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Secretory IgM exacerbates tumor progression by inducing accumulations of MDSCs in mice

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Abstract

Chronic lymphocytic leukemia (CLL) cells can secrete immunoglobulin M. However, it is not clear whether secretory IgM (sIgM) plays a role in disease progression. We crossed the E μ -TCL1 mouse model of CLL, in which the expression of human TCL1 oncogene was driven by the V(H) promoter-Ig(H)-E μ enhancer, with MD4 mice whose B cells produced B cell receptor (membrane-bound IgM) and sIgM with specificity for hen egg lysozyme (HEL). CLL cells that developed in these MD4/E μ -TCL1 mice reactivated a parental Ig gene allele and secreted IgM, and did not recognize HEL. The MD4/E μ -TCL1 mice had reduced survival, increased myeloid-derived suppressor cells (MDSCs), and decreased numbers of T cells. We tested whether sIgM could contribute to the accumulation of MDSCs by crossing μ S^{-/-} mice, which could not produce sIgM, with E μ -TCL1 mice. The μ S^{-/-}/E μ -TCL1 mice survived longer than E μ -TCL1 mice and developed decreased numbers of MDSCs which were less able to suppress proliferation of T cells. We targeted the synthesis of sIgM by deleting the function of XBP-1s and showed that targeting XBP-1s genetically or pharmacologically could lead to decreased sIgM, accompanied by decreased numbers and reduced functions of MDSCs in MD4/E μ -TCL1 mice. Additionally, MDSCs from μ S^{-/-} mice grafted with Lewis lung carcinoma were inefficient suppressors of T cells, resulting in slower tumor growth. These results demonstrate that sIgM produced by B cells can upregulate the functions of MDSCs in tumor-bearing mice to aggravate cancer progression.

Keywords

endoplasmic reticulum; XBP-1; B lymphocyte; CLL; myeloid-derived suppressor cells

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Introduction

Chronic lymphocytic leukemia (CLL) cells use restricted immunoglobulin variable heavy and light chain genes to manufacture B cell receptors (BCRs) (1,2), suggesting their need for BCR signaling through binding to common antigens. CLL cells carrying such BCRs can respond to *in vitro* anti-IgM stimulation by robustly activating BCR signaling (3,4). BCR signaling supports CLL survival. Therapies that target BCR signaling molecules, such as spleen tyrosine kinase (Syk) or Bruton's tyrosine kinase (BTK), have proven useful in the control of human and mouse CLL (5–7).

The proto-oncoprotein TCL1 is expressed in 90% of human CLL patients (8,9). Clinically, TCL1 overexpression is associated with constitutive BCR signaling, which allows CLL cells to proliferate rapidly (8,10). To reproduce this phenomenon in a transgenic mouse model, E μ -TCL1 mice were established, in which the expression of human TCL1 is driven by an immunoglobulin heavy chain promoter/enhancer, E μ (11). These mice develop CD19⁺/IgM⁺/B220^{low}/CD5⁺ CLL cells in the blood, spleens, lymph nodes, and bone marrow, and progress to full-blown monoclonal CLL with all clinical features of aggressive human CLL (11,12). CLL progresses more slowly in E μ -TCL1/IgHEL mice in which E μ -TCL1 B cells also express the MD4 transgene that encodes a monoclonal BCR against hen egg lysozyme (HEL) (13). The MD4 transgene allows E μ -TCL1 B cells to produce not only HEL-reactive monoclonal BCR but also secretory IgM (sIgM). The role of sIgM in the progression of CLL remains unclear.

Solid tumor growth decelerates in C57BL/6 $\times$ C3H F1 mice in which B cells are depleted (14). Similarly, when comparing SCID mice reconstituted with T cells or with both T and B cells, tumors grow slower in and are rejected more frequently by mice lacking B cells (15). Mice carrying a deletion of an exon of the IgM heavy chain gene are incapable of producing B cells (16). When these mice lacking B cells were implanted with EL4 thymoma, MC38 colon carcinoma or B16 melanoma, slower growth of all three tumors were observed (17). By crossing the squamous cell carcinoma mouse model (K14-HPV16) with RAG-1^{-/-} mice lacking mature B and T cells, the growth of skin cancer is significantly slowed in HPV16/RAG-1^{-/-} mice. Transfer of B cells or serum from HPV16 mice into HPV16/RAG-1^{-/-} mice restores skin cancer growth (18). Although B cells do not infiltrate premalignant HPV16 skin (18), IgG engages IgG receptors (Fc γ Rs) on mast cells and macrophages to promote squamous carcinogenesis (19). Although dendritic cells and myeloid-derived suppressor cells (MDSCs) express Fc γ Rs, they do not exhibit immunosuppressive effects in this skin cancer model (19). Thus, although B cells can mediate immunosuppression, it is unknown whether Ig can orchestrate an immunosuppressive microenvironment by recruiting MDSCs into different tumor models.

MDSCs are pathologically activated immunosuppressive myeloid cells (20,21). Monocytic MDSCs (M-MDSCs) are morphologically and phenotypically similar to monocytes. Granulocytic MDSCs (G-MDSCs), also known as polymorphonuclear MDSC (PMN-MDSC), are morphologically and phenotypically similar to neutrophils. In mice, M-MDSCs and G-MDSCs are CD11b⁺/Ly6C⁺/Ly6G⁻ and CD11b⁺/Ly6C^{low}/Ly6G⁺ populations, respectively. MDSC-mediated immunosuppressive effects are localization-dependent (22).

Evidence supports an association between MDSC accumulation and clinical outcomes in human patients with various types of cancer (23), including CLL (24). Although MDSCs can suppress the functions of immune cells, data in two studies suggest that MDSCs can be regulated by tumor-associated B cells (25) or CLL cells (26). It is unclear whether sIgM produced by B cells or CLL cells can contribute to the accumulation of MDSCs in tumor models. Here, we establish that sIgM upregulates MDSCs to promote tumor growth.

Materials and Methods

Mice and study approval

E μ -TCL1^{+/+}, MD4^{+/-}, MD4^{+/-}/E μ -TCL1^{+/+}, μ S^{-/-}, μ S^{-/-}/E μ -TCL1^{+/+}, XBP-1^{f/f}/MD4^{+/-}/E μ -TCL1^{+/+}, and CD19Cre/XBP-1^{f/f}/MD4^{+/-}/E μ -TCL1^{+/+} mice were maintained at our animal facility following guidelines provided by the Wistar Institute Committee on Animal Care. All strains carrying E μ -TCL1^{+/+} had been backcrossed to the B6C3 background for more than 10 generations. All experiments involving the use of mice were performed following protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Wistar Institute.

Flow cytometric analysis and gating strategies to analyze granulocytic and monocytic MDSCs

Single cell suspensions from spleens, bone marrow or peripheral lymph nodes were blocked for 30 minutes using FBS. Cell surface staining was achieved by incubating cells at 4°C for 30 minutes with the following anti-mouse antibodies: CD3-APC-Cy7 (145-2C11; Biolegend); IgM-PE-Cy7 (RMM-1; Biolegend); B220-FITC (RA3-6B2; Biolegend); CD5-APC (53-7.3; e-Bioscience); CD11c-BV421 (N418; Biolegend); CD11b-PE (M1/70; Biolegend); Ly6C-Alexa-488 (HK1.4; Biolegend); Ly6G-Alexa-647 (1A8; Biolegend); CD4-BV605 (RM4-5; Biolegend); CD8 α -PE-Cy7 (53-6.7; Biolegend); CD45-PE (30-F11; BD Biosciences); CD11b-BV605 (M1/70; Biolegend); and Arg-1-PE (pAB; R&D). Viability staining was accomplished using DAPI exclusion during acquisition. Acquisition of cell populations was performed on a LSRII cytometer (BD Biosciences) harboring a custom configuration for the Wistar Institute. Cytometry data was analyzed using FlowJo software version 7.6.1 (Tree Star Inc.). To examine the presence of MDSCs in lymphoid organs and tissues, we treated mouse blood cells, splenocytes and bone marrow cells with RBC lysis buffer, stained cells with CD11c-BV421, CD11b-PE, Ly6C-Alexa 488 and Ly6G-Alexa 647, and gated CD11c⁻/CD11b⁺ populations to analyze for Ly6C^{intermediate}/Ly6G⁺ granulocytic and Ly6C⁺/Ly6G⁻ monocytic populations. To examine the presence of MDSCs in tumors, tumors dissected from wild-type and μ S^{-/-} mice were cut into pieces, digested with the mouse tumor dissociation kit, and stained with CD45-PE, CD11b-BV605, Ly6C-Alexa-488 and Ly6G-Alexa-647. Gated CD45⁺ hematopoietic cells were further gated for CD11b⁺ myeloid populations, which were then analyzed for Ly6C^{intermediate}/Ly6G⁺ granulocytic MDSCs and Ly6C⁺/Ly6G⁻ monocytic MDSCs.

Antibodies and reagents

Polyclonal antibodies against Ig α , Derlin-1, Derlin-2, BiP, and PDI were generated in rabbits. Antibodies against TCL1 (Cell Signaling), IRE-1 (Cell Signaling), XBP-1 (Cell

Signaling), Syk (Cell Signaling), phospho-Syk (Tyr525/526) (Cell Signaling), AKT (Cell Signaling), phospho-AKT (Ser473) (Invitrogen), ERK1/2 (Cell Signaling), phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling), GRP94 (Stressgen), p97 (Fitzgerald), actin (Sigma), phospho-Igα (Tyr182) (Cell Signaling), mouse μ (SouthernBiotech), human μ (SouthernBiotech), and phosphotyrosine (4G10; Millipore) were obtained commercially. Anti-μ Fab and F(ab')₂ were purchased from SouthernBiotech. LPS (Sigma), CpG-1826 oligodeoxynucleotides (TIB-Molbiol), CFSE (Biolegend), and Ultra-LEAFTM purified anti-mouse CD3ε (145-2C11) and anti-mouse CD28 (37.51) antibodies were also obtained commercially. We developed and chemically synthesized the IRE-1 RNase inhibitor, B-I09 (27).

Crosslinking of hen egg lysozyme (HEL) and purification of oligomeric HEL

Hen egg lysozyme (Sigma) was dissolved in PBS (pH 7.4), cross-linked with glutaraldehyde (Fisher) for 30 min at room temperature, and quenched with 1 M glycine. The insoluble precipitates were removed by centrifugation. Soluble proteins in the supernatant were precipitated by the addition of ammonium sulfate, and the precipitate was dissolved in a buffer containing 50 mM Tris-HCl (pH 7.4) and 8 M urea. The HEL monomers, dimers and oligomers were then separated on a Superdex 75 preparation column (GE Healthcare) equilibrated with 50 mM Tris-HCl (pH 7.4), 5 M urea and 300 mM NaCl. Monomeric, dimeric, and oligomeric HEL conjugates were pooled, dialyzed against PBS, and analyzed by SDS-PAGE followed by Coomassie blue staining.

Purification of mouse B cells, CLL cells and MDSCs

Splenocytes were obtained from mice by mashing the spleens through cell strainers followed by RBC lysis (Sigma). Mouse B cells and CLL cells were purified from mouse spleens by negative selection using CD43 (Ly48) or Pan-B magnetic beads (Miltenyi Biotech), respectively, following the manufacturer's instructions. MDSCs were purified from spleens, bone marrow or LLC tumors (digested with the mouse tumor dissociation kit purchased from Miltenyi Biotech) by positive selection using myeloid-derived suppressor cell isolation kit (Miltenyi Biotech).

Cell culture

Purified mouse B cells or CLL cells were cultured in the RPMI 1640 media (Corning) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma), 2 mM L-glutamine, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 0.1 mM β-mercaptoethanol (β-ME). Lewis lung carcinoma (LLC) cells were obtained from ATCC, cultured in DMEM (Corning Incorporated) supplemented with 10% FBS, and regularly tested negative for mycoplasma. Cells were incubated in a 37°C and 5% CO₂ incubator. 70–80% confluent cells were harvested using 0.25% Trypsin (Thermo Fisher Scientific Inc.) and passaged or used for experiments.

BCR activation

Mouse MD4^{+/-}, MD4^{+/-}/E μ -TCL1^{+/+}, and E μ -TCL1^{+/+} B cells were suspended in RPMI serum-free media supplemented with 25 mM Hepes; stimulated with Fab or F(ab')₂ fragments of the goat anti-mouse IgM antibody (20 μ g/mL) (SouthernBiotech) or with monomeric, dimeric or oligomeric HEL (5 μ g/mL) for indicated times; and lysed immediately by adding ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% Triton X-100; 1 mM EDTA) supplemented with protease inhibitor cocktail (Roche), 4 mM sodium pyrophosphate, 2 mM sodium vanadate and 10 mM sodium fluoride. The lysates were analyzed by SDS-PAGE and immunoblotted for molecules of interest using specific antibodies.

Protein isolation and immunoblotting

Cells were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM EDTA) supplemented with protease inhibitors (Roche) and phosphatase inhibitors. Protein concentrations were determined by BCA assays (Pierce). Proteins were boiled in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.1% bromophenol blue) with β -ME, analyzed by SDS-PAGE, and transferred to nitrocellulose membranes, which were subsequently blocked in 5% non-fat milk (wt/vol in PBS), and immunoblotted with indicated primary antibodies and appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoblots were developed using Western Lighting Chemiluminescence Reagent (Perkin-Elmer).

Tumor bearing mice and treatment with an antibody to mouse CD8 α

LLC cells were harvested, suspended in DPBS (Corning) as 200 μ L containing 5×10^5 cells, and then injected subcutaneously into wild-type and μ S^{-/-} mice. The in vivo anti-mouse CD8 α monoclonal antibody (53-6.7, Rat IgG2a) and isotype control monoclonal antibody (2A3, Rat IgG2a) were procured from BioXCell. Two days before LLC cells were subcutaneously injected on day 0, eight-week-old wild-type and μ S^{-/-} mice were injected intraperitoneally with the anti-mouse CD8 α monoclonal antibody (100 μ g per mouse) or the isotype control monoclonal antibody (100 μ g per mouse). These mice received 5 subsequent antibody injections on days 1, 5, 8, 12, and 15. The size of tumor was measured and recorded starting from day 11 to day 22, and data were plotted as means $\pm$ SEM.

Patient Samples

Primary human CLL cells were obtained by Dr. Mato from patients at the Abramson Cancer Center of the University of Pennsylvania following the approved IRB guidelines from the University of Pennsylvania and the Wistar Institute, with informed consent in accordance with the Declaration of Helsinki. All patients signed a written consent form.

Pulse chase experiments and immunoprecipitation

Human CLL cells were starved in methionine- and cysteine-free media containing dialyzed fetal bovine serum for 1 h, and pulse-labeled with 250 μ Ci/ml [³⁵S]-methionine and [³⁵S]-cysteine (Perkin-Elmer) for indicated times. After labeling, cells were incubated in the chase medium containing unlabeled methionine (2.5 mM) and cysteine (0.5 mM). At the end of

each chase interval, cells were lysed in RIPA buffer containing protease inhibitors. Pre-cleared lysates were incubated with an anti-human Ig μ heavy chain antibody (SouthernBiotech), together with Protein G-sepharose beads. Immunoprecipitates were boiled in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.1% bromophenol blue) with β -ME, analyzed by SDS-PAGE and visualized by autoradiography.

Enzyme-linked immunosorbent assay (ELISA)

ELISA analyses of IgM (captured by goat anti-mouse μ chain IgG) or anti-HEL IgM (captured by HEL) in mouse sera were achieved using an HRP-conjugated antibody to mouse IgM (SouthernBiotech) and 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate system (Sigma).

MDSC-mediated T-cell suppression assay

MDSCs were purified from the spleens, bone marrow, or tumors. The purity of cell populations was >95%. The PMEL-1 and OT-I responder mice have CD8⁺ T cells which recognize gp100-derived and OVA-derived peptides, respectively. Splenocytes from PMEL-1 or OT-I mice were mixed with splenocytes from naïve mice at the 1:4 ratio in the complete RPMI media, and then plated into 96-well U-bottom plates at 10^5 cells per well. CD11b⁺/Ly6G⁺ or CD11b⁺/Ly6C⁺ MDSCs were added to the wells at 0.25, 0.5 or 1×10^5 cells per well. The murine gp100 peptide (amino acids 25~33), EGSRNQDWL (AnaSpec), or OVA peptide (amino acids 257~264), SIINFEKL (AnaSpec) was dissolved in pure water, diluted with the RPMI complete media, and then added into the wells at the final concentration of 0.1 μ g/mL. After incubation for 48 h, cells were radiolabeled with ³H-thymidine (1 μ Ci per well; GE Healthcare) for 6 h. The uptake of ³H-thymidine was measured as counts per minute (CPM) using a liquid scintillation counter. The percentage of proliferation in comparison to positive controls (the wells with responder cells and the corresponding peptide) was calculated.

Statistics

The Kaplan-Meier analysis was used to evaluate mouse survival data. For comparison of percentages of cell populations among experimental groups, data were graphed as means $\pm$ SEM and analyzed by unpaired two-tailed Student's *t* test. A *p* value of <0.05 was considered significant.

Results

CLL cells developed in MD4^{+/-}/E μ -TCL1^{+/-} mice fail to recognize HEL

To investigate the role of a monoclonal BCR in the progression of CLL in mice, we generated MD4/E μ -TCL1 mice by crossing E μ -TCL1^{+/-} mice, which spontaneously develop CLL (11), with MD4^{+/-} transgenic mice, which produce a monoclonal BCR against HEL (28). To maintain consistent numbers of BCR and equal doses of TCL1 transgene, MD4^{+/-}/E μ -TCL1^{+/-} mice were crossed with E μ -TCL1^{+/-} mice since the establishment of this colony. To enumerate HEL-positive B or CLL cells, we conjugated HEL with Alexa-568 for cell surface staining. We stained splenocytes harvested from MD4^{+/-}/E μ -TCL1^{+/-} mice of

different age groups with CD3-APC-Cy7, IgM-PE-Cy7, B220-FITC, CD5-APC and HEL-Alexa-568. The IgM⁺ cells were gated to analyze for B220^{hi}/CD5⁻ precancerous B cells and B220^{lo}/CD5⁺ CLL cells (Fig. 1A). MD4^{+/-}/Eμ-TCL1^{+/+} mice developed CLL with enlarged spleens (Fig. 1A–B), and all IgM⁺/B220^{lo}/CD5⁺ CLL cells developed in older MD4^{+/-}/Eμ-TCL1^{+/+} mice failed to recognize HEL (Fig. 1A).

MD4^{+/-}/Eμ-TCL1^{+/+} B cells and CLL cells respond to BCR crosslinking with BCR signaling

To activate the BCR via antigen-binding sites instead of constant regions, we generated dimeric and oligomeric HEL by chemically crosslinking HEL using glutaraldehyde (Supplementary Fig. S1A), and separated monomeric, dimeric and oligomeric HEL by size exclusion column chromatography (Supplementary Fig. S1 B–D). When Eμ-TCL1^{+/+} and MD4^{+/-}/Eμ-TCL1^{+/+} B cells were exposed to goat Fab or F(ab')₂ anti-mouse IgM (used as negative or positive controls, respectively), monomeric, dimeric or oligomeric HEL for 2 min, only MD4^{+/-}/Eμ-TCL1^{+/+} B cells responded to HEL by activating BCR signaling, as shown by increased tyrosine phosphorylation of BCR signaling molecules such as Syk (Supplementary Fig. S2). Dimeric or oligomeric HEL was more effective than monomeric HEL in activating BCR signaling in MD4^{+/-}/Eμ-TCL1^{+/+} B cells (Supplementary Fig. S2B). MD4^{+/-} and MD4^{+/-}/Eμ-TCL1^{+/+} B cells responded to oligomeric HEL within 30 sec, reached the maximal response at 2 min, and began to downregulate the BCR signaling at 5 min (Supplementary Fig. S3). Activating the same cells via crosslinking the constant regions of the BCR using goat anti-mouse IgM F(ab')₂ resulted in slower but more persistent BCR signaling, as shown by phosphorylated Igα, Syk, AKT and ERK (Supplementary Fig. S3). Thus, activation of the BCR by antigen-binding sites or by constant regions produces different responses.

BCR signaling promotes CLL survival. To evaluate BCR signaling in precancerous B cells developed in MD4^{+/-}/Eμ-TCL1^{+/+} mice, we isolated and stimulated IgM⁺/B220^{hi}/CD5⁻ B cells from spleens of 6-week-old and 3-month-old MD4^{+/-} and MD4^{+/-}/Eμ-TCL1^{+/+} mice with oligomeric HEL for 30 min. We found that precancerous B cells from MD4^{+/-}/Eμ-TCL1^{+/+} mice were more responsive to oligomeric HEL stimulation than were B cells from age-matched MD4^{+/-} counterparts (Fig. 2A–B). Since IgM⁺/B220^{lo}/CD5⁺ CLL cells developed in MD4^{+/-}/Eμ-TCL1^{+/+} mice did not recognize HEL (Fig. 1A), we examined whether these CLL cells still expressed the anti-HEL IgM encoded by the MD4 transgene. We found that IgM⁺/B220^{lo}/CD5⁺ CLL cells in MD4^{+/-}/Eμ-TCL1^{+/+} consistently shut down the expression of the MD4 transgene and reactivated the non-MD4 Ig gene allele to express both membrane-bound and secretory IgM. When these CLL cells were stimulated with goat anti-mouse IgM F(ab')₂, they responded by eliciting stronger phosphorylation of Igα, Syk and ERK1/2 than precancerous B cells isolated from 6-week-old MD4^{+/-}/Eμ-TCL1^{+/+} mice (Fig. 2C). In addition, when IgM⁺/B220^{lo}/CD5⁺ CLL cells isolated from MD4^{+/-}/Eμ-TCL1^{+/+} mice were stimulated with lipopolysaccharide (LPS, a TLR4 ligand) or CpG-1826 (a TLR9 ligand), they began to produce large quantities of secretory IgM (sIgM) encoded from the non-MD4 Ig gene allele. Reactivation of the non-MD4 transgene Ig allele did not occur in B cells isolated from MD4^{+/+} mice at any age group or in precancerous B cells isolated from young MD4^{+/-}/Eμ-TCL1^{+/+} mice (Fig. 2D). The abundant production of sIgM

was accompanied by the enhanced endoplasmic reticulum (ER) stress response, as shown by activation of the IRE-1/XBP-1 pathway and increased expression levels of BiP (Fig. 2D).

Accumulation of CD11b⁺/Ly6G⁺ granulocytic cells in Eμ-TCL1^{+/+} and MD4^{+/-}/Eμ-TCL1^{+/+} mice

Our blood count analyses of approximately 6-month old MD4^{+/-}/Eμ-TCL1^{+/+} and Eμ-TCL1^{+/+} mice showed that CLL developed in both mouse models (Fig. 3A). We also observed significantly increased numbers of granulocytic cells in the blood of MD4^{+/-}/Eμ-TCL1^{+/+} mice (Fig. 3B). Although there was an increase in the mean of monocytic cells in MD4^{+/-}/Eμ-TCL1^{+/+} mice, the data were not statistically significant (Fig. 3C). We next stained peripheral blood cells from age-matched Eμ-TCL1^{+/+} and MD4^{+/-}/Eμ-TCL1^{+/+} mice with CD11c-BV421, CD11b-PE, Ly6C-Alexa-488 and Ly6G-Alexa-647, and gated CD11b⁺ myeloid populations to analyze for Ly6C⁺ monocytic and Ly6G⁺ granulocytic populations (Fig. 3D). We discovered significantly higher percentages of CD11b⁺ myeloid populations and CD11b⁺/Ly6G⁺ granulocytic cells in the peripheral blood of MD4^{+/-}/Eμ-TCL1^{+/+} mice than those in Eμ-TCL1^{+/+} mice in age-matched 4-month-old (Fig. 3D), 6-month-old (Fig. 3D–G) and 8-month-old (Fig. 3H–J) groups. CD11b⁺/Ly6G⁺ granulocytic cells reached the highest percentages in the blood of MD4^{+/-}/Eμ-TCL1^{+/+} mice at the age of approximately 6 months (Fig. 3D–G).

Since 6-month-old wild-type and MD4^{+/-} mice did not accumulate CD11b⁺/Ly6G⁺ granulocytic cells in the peripheral blood (Supplementary Fig. S4), we hypothesized that such phenotypes found in Eμ-TCL1^{+/+} and MD4^{+/-}/Eμ-TCL1^{+/+} mice were associated with CLL progression driven by the 14-kDa TCL1 proto-oncoprotein. The introduction of the MD4 transgene in Eμ-TCL1^{+/+} mice forced precancerous B cells in MD4^{+/-}/Eμ-TCL1^{+/+} mice to produce not only HEL-reactive BCR on the B cell surface (Figs. 1A, 2A, 2B, Supplementary Figs. S2B and S3B) but also sIgM against HEL in mouse sera (Supplementary Fig. S5A). Although CLL cells developed in MD4^{+/-}/Eμ-TCL1^{+/+} mice were no longer HEL-reactive (Figs. 1A, 2C and 2D), they still produced large quantities of sIgM (Fig. 2D). Because of the data showing that CLL cells from Eμ-TCL1^{+/+} mice also produced large quantities of sIgM and increased levels of ER chaperones and ER-associated misfolded protein degradation machineries (Supplementary Fig. S5B) (9), we hypothesized that sIgM might induce CD11b⁺/Ly6G⁺ granulocytic cells to accumulate in Eμ-TCL1^{+/+} and MD4^{+/-}/Eμ-TCL1^{+/+} mice. To establish the relevance of sIgM in human CLL, we performed pulse-chase experiments using fresh CLL cells from human patients, and immunoprecipitated IgM from cell lysates and culture media using an antibody to human Ig μ chain. Human CLL cells from patients could secrete IgM (Supplementary Fig. S5 C–D).

Secretory IgM drives accumulation of CD11b⁺/Ly6G⁺ granulocytic cells in Eμ-TCL1^{+/+} mice

To examine the role of sIgM in inducing the accumulation of CD11b⁺/Ly6G⁺ granulocytic cells in Eμ-TCL1^{+/+} mice, we crossed Eμ-TCL1^{+/+} mice with μS^{-/-} mice (29), in which the Ig μ chain gene allele was genetically manipulated to allow for the expression of membrane-bound IgM but not sIgM. When B cells purified from 6-week-old μS^{-/-}, μS^{-/-}/Eμ-TCL1^{+/+}, and Eμ-TCL1^{+/+} mice were stimulated with LPS, those from μS^{-/-} and μS^{-/-}/Eμ-TCL1^{+/+} did not produce sIgM (Supplementary Fig. S5E). In addition, μS^{-/-}/Eμ-TCL1^{+/+} B cells

produced more membrane-bound IgM and ER chaperones than did $\mu S^{-/-}$ B cells (Supplementary Fig. S5E). Compared with $E\mu$ -TCL1^{+/+} mice, $\mu S^{-/-}/E\mu$ -TCL1^{+/+} mice did not produce sIgM in the sera (Fig. 4A). IgM⁺/CD5⁺ CLL cells purified from $\mu S^{-/-}/E\mu$ -TCL1^{+/+} mice also did not respond to LPS by producing sIgM (Fig. 4B). When compared with age-matched 4-month-old and 6-month-old $E\mu$ -TCL1^{+/+} mice, in the peripheral blood of $\mu S^{-/-}/E\mu$ -TCL1^{+/+} mice, CD11b⁺ myeloid cells and CD11b⁺/Ly6G⁺ granulocytic cells but not CD11b⁺/Ly6C⁺ monocytic cells were decreased (Fig. 4C–F). The $\mu S^{-/-}/E\mu$ -TCL1^{+/+} mice survived longer than $E\mu$ -TCL1^{+/+} mice, whereas MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice were more short-lived than $E\mu$ -TCL1^{+/+} mice (Fig. 4G). Such differences in survival were observed in both sexes (Fig. 4H–I).

CD11b⁺/Ly6G⁺ cells from spleens of MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice suppress T-cell proliferation

CLL cells proliferate and survive via interactions with other types of immune cells in secondary lymphoid organs. We hypothesized that the accumulations of CD11b⁺/Ly6G⁺ granulocytic cells in spleens of MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice might play a role in suppressing the antitumor T-cell function, leading to decreased numbers of CD3⁺ T cells in spleens of CLL-bearing MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice (Fig. 1A). The spleens of 6-month-old wild-type, MD4^{+/+} and $\mu S^{-/-}$ mice contained only low percentages of CD11b⁺/Ly6G⁺ granulocytic cells and CD11b⁺/Ly6C⁺ monocytic cells (Supplementary Fig. S4). When we examined the granulocytic cells and monocytic cells in spleens of 6-month-old MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice, we found significantly higher percentages of these cells in MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice than in age-matched MD4^{+/+} (Supplementary Fig. S4) and $E\mu$ -TCL1^{+/+} mice (Fig. 5A–D). Deleting the ability of B cells to produce sIgM in $E\mu$ -TCL1^{+/+} mice reduced numbers of granulocytic cells but not monocytic cells in spleens of 6-month-old $\mu S^{-/-}/E\mu$ -TCL1^{+/+} mice (Fig. 5A–D) and also 6-week-old $\mu S^{-/-}/E\mu$ -TCL1^{+/+} mice (Supplementary Fig. S6 A–B). In the bone marrow, we found significantly higher percentages of granulocytic cells in 6-month-old MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice than those in age-matched $E\mu$ -TCL1^{+/+} mice, and significantly lower percentages of both granulocytic and monocytic cells in 6-month-old $\mu S^{-/-}/E\mu$ -TCL1^{+/+} mice than those in age-matched $E\mu$ -TCL1^{+/+} mice (Supplementary Fig. S6 C–F). Although there was no difference of granulocytic and monocytic cells in the bone marrow between 6-week-old $\mu S^{-/-}/E\mu$ -TCL1^{+/+} mice and $E\mu$ -TCL1^{+/+} mice, both populations increased significantly in the bone marrow of 6-week-old MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice (Supplementary Fig. S6 G–H).

We next tested whether CD11b⁺/Ly6G⁺ granulocytic cells could suppress proliferation of T cells, thus functionally qualifying these cells as MDSCs. It is documented that tumor-associated MDSCs are capable of inhibiting T-cell function. CLL cells proliferate in the spleens and circulate in the peripheral blood. We thus purified CD11b⁺/Ly6G⁺ granulocytic cells from spleens and peripheral blood of MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice (Supplementary Fig. S7A). CD11b⁺/Ly6G⁺ granulocytic cells purified from peripheral blood of MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice did not suppress gp100-loaded class I MHC-mediated proliferation of CD8⁺ T cells from PMEL-1 mice (Supplementary Fig. S7B). CD11b⁺/Ly6G⁺ granulocytic cells purified from spleens of MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice could suppress CD3/CD28-stimulated proliferation of CFSE-stained CD8⁺ T lymphocytes (Fig. 5E). We also demonstrated that both CD11b⁺/Ly6G⁺ granulocytic cells and CD11b⁺/Ly6C⁺ monocytic cells purified from

spleens of MD4^{+/-}/Eμ-TCL1^{+/+} mice could suppress gp100-loaded class I MHC-mediated proliferation of CD8⁺ T cells from PMEL-1 mice (Fig. 5F–G). In addition, when compared with CD11b⁺/Ly6G⁺ granulocytic cells purified from spleens of 8-month-old Eμ-TCL1^{+/+} mice, those cells from spleens of age-matched μS^{-/-}/Eμ-TCL1^{+/+} mice were less capable of suppressing proliferation of CD8⁺ T cells (Fig. 5H), suggesting that sIgM could mediate immunosuppressive functions of MDSCs.

Targeting XBP-1s reduces sIgM as well as the numbers and functions of CD11b⁺/Ly6G⁺ MDSCs

XBP-1-deficient B cells and CLL cells produce less sIgM (27,30). Activation of regulated IRE-1-dependent decay cleaves and degrades μS mRNA (31,32). To test whether deleting the XBP-1 gene from MD4^{+/-}/Eμ-TCL1^{+/+} CLL cells could reduce the production of sIgM and lead to decreased numbers of MDSCs in MD4^{+/-}/Eμ-TCL1^{+/+} mice, we crossed B cell-specific XBP-1^{KO} mice with MD4^{+/-}/Eμ-TCL1^{+/+} mice. Compared with MD4^{+/-}/Eμ-TCL1^{+/+} mice, XBP-1^{KO}/MD4^{+/-}/Eμ-TCL1^{+/+} mice produced less serum IgM (Fig. 6A). As a result, we detected significantly decreased percentages of CD11b⁺ myeloid cells and CD11b⁺/Ly6G⁺ granulocytic MDSCs in the peripheral blood of XBP-1^{KO}/MD4^{+/-}/Eμ-TCL1^{+/+} mice (Fig. 6B–D). When compared with granulocytic MDSCs in MD4^{+/-}/Eμ-TCL1^{+/+} mice bearing similar burden of CLL, such MDSCs in XBP-1^{KO}/MD4^{+/-}/Eμ-TCL1^{+/+} mice expressed significantly decreased levels of arginase-1 (Arg-1) and were less able to suppress T-cell proliferation (Fig. 6E–G).

We have shown previously that a small-molecule inhibitor, B-I09, can inhibit the expression of XBP-1s in CLL cells and retard CLL growth in Eμ-TCL1^{+/+} mice (27). Similar to XBP-1-deficient B cells, B-I09-treated B cells are inefficient in producing sIgM (27). We thus hypothesized that treatment with B-I09 might alter the function of MDSCs. We intraperitoneally injected MD4^{+/-}/Eμ-TCL1^{+/+} mice with B-I09, and observed leukemic regression (Fig. 6H and Supplementary Fig. S7C), consistent with results of injecting Eμ-TCL1^{+/+} mice with B-I09 (27). When compared with CD11b⁺/Ly6G⁺ granulocytic MDSCs in DMSO-injected MD4^{+/-}/Eμ-TCL1^{+/+} mice, such MDSCs in B-I09-injected MD4^{+/-}/Eμ-TCL1^{+/+} mice expressed decreased levels of Arg-1 (Fig. 6I). Although purified CD11b⁺/Ly6G⁺ granulocytic MDSCs from un-injected MD4^{+/-}/Eμ-TCL1^{+/+} mice could suppress proliferation of T cells, those purified from B-I09-injected MD4^{+/-}/Eμ-TCL1^{+/+} mice lost their immunosuppressive function (Fig. 6J).

Secretory IgM is critical for the function of MDSCs in suppressing T-cell proliferation

To investigate whether sIgM induces accumulation of MDSCs in solid tumor microenvironments, we grafted eight-week-old wild-type and μS^{-/-} mice with mouse Lewis lung carcinoma (LLC) and found significantly reduced tumor growth in μS^{-/-} mice (Fig. 7A–B). There was no detectable serum IgM in μS^{-/-} mice grafted with LLC (Supplementary Fig. S8A). We lysed tumors, stained cells with DAPI, CD45-PE, CD11b-BV605, Ly6C-Alexa-488 and Ly6G-Alexa-647. Live DAPI⁻/CD45⁺ immune cells were gated for CD11b⁺ myeloid cell populations, which were analyzed for their expression of Ly6C and Ly6G (Fig. 7C). Despite a significant decrease of tumor-infiltrating CD11b⁺ myeloid cells in LLC-grafted μS^{-/-} mice, there was no significant difference in the percentages of tumor-

infiltrating CD11b⁺/Ly6G⁺ granulocytic or CD11b⁺/Ly6C⁺ monocytic MDSCs between LLC-grafted wild-type and $\mu S^{-/-}$ mice (Fig. 7D–F). Although both monocytic and granulocytic MDSCs residing in LLC tumors of wild-type mice suppressed gp100-stimulated T-cell proliferation, MDSCs residing in tumors of $\mu S^{-/-}$ mice lost their immunosuppressive functions (Fig. 7G–H), confirming the role of sIgM in mediating the functions of MDSCs. We did not detect a difference in the percentages of infiltrating B cells between tumors in wild-type and $\mu S^{-/-}$ mice (Supplementary Fig. S8B), suggesting that the physical presence of B cells did not regulate functions of MDSCs. In addition, CD11b⁺/Ly6G⁺ granulocytic MDSCs purified from the spleens or bone marrow of LLC-grafted wild-type and $\mu S^{-/-}$ mice had a small or no effect in suppressing gp100-stimulated T-cell proliferation (Supplementary Fig. S8 C–D). To ascertain whether the decreased LLC tumor growth in $\mu S^{-/-}$ mice was resulted from decreased capabilities of MDSCs in suppressing antitumor CD8⁺ T cells in mice, we intraperitoneally injected LLC-grafted wild-type and $\mu S^{-/-}$ mice with anti-CD8 monoclonal antibodies twice weekly to deplete CD8⁺ T cells. When antitumor CD8⁺ T cells were depleted, tumor growth in LLC-grafted $\mu S^{-/-}$ mice could no longer be suppressed (Fig. 7I–J).

Discussion

MD4^{+/-}/E μ -TCL1^{+/+} mice on the B6C3 background lived to a median age of approximately 8 months. Approximately 50% E μ -TCL1/IgHEL (MD4) mice on the C57BL/6 background in a previous Kaplan-Meier survival study can live longer than 16 months (13). The different genetic background of the two models may account for the distinct survival outcomes. Additional causes for the difference may include our choice to maintain the MD4 transgene in heterozygous status in our model to avoid the possibility of comparing heterozygous MD4^{+/-} mice with homozygous MD4^{+/+} mice. The MD4 transgene dosage cannot be determined by routine PCR-based genotyping. Despite the difference in genetic background and potentially in MD4 transgene dosage, the CD5⁺ CLL cells developed in both models failed to recognize HEL (13).

We demonstrated that sIgM had immunosuppressive activity by inducing accumulation and upregulating functions of MDSCs in tumor-bearing mice, and that targeting the synthesis and/or secretion of sIgM in tumor-bearing mice could be useful in decelerating tumor progression. Tumor-associated monocytic and granulocytic MDSCs from LLC-grafted $\mu S^{-/-}$ mice lost their ability to suppress T-cell proliferation. Such data suggest that sIgM may promote suppressive activities of MDSCs. Since sIgM binds to polymeric immunoglobulin receptor (pIgR), Fc α / μ receptor (Fc α / μ R or CD351), Fc μ receptor (Fc μ R or TOSO or FAIM3), complement receptors, and sialic acid-binding immunoglobulin-like lectins, it is likely that sIgM may recruit MDSCs into tumors and upregulate their suppressive functions using one or some of these receptors. Because pIgR is exclusively expressed by epithelial cells in guts and lungs, where it mediates transcellular transport of sIgM and sIgA (33,34), there is little chance that sIgM would induce MDSCs using pIgR. Fc α / μ R, which binds to the Fc regions of sIgM and sIgA to mediate endocytosis of antibody-coated pathogens, is expressed on B cells, follicular dendritic cells and macrophages but not granulocytes, T cells or NK cells (35,36). Thus, Fc α / μ R is unlikely to be the receptor for recruiting and activating MDSCs. Fc μ R is also an Fc receptor for sIgM (37,38). Although there was a debate on

whether myeloid cells express Fc μ R (39,40), Fc μ R-deficient granulocytes produce more reactive oxygen species (ROS), and both Fc μ R-deficient granulocytes and monocytes show reduced phagocytosis (41). Such results suggest that activation of Fc μ R through binding by sIgM will suppress, instead of activate, the function of MDSCs in producing ROS to inhibit antitumor T-cell functions (20). The other two types of receptors are more likely to be used to engage MDSCs. First, Siglec-G and CD22 are both sialic acid-binding immunoglobulin-like lectins, and they can bind to sialic acid residues on sIgM (42–44). CD22 is primarily expressed by B cells (45). Although myeloid DCs express Siglec-G, it is not clear whether MDSCs express Siglec-G. However, engagement of Siglec-3 by S100A9 causes MDSC accumulation (46). Second, sIgM-antigen complexes can bind to complement receptors via activated complement molecules, and some complement cascade components and complement receptors, such as C5a/C5aR, iC3b and C3d, promote tumor progression by recruiting and upregulating the suppressive function of MDSCs (47–50). Future studies will be required to test whether Siglec, complement receptor or other molecules on the surface of MDSCs can be activated by sIgM to promote immunosuppressive functions of MDSCs. It is also possible that sIgM does not upregulate the functions of MDSCs by binding to a receptor on the surface of MDSCs. One possible scenario is that sIgM may stimulate non-MDSC immune cells or stromal cells within tumors to increase production of cytokines or chemokines, contributing to the expansion and upregulated immunosuppressive functions of MDSCs.

CD11b⁺/Ly6G⁺ granulocytic MDSCs isolated from spleens, where CLL develop, but not from peripheral blood, of CLL-bearing MD4^{+/-}/E μ -TCL1^{+/-} mice suppressed proliferation of T cells. Additionally, CD11b⁺/Ly6G⁺ granulocytic MDSCs purified from tumors of LLC-grafted mice were more effective at suppressing T-cell proliferation than those purified from the spleens and bone marrow of the same mice. These data are consistent with the previous reports showing that MDSCs residing within tumors are more effective at suppressing antitumor T cells, due to upregulating the expression of various immunosuppressive molecules within tumor microenvironments (22).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Summary

In a mouse model of chronic lymphocytic leukemia, production of secretory IgM led to more MDSCs, fewer T cells, and shorter survival times for the mice. Thus, secretory IgM may aggravate the progression of this cancer.

Figure 1. CLL cells developed in MD4^{+/-}/Eμ-TCL1^{+/+} mice fail to recognize HEL

(A) Splenocytes from MD4^{+/-}/Eμ-TCL1^{+/+} mice of indicated age groups were stained with indicated antibodies, HEL-Alexa-568 and DAPI. Live splenocytes were analyzed for precancerous CD5⁻/B220⁺ B cells and CD5⁺/B220⁺ CLL cells on gated CD3⁻/IgM⁺ B cell populations. CD5⁻/B220⁺ B cells (red line) and CD5⁺/B220^{lo} CLL cells (blue line) were further analyzed for their HEL-binding capability, shown in the histograms. (B) A representative enlarged spleen of 8-month-old MD4^{+/-}/Eμ-TCL1^{+/+} mice (right), compared with a representative spleen from 6-week-old MD4^{+/-}/Eμ-TCL1^{+/+} mice (left). Data are representative of three independent experiments.

Figure 2. MD4^{+/}/Eμ-TCL1^{+/} CLL cells can be activated by F(ab')₂ and secrete IgM
(A) CD5⁻/B220⁺ B cells from 6-week-old MD4^{+/} and MD4^{+/}Eμ-TCL1^{+/} mice were stimulated with oligomeric HEL (5 μg/mL) for indicated times, and lysed for immunoblot analysis. **(B)** CD5⁻/B220⁺ B cells from 3-month-old MD4^{+/} and MD4^{+/}Eμ-TCL1^{+/} mice were stimulated with oligomeric HEL for indicated times, and lysed for immunoblot analysis. **(C)** CD5⁻/B220⁺ B cells from 6-week-old MD4^{+/}Eμ-TCL1^{+/} mice and CD5⁺/B220⁺ CLL cells from 11-month-old CLL-bearing MD4^{+/}Eμ-TCL1^{+/} mice were stimulated with F(ab')₂ (20 μg/mL) for indicated time, and lysed for immunoblot analysis. **(D)** CD5⁻/B220⁺ B cells were purified from 7-month-old MD4^{+/} mice and 8-week-old MD4^{+/}Eμ-TCL1^{+/} mice. CD5⁺/B220⁺ CLL cells were purified from 7-month-old CLL-bearing MD4^{+/}Eμ-TCL1^{+/} mice. These cells were treated with CpG-1826 (0.5 μM) or LPS (20 μg/mL) for 3 days, and lysed for immunoblot analysis. Data are representative of three independent experiments.

Figure 3. MD4^{+/-}/E μ -TCL1^{+/+} CLL mice generate significantly increased numbers of CD11b⁺/Ly6G⁺ granulocytic cells

(A) Lymphocyte, (B) granulocyte and (C) monocyte counts in the peripheral blood of approximately 6-month-old E μ -TCL1^{+/+} and MD4^{+/-}/E μ -TCL1^{+/+} mice were analyzed using a HemaTrue Hematology Analyzer. Leukemia in mice: >5000 lymphocytes per μ L blood. Data were plotted as means $\pm$ SEM. (D) Blood cells from E μ -TCL1^{+/+} and MD4^{+/-}/E μ -TCL1^{+/+} mice were stained and analyzed for CD11c⁻/CD11b⁺ myeloid, CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic and CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic cells. (E–G) Percentages of CD11b⁺ myeloid cells (E), CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic cells (F), and CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic cells (G) in the blood of age-matched 6-month-old E μ -TCL1^{+/+} and MD4^{+/-}/E μ -TCL1^{+/+} mice were plotted as means $\pm$ SEM. (H–J) Percentages of CD11b⁺ myeloid cells (H), CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic cells (I), and CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic cells (J) in the blood of age-matched 8-month-old E μ -TCL1^{+/+} and MD4^{+/-}/E μ -TCL1^{+/+} mice were plotted as means $\pm$ SEM.

Figure 4. Deleting the capability of $E\mu$ -TCL1^{+/+} mice to produce sIgM leads to decreased numbers of CD11b⁺/Ly6G⁺ granulocytic cells and prolonged survival of the mice
(A) Serum IgM levels in $E\mu$ -TCL1^{+/+} and $\mu S^{-/-}E\mu$ -TCL1^{+/+} mice were determined by ELISA. **(B)** CD5⁺/B220⁺ CLL cells from CLL-bearing $E\mu$ -TCL1^{+/+} and $\mu S^{-/-}E\mu$ -TCL1^{+/+} mice were stimulated with LPS for 3 days and lysed for immunoblot analysis. Data are representative of three independent experiments. **(C)** Blood cells from $E\mu$ -TCL1^{+/+} and $\mu S^{-/-}E\mu$ -TCL1^{+/+} mice were stained and analyzed for CD11c⁻/CD11b⁺ myeloid, CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic and CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic cells. **(D–F)** Percentages of CD11b⁺ myeloid cells (D), CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic cells (E), and CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic cells (F) in the blood of age-matched 6-month-old $E\mu$ -TCL1^{+/+} and $\mu S^{-/-}E\mu$ -TCL1^{+/+} mice were plotted as means $\pm$ SEM. **(G)** The Kaplan-Meier survival analysis of $E\mu$ -TCL1^{+/+}, $\mu S^{-/-}E\mu$ -TCL1^{+/+} and MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice. **(H–I)** The Kaplan-Meier survival analyses of female (H) and male (I) $E\mu$ -TCL1^{+/+}, $\mu S^{-/-}E\mu$ -TCL1^{+/+} and MD4^{+/+}/ $E\mu$ -TCL1^{+/+} mice.

Figure 5. CD11b⁺/Ly6G⁺ granulocytic cells from spleens of MD4^{+/-}/Eμ-TCL1^{+/-} and Eμ-TCL1^{+/-} mice suppress T-cell proliferation

(A) Splenocytes from age-matched 6-month-old Eμ-TCL1^{+/-}, MD4^{+/-}/Eμ-TCL1^{+/-}, and μS^{-/-}/Eμ-TCL1^{+/-} mice were stained and analyzed for CD11c⁻/CD11b⁺ myeloid, CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic and CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic cells. (B–D) Percentages of CD11b⁺ myeloid cells (B), CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic cells (C), and CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic cells (D) in spleens of age-matched 6-month-old Eμ-TCL1^{+/-}, MD4^{+/-}/Eμ-TCL1^{+/-} and μS^{-/-}/Eμ-TCL1^{+/-} mice were plotted as means ± SEM. (E) CFSE-stained mouse splenocytes (1 × 10⁵ cells) were unstimulated (left panel), or stimulated with antibodies against mouse CD3e (1 μg/mL) and CD28 (0.5 μg/mL) for 72 h in the absence (middle panel) or presence (right panel) of 1 × 10⁵ CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic cells from spleens of MD4^{+/-}/Eμ-TCL1^{+/-} mice, and subsequently stained with CD4-BV605 and CD8-PE-Cy7. CD8⁺ T cells were gated and analyzed for the frequency of cell division by CFSE dye dilution. (F–G) Mixed PMEL-1/naïve splenocytes were incubated at indicated ratios with CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic cells (F) or CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic cells (G) from MD4^{+/-}/Eμ-TCL1^{+/-} mouse spleens in the absence or presence of the gp100 peptide. After incubation for 48 h, cells were radiolabeled with ³H-thymidine for 6 h. The ³H-thymidine uptake was plotted as means ± SD. (H) Mixed PMEL-1/naïve splenocytes were incubated at indicated

ratios with CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic cells from spleens of 8-month-old Eμ-TCL1^{+/+} and μS^{-/-}/Eμ-TCL1^{+/+} mice in the presence of the gp100 peptide for 48 h, and radiolabeled for 6 h. The percentages of proliferation were plotted as means ± SEM.

Figure 6. Targeting XBP-1s in CLL cells of MD4^{+/-}/Eμ-TCL1^{+/-} mice leads to reduced sIgM, and decreased numbers and functions of granulocytic MDSCs

(A) Serum IgM levels in MD4^{+/-}/Eμ-TCL1^{+/-} and XBP-1^{KO}/MD4^{+/-}/Eμ-TCL1^{+/-} mice were determined by ELISA. (B) Blood cells from MD4^{+/-}/Eμ-TCL1^{+/-} and XBP-1^{KO}/MD4^{+/-}/Eμ-TCL1^{+/-} mice were stained and analyzed for CD11c⁻/CD11b⁺ myeloid, CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic and CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic cells. (C–D) Percentages of CD11b⁺ myeloid cells (C) and CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic MDSCs (D) in the blood of MD4^{+/-}/Eμ-TCL1^{+/-} and XBP-1^{KO}/MD4^{+/-}/Eμ-TCL1^{+/-} mice were plotted as means ± SEM. (E) Splenocytes from MD4^{+/-}/Eμ-TCL1^{+/-} and XBP-1^{KO}/MD4^{+/-}/Eμ-TCL1^{+/-} mice were stained with CD11b-BV605, Ly6C-Alexa-488, Ly6G-Alexa-647 and Arg-1-PE. Gated CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic MDSCs were analyzed for the expression of Arg-1. (F) Mean fluorescence intensity (MFI) of Arg-1 in gated CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic MDSCs from spleens of 5 MD4^{+/-}/Eμ-TCL1^{+/-} and 5 XBP-1^{KO}/MD4^{+/-}/Eμ-TCL1^{+/-} mice was plotted as means ± SEM. (G) Mixed OT-I/naïve splenocytes were incubated at indicated ratios with CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic cells from spleens of 5 MD4^{+/-}/Eμ-TCL1^{+/-} and 5 XBP-1^{KO}/MD4^{+/-}/Eμ-TCL1^{+/-} mice in the presence of the OVA peptide for 48 h, and radiolabeled for 6 h. The percentages of proliferation were plotted as means ±

SEM. **(H)** MD4^{+/-}/Eμ-TCL1^{+/+} mice with high percentage of CLL cells in the blood were identified and injected intraperitoneally with B-I09 (50 mg/kg; n = 4) on days 1 to 5 and days 8 to 12. The percentage of CLL cells in PBMCs for each mouse was determined by flow cytometry on day 6 and day 13, and compared with initial CLL burden data on day 0 (100%). Data from 4 mice receiving the same treatment were plotted as means ± SEM. **(I)** MFI of Arg-1 in gated CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic MDSCs from spleens of 4 DMSO-treated and 4 B-I09-treated MD4^{+/-}/Eμ-TCL1^{+/+} mice was plotted as means ± SEM. **(J)** Mixed OT-I/naïve splenocytes were incubated at indicated ratios with CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic cells from spleens of untreated or B-I09-treated MD4^{+/-}/Eμ-TCL1^{+/+} mice in the presence of the OVA peptide for 48 h, and radiolabeled for 6 h. The percentages of proliferation were plotted as means ± SEM.

Figure 7. Reduced LLC growth in $\mu S^{-/-}$ mice is resulted from reduced capabilities of MDSCs in suppressing proliferation of antitumor CD8⁺ T cells

(A) Eight-week-old wild-type and $\mu S^{-/-}$ mice were subcutaneously injected with LLC cells on day 0. Tumor size was measured on days 11, 14, 18 and 24, and data were plotted as means $\pm$ SEM. (B) Tumor weights from day 24 were plotted as means $\pm$ SEM. (C) Tumors dissected from wild-type and $\mu S^{-/-}$ mice on day 24 were dissociated and stained. CD45⁺ cells were gated to analyze for CD11b⁺ myeloid cells, and CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic and CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic MDSCs. (D–F) Percentages of CD11b⁺ myeloid cells (D), CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic MDSCs (E), and CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic MDSCs (F) in LLC tumors of wild-type and $\mu S^{-/-}$ mice were plotted as means $\pm$ SEM. (G–H) Mixed PMEL-1/naïve splenocytes were incubated at indicated ratios with CD11b⁺/Ly6C⁺/Ly6G⁻ monocytic (G) or CD11b⁺/Ly6C^{intermediate}/Ly6G⁺ granulocytic (H) MDSCs from LLC tumors in wild-type and $\mu S^{-/-}$ mice in the presence of the gp100 peptide for 48 h, and radiolabeled for 6 h. The percentages of proliferation were plotted as means $\pm$ SEM. (I) Effect of T-cell depletion with an antibody to mouse CD8 α on tumor size. The size of tumor was measured and recorded starting from day

11 to day 22, and data were plotted as means $\pm$ SEM. (J) Tumor weights from (I) on day 22 were plotted as means $\pm$ SEM.

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