Hrs is required for peripheral development, activation and humoral immune responses of B-cells.

2. Materials and methods

2.1. Mice

$Hrs^{flox/flox}$ mice were generated as described previously [24]. To generate B-cell-specific Hrs-deficient mice, $Hrs^{flox/flox}$ mice on a C57BL/6 background were bred with $mb1^{cre/+}$ mice on a C57BL/6 background [25], and the progeny were crossed to yield homozygous knock-out (KO) ($Hrs^{flox/flox}$;$mb1^{cre/+}$:Hrs-cKO) and control ($Hrs^{+/+}$;$mb1^{cre/+}$:WT) mice, which were genotyped using the polymerase chain reaction (PCR) to amplify genomic DNA obtained from the ear. Mice were bred and maintained under specific pathogen-free conditions at the Institute for Animal Experimentation, Iwaki Meisei University. All procedures were performed according to protocols approved by the Institutional Committee for the Use and Care of Laboratory Animals of Iwaki Meisei University.

2.2. Reagents and antibodies

Goat anti-mouse $F(ab')_2$ IgM Ab and lipopolysaccharide (LPS) from *Escherichia coli* serotype O55:B5 used to stimulate B-cells were purchased from Jackson ImmunoResearch and Sigma–Aldrich, respectively. Abs used were as follows: FITC-conjugated anti-mouse CD21 (7G6), biotin-conjugated anti-mouse CD23 (B3B4) (BD PharMingen), FITC-conjugated anti-mouse IgM (RMM-1), FITC-conjugated anti-mouse CD3 (145-2C11) and biotin-conjugated anti-mouse B220 (RA3-6B2) (BioLegend), biotin-conjugated anti-mouse IgD [11–26], FITC-conjugated anti-mouse CD19 (MB19-1) (eBio Science). Anti-mouse Hrs (C-9) (Santa Cruz Biotechnology, Inc.) was used Western blotting.

2.3. Reverse transcriptase (RT)-PCR

RT-PCR was performed as described previously [24].

2.4. ELISA of serum Ig molecules

The serum levels of each Ig subclass were assayed using a quantitative ELISA as described previously [26]. In brief, diluted sera were added to a plate containing 96 wells coated with goat anti-mouse Ig Abs (Southern Biotechnology Associates) and incubated for 1 h at room temperature. After washing, bound Abs were detected by incubation with goat anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, IgG3 or IgA conjugated to alkaline phosphatase (Southern Biotechnology Associates). Colour reactions generated using alkaline phosphatase (Sigma–Aldrich) were then evaluated by determining OD_{450}. Values were within the linear range of the assay.

2.5. Proliferation assay

B-cells were purified from splenocytes using anti-B220 microbeads and magnetic-activated cell sorting (Milteny Biotec). Purified B-cells were treated with goat anti-mouse $F(ab')_2$IgM or LPS. For cell proliferation assays, 1×10^5 cells were cultured in triplicate for 48 h in 96-well flat-bottom plates and pulsed with bromodeoxyuridine (BrdU) for 2 h. Incorporated BrdU (Roche) was detected by incubation with peroxidase-conjugated anti-BrdU Ab. Colour reactions generated using peroxidase were then evaluated by determining the OD_{450}.

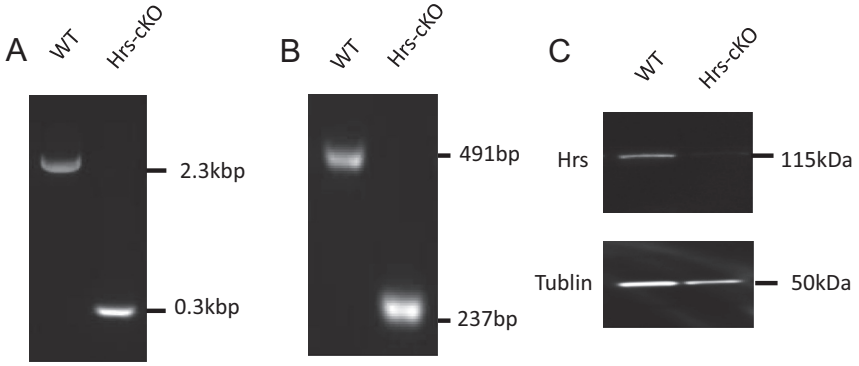

Fig. 1. Targeted disruption of *Hrs*. (A) PCR analysis of *Hrs* genomic DNA to evaluate cre-mediated deletion of the floxed gene in spleen B-cells isolated from $hrs^{+/+}$*mb1-Cre* (WT) and $hrs^{flox/flox}$ *mb1-Cre* (Hrs-cKO) mice. (B) RT-PCR analysis of total RNA from the spleen B-cells of WT and Hrs-cKO mice. (C) Western blotting of Hrs expression. Lysates from WT and Hrs-cKO spleen B-cells were separated using sodium dodecyl sulphate–polyacrylamide gel electrophoresis and blotted with an anti-Hrs antibody.

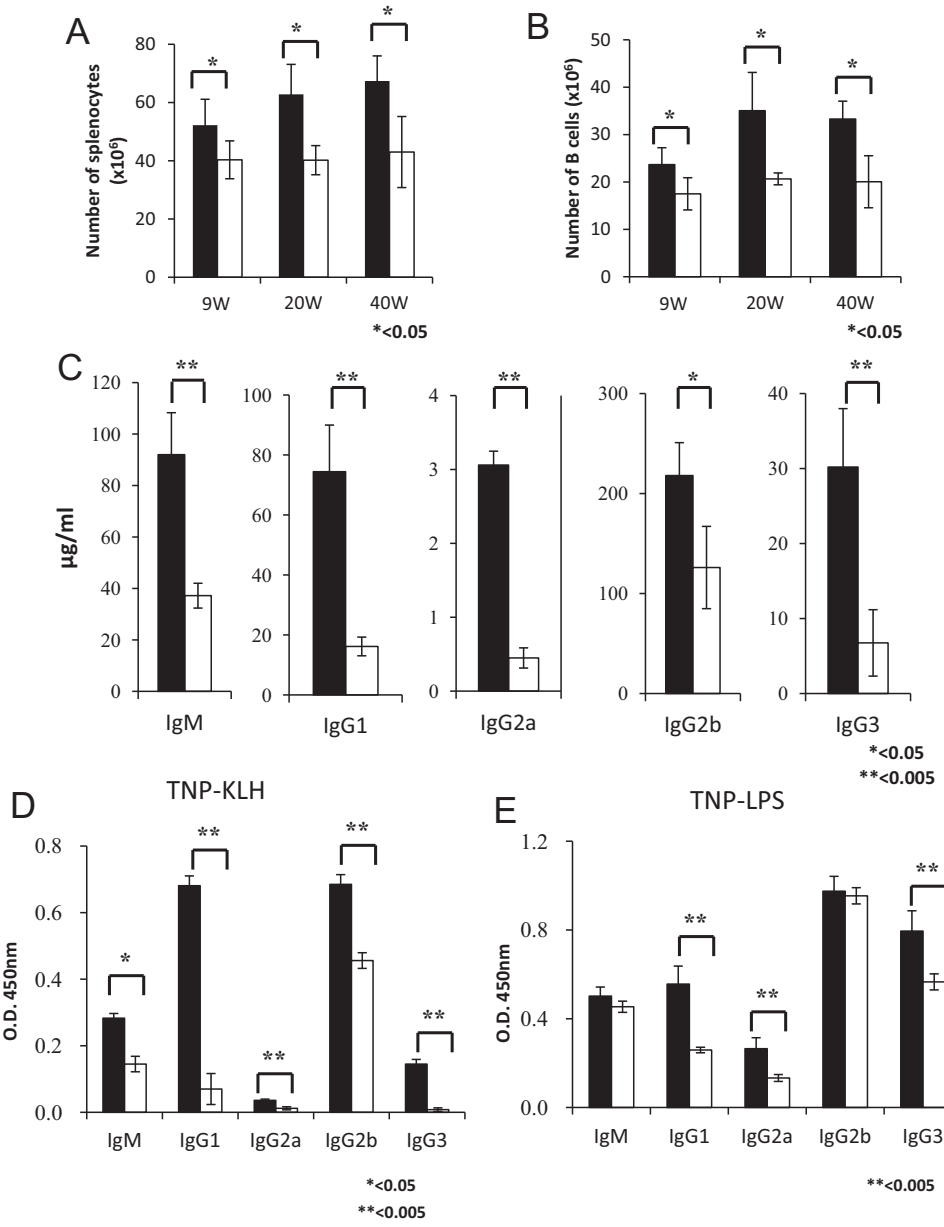

Fig. 2. Reduction in serum IgG levels and Ig production by Hrs-cKO mice. (A) and (B) Number of splenocytes and B-cells from 9-, 20- and 40-week-old WT and Hrs-cKO mice. Data are presented as the mean ± S.D. of six mice. $^{*}p < 0.05$. (C) Serum concentrations of immunoglobulins (Ig) of different Ig isotypes. Data were acquired for WT (■) and Hrs-cKO (□) mice at 8–11 weeks of age. Data are presented as the mean ± S.D. of 10–12 mice. $^{*}p < 0.05$; $^{**}p < 0.005$. (D) and (E) WT (■) and Hrs-cKO (□) mice were intraperitoneally immunized with TNP-KLH (D) or TNP-LPS (E) for 2 weeks, and then reimmunized. Sera were collected after 2 weeks and analysed using ELISAs to determine the titre of TNP-specific antibody isotypes. Data were collected from WT (■) and Hrs-cKO (□) mice 8–11 weeks of age. Data are presented as mean ± S.D. of six mice. $^{*}p < 0.05$; $^{**}p < 0.005$.

2.6. Flow cytometry

Red blood cells were extracted from bone marrow and spleen samples, and single-cell suspensions were prepared and incubated with 2.4G2 Ab for 20 min at 4 °C to block Fc receptors. The cell suspensions were then incubated with the Abs indicated above. Surface staining was analysed using a FACS CantoII Flow Cytometer (BD Biosciences) in two-colour mode using the FACS Diva software (BD Biosciences).

2.7. Western blotting

Immunoblotting was carried out as described previously [12].

2.8. Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using either paired or unpaired Students *t* tests. Statistical significance was defined as *p* values < 0.05 (*) and <0.005 (**).

3. Results

3.1. B-cell-specific disruption of Hrs

To study Hrs function in B-cells, we conditionally inactivated *Hrs* in developing B lymphocytes by breeding $Hrs^{flox/flox}$-conditional

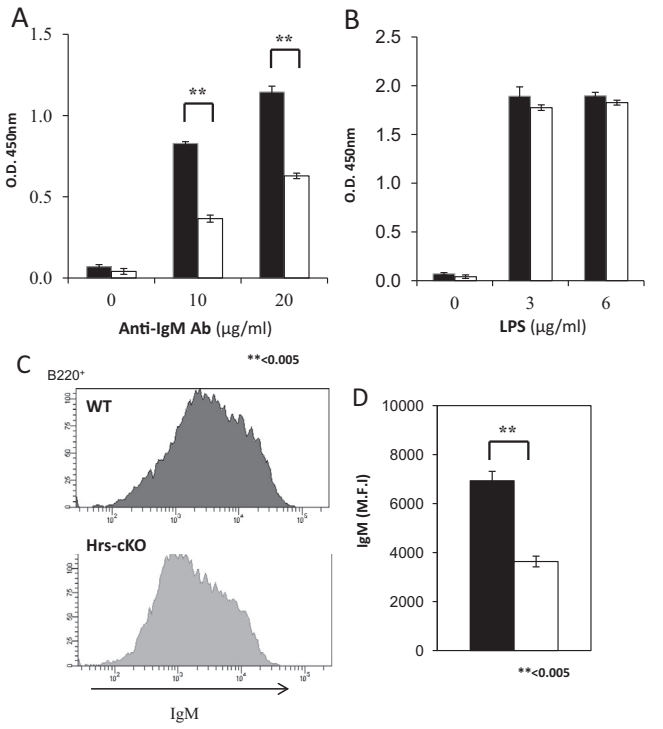

Fig. 3. Reduction of proliferative responses and surface BCR levels in *Hrs*-deficient B-cells. (A) and (B) Spleen B-cells from WT (■) and Hrs-cKO (□) were cultured for 48 h either in complete medium or in complete medium supplemented with anti-IgM (10 or 20 μg/ml) (A) and LPS (3 or 6 μg/ml) (B). Cell proliferation was measured using ELISA to detect BrdU incorporated into DNA. Data were collected from three WT (■) and Hrs-cKO (□) mice aged 8–11 weeks. Data are presented as the mean ± S.D. for three animals. Values represent three independent experiments. (C) The expression of surface IgM on B220$^+$ B-cells from WT (upper panel) and Hrs-cKO (lower panel). (D) MFI (mean fluorescence intensity) of surface IgM on B220$^+$ B-cells from WT (■) and Hrs-cKO (□). Data are presented as the mean ± S.D. for three animals.

mice [24] with *mb1*$^{cre/+}$-knock-in mice [25] because *Hrs*-deficient mice die on embryonic day 10.5 [23]. PCR analyses of spleen B-cells confirmed the cre-mediated deletion of floxed *Hrs* (Fig. 1A) as well as the deletion of *Hrs* mRNA from spleen B-cells of *Hrs*$^{flox/flox}$;-*mb1*$^{cre/+}$ (Hrs-cKO) mice (Fig. 1B). We also confirmed the conditional deletion of Hrs using Western blotting (Fig. 1C).

3.2. Hrs mediates Ab production in vivo

The Hrs-cKO mice developed normally and were fertile (data not shown). First, we examined the effects of B-cell-specific Hrs deficiency on the immune system. The total number of splenocytes and the absolute number of B-cells decreased in the absence of Hrs compared with that observed in the wild-type (WT) mice (Fig. 2A and B). Hrs-cKO mice apparently showed reduced levels of serum IgM, IgG1, IgG2a, IgG2b and IgG3 when compared with WT mice (Fig. 2C). We next examined how the loss of Hrs affected Ag-specific Ab responses by immunizing the mice with trinitrophenol (TNP)-conjugated T-independent (LPS) and T-dependent (keyhole limpet hemocyanin, KLH) Ags. The production of IgM, IgG1, IgG2a, IgG2b and IgG3 in response to TNP-KLH was reduced in Hrs-cKO mice compared with WT mice (Fig. 2D). T-cell-independent Ag production, except for IgM and IgG2b, was significantly reduced by Hrs deficiency (Fig. 2E). Taken together with the data presented in (Fig. 2), we conclude that Hrs plays a critical role in regulating Ab responses *in vivo*.

3.3. Hrs alters BCR-induced B-cell proliferation in vitro and IgM expression

To further elucidate B-cell function in the absence of Hrs, purified B-cells were stimulated with an anti-IgM Ab or LPS *in vitro*. Hrs-deficient B-cells were hypoproliferative in response to anti-IgM Ab compared with WT B-cells (Fig. 3A). However, the Hrs-deficient and WT B-cells showed comparable proliferative responses to LPS (Fig. 3B). Because Hrs deficiency influenced B-cell proliferation, we determined the surface expression of BCR in the Hrs-cKO mice. The expression of IgM on the B220$^+$ B-cells decreased in splenocytes isolated from Hrs-deficient mice compared with the WT mice (Fig. 3C and D).

3.4. Altered B-cell development in Hrs-cKO mice

To determine the effect of Hrs-deficiency on B-cell development, we performed multi-parameter flow cytometric analyses of bone marrow and spleen cells from WT and Hrs-cKO mice (Fig. 4). Analysis of bone marrow lymphocytes revealed a normal frequency and absolute numbers of immature pro- and pre-B-cells (B220^{low} IgM$^-$) and immature (B220^{low} IgM$^+$) B-cells (Fig. 4A and B). In the absence of Hrs, abnormalities of early development of B-cells in the bone marrow were not detected. To examine as to how the loss of Hrs affected peripheral B-cell maturation, we further analysed spleen B-cell populations. Immature B-cells differentiate through transitional stages (from T1 to T2) and eventually become follicular mature B-cells or MZ B-cells (17). However, Hrs-cKO mice demonstrated a marked reduction in the numbers of T1 (B220$^+$, IgM^{high} and IgD^{low}) (Fig. 4C and D), T2 B-cells (B220$^+$, IgM^{high} and IgD^{high}) (Fig. 4C and D) and MZ B-cells (B220$^+$, CD21$^+$ and CD23^{low}) (Fig. 4E and F). Taken together, these results indicate an important role for Hrs in promoting the peripheral development of B-cells.

4. Discussion

The present study demonstrates that the ESCRT-0 protein Hrs expressed by B-cells is required to regulate peripheral B-cell development and responsiveness to Ag stimulation.

Hrs is expressed by B-cells, and its deficiency leads to decreased numbers of B-cells accompanied through the impaired development of T1, T2 and MZ B-cells in the spleen. However, the early development of B-cells in the bone marrow was not detectably abnormal in the absence of Hrs; therefore, our data suggest that Hrs regulates peripheral B-cell development. Signalling through the BCR is required for normal lymphocyte development [20] and tonic BCR signalling is permissive for T2 B-cell differentiation [27]. The decreased BCR levels on mature B-cells and the reduced proliferation through BCR stimulation observed in Hrs-deficient B-cells may explain the diminished capability of these cells to differentiate into T2 B-cells. This mechanism is supported by our findings of reduced BCR levels on T1 B-cells.

These data indicate that Hrs plays a conserved role in regulating Ag receptor levels and signal strength during B-cell development. The immunoglobulin heavy-chain subunit of the BCR is ubiquitinated [15]. The BCR is constitutively ubiquitinated in resting B-cells and is then internalized, and monomeric and multimeric receptor complexes traverse a common endocytic route to late endosomes [22]. Hrs, which binds to ubiquitinated proteins, may ubiquitinate the BCR, thus contributing to its internalization.

Signal-transducing adaptor molecule (STAM) 1 and STAM2 (also known as EAST/Hbp) are involved in the vesicular transport of ubiquitinated cargo. Formation of complexes between STAMs and Hrs is essential for the formation of the functional ESCRT-0 complex. We previously reported that STAM1 and STAM2 T-cell-specific double-KO mice are severely impaired in thymic T-cell development, resulting in the complete absence of peripheral T-cells [28]. These findings suggest that the ESCRT-0 complex plays a crucial role in early T-cell development. Furthermore, T-cell-specific mice that conditionally express Hrs are deficient in T-cells (Murata and Ishii, unpublished

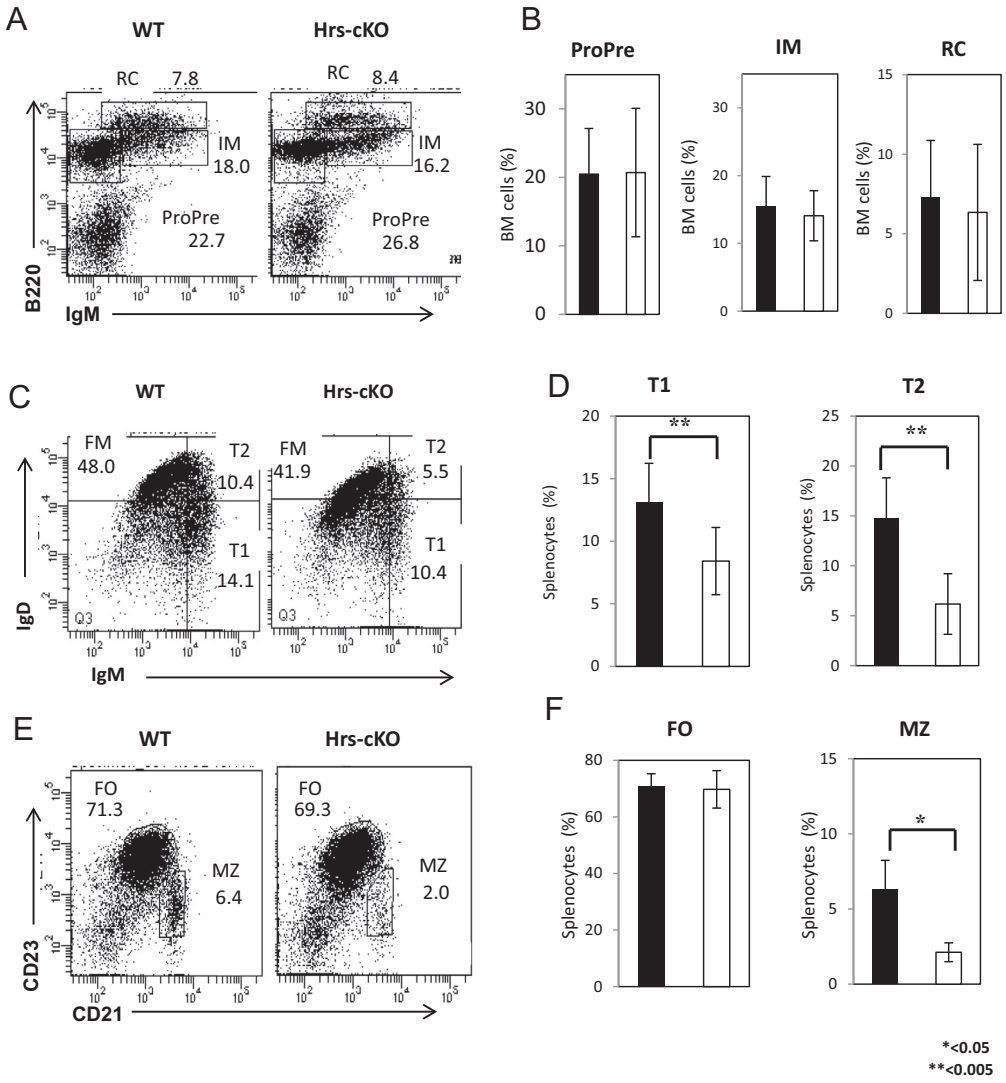

Fig. 4. Flow cytometric and statistical analyses of B-cell subsets of Hrs-cKO mice. (A) and (B) Bone marrow cells from WT and Hrs-cKO mice were stained with fluorescent anti-IgM and B220 and subjected to flow cytometry to analyse the percentage of cells during early developmental stages as follows: early (ProPre), immature (IM) and recirculating (RC) B-cells. (C) (D) and (E) (F) Splenocytes from WT and Hrs-cKO mice were stained with the indicated antibodies and analysed by flow cytometry to determine the percentage of different subpopulations of B-cells as follows: T1, transitional 1 ($IgM^{hi}IgD^{lo}$), T2, transitional 2 ($IgM^{hi}IgD^{hi}$), MZB, marginal zone ($CD21^{hi}CD23^{lo}$) and FO, follicular ($CD21^{int}CD23^{hi}$). Data were acquired for WT and Hrs-cKO mice at 8–10 weeks of age. Data are presented as mean ± the S.D. of seven mice.

results). Therefore, we postulate that Hrs plays an important role in early B-cell development. However, the present results demonstrate normal early development of Hrs-cKO B-cells, suggesting distinct roles for the ESCRT complex that depend on the activities of Hrs and STAMs during the development of T- and B-cells. Further research is required to support this possibility.

This study demonstrates, for the first time, that the ESCRT complex is critically involved in B-cell development and function. Because ESCRT-mediated protein trafficking plays important roles in immunoreceptor internalization and degradation by lysosomes, and possibly in antigen processing and presentation, it is important to establish these and other roles of the ESCRT complex in the immune system.

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