CD160 Stimulates CD8$^+$ T Cell Responses and Is Required for Optimal Protective Immunity to *Listeria monocytogenes*

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RESEARCH ARTICLE

Adaptive Immunity

CD160 Stimulates CD8$^+$ T Cell Responses and Is Required for Optimal Protective Immunity to *Listeria monocytogenes*

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ABSTRACT

CD160 promotes NK cell cytotoxicity and IFN-γ production, but the function of CD160 on CD8$^+$ T cells remains unclear with some studies supporting a coinhibitory role and others a costimulatory role. In this study, we demonstrate that CD160 has a costimulatory role in promoting CD8$^+$ T cell effector functions needed for optimal clearance of oral *Listeria monocytogenes* infection. CD160$^{-/-}$ mice did not clear oral *L. monocytogenes* as efficiently as wild type (WT) littermates. WT RAG$^{-/-}$ and CD160$^{-/-}$ RAG$^{-/-}$ mice similarly cleared *L. monocytogenes*, indicating that CD160 on NK cells does not contribute to impaired *L. monocytogenes* clearance. Defective *L. monocytogenes* clearance is due to compromised intraepithelial lymphocytes and CD8$^+$ T cell functions. There was a reduction in the frequencies of granzyme B–expressing intraepithelial lymphocytes in *L. monocytogenes*–infected CD160$^{-/-}$ mice as compared with WT littermate controls. Similarly, the frequencies of granzyme B–expressing splenic CD8$^+$ T cells and IFN-γ and TNF-α double-producer CD8$^+$ T cells were significantly reduced in *L. monocytogenes*–infected CD160$^{-/-}$ mice compared with WT littermates. Adoptive transfer studies showed that RAG$^{-/-}$ recipients receiving CD160$^{-/-}$ CD8$^+$ T cells had a higher mortality, exhibited more weight loss, and had a higher bacterial burden compared with RAG$^{-/-}$ recipients receiving WT CD8$^+$ T cells. These findings demonstrate that CD160 provides costimulatory signals to CD8$^+$ T cells needed for optimal CD8$^+$ T cell responses and protective immunity during an acute mucosal bacterial infection. *ImmunoHorizons*, 2018, 2: 238–250.

INTRODUCTION

CD160 is an Ig-like, GPI-anchored cell membrane protein expressed on activated and exhausted CD8$^+$ T cells, some CD4$^+$ T cells, intraepithelial lymphocytes (IELs), intraepithelial innate lymphoid cell type 1 (ILC1) cells, NK cells, NKT cells, and myeloid cells (1–3). CD160 binds to MHC class I molecules with low affinity and to herpes virus entry mediator (HVEM), a TNF receptor family member, with much higher affinity (4, 5). HVEM can negatively or positively regulate T cells depending on the ligands/receptors engaged, thus serving as a bidirectional switch for T cell regulation. Binding of HVEM to B and T lymphocyte attenuator is inhibitory, whereas binding of HVEM to lymphotoxin (LT-α) or LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes; it is also known as TNFSF14) delivers a costimulatory signal (5–7). Recent studies indicate a critical role for HVEM–CD160 interactions in

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Abbreviations used in this article: ES, embryonic stem; GF, germ-free; HVEM, herpes virus entry mediator; IEL, intraepithelial lymphocyte; ILC1, intraepithelial innate lymphoid cell type 1; LCMV, lymphocytic choriomeningitis virus; mLN, mesenteric lymph node; SPF, specific pathogen-free; WT, wild type.

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mediating intestinal immunity (8, 9). However, the function of CD160 remains unclear and may be cell type– and context-specific given the multiple binding partners for CD160 and HVEM.

There are data to support both coinhibitory and costimulatory functions for CD160 (4, 5, 10–14). Human CD160$^+$ CD4$^+$ T cells received an inhibitory signal upon binding HVEM in vitro (5). CD160 is highly expressed by exhausted CD8$^+$ T cells, and cytotoxicity of CD8$^+$ T cells from mice infected with lymphocytic choriomeningitis virus (LCMV) clone 13 was enhanced by anti-CD160 in vitro (15). These findings point to an inhibitory function for CD160. However, there are also studies showing that CD160 can stimulate NK cytotoxicity and IFN-γ production and enhance T cell activity (4, 11–13). The interpretation of studies with anti-CD160 mAbs and CD160Ig as agonists or antagonists is complex given the multiple binding partners for CD160 and HVEM and the notion that HVEM can serve as both a receptor and ligand for its binding partners, resulting in proinflammatory or inhibitory signals. In this study, we investigate the expression and immunoregulatory functions of CD160 on CD8$^+$ T cells in the intestinal microenvironment. Using CD160-deficient mice, we demonstrate that CD160 promotes CD8$^+$ T cell cytotoxicity and cytokine production and is critical for optimal bacterial clearance during oral *Listeria monocytogenes* infection.

MATERIALS AND METHODS

Mice

C57BL/6J (000664) wild type (WT) mice, B6.129S7-*Rag1*^{tm1Mom}/*J* (002216) (also known as *RAG1*$^{-/-}$) mice, and B6.129P2-*Il10*^{tm1Cgn}/J (002251) (also known as *IL10*$^{-/-}$) mice were obtained from The Jackson Laboratory. B6.Cg-*Tcra*^{tm1Mom} Tg (TcrLCMV)327Sdz (4138), also known as P14 transgenic, mice were obtained from Taconic Biosciences. The P14 mice carry a transgene that encodes a TCR that is specific for a peptide (gp33) from the LCMV. Germ-free (GF) mice were a gift from D. Kasper. The mice used in these studies were between 8 and 12 wk old, and littermates were used as controls. All mice were maintained in a specific pathogen-free (SPF) facility and used according to Harvard Medical School and National Institutes of Health guidelines. Harvard Medical School is accredited by the American Association of Accreditation of Laboratory Animal Care.

Generation of CD160-deficient mice

To generate CD160$^{-/-}$ mice, we designed a CD160-targeting vector containing the neo gene (Supplemental Fig. 1A). The flanking regions of the CD160 gene were cloned from a CD160 containing bacterial artificial chromosome using standard techniques. Linearized vector DNA was electroporated into Bruce 4 C57BL/6 embryonic stem (ES) cells, and the resulting neomycin-resistant ES cells were screened for homologous recombination by Southern blotting using Bgl I digest with a 5′ external probe and Bgl II digest with a 3′ external probe. ES cells carrying the desired recombinant event were microinjected into blastocysts, and the resulting chimeric mice gave germline transmission of the targeted CD160 allele. The resulting CD160$^{-/-}$ mice lack exons 2 and 3 encoding the signal sequence and Ig-V, respectively. Routine genotyping was performed by PCR using primers 5′-CTTCCTA GAATCGATCCTAGACCG-3′ and 5′-GGCCCTTTATAAAGC TTGA-3′ at an annealing temperature of 52°C, which produced a PCR product of 602 bp. Loss of CD160 transcription was verified by RT-PCR and loss of protein expression by flow cytometry. CD160$^{-/-}$ RAG$^{-/-}$ mice were generated by crossing CD160$^{-/-}$ mice with B6.129S7-*Rag1*^{tm1Mom}/*J* (002216), also known as RAG1$^{-/-}$ mice, purchased from The Jackson Laboratory.

L. monocytogenes *infection and determination of CFU*

Streptomycin-resistant *L. monocytogenes* In1A^M strain 10403s expressing a truncated form of OVA (16) was grown in brain–heart infusion broth (BD Biosciences) overnight. *L. monocytogenes* was subcultured in the morning into brain–heart infusion broth (1:50 dilution) and incubated at 37°C until OD_{600} reached 0.8; 20 ml of *L. monocytogenes* culture of OD_{600} at 0.8 will yield 2×10^{10} *L. monocytogenes*. The *L. monocytogenes* culture was centrifuged and resuspended in PBS. Mice were pretreated with 100 μl of 10% sodium bicarbonate 10 min prior to infection by oral gavage with 2×10^9 *L. monocytogenes* using a 20-gauge curved needle. The infectious dose was confirmed by plating dilutions of the inoculum on brain–heart infusion agar plates supplemented with 50 μg/ml streptomycin. Organs were homogenized and lysed in PBS with 0.05% Triton X-100, serial dilutions of homogenates were plated on brain–heart infusion agar plates supplemented with 50 μg/ml streptomycin, and colonies were counted after incubation at 37°C for 24–48 h.

Citrobacter rodentium *infection and determination of CFU*

Citrobacter rodentium strain DBS100 resistant to chloramphenicol (51495; American Type Culture Collection) was used. *C. rodentium* was grown overnight in Luria–Bertani broth (Sigma-Aldrich) with shaking at 37°C. Bacterial cultures were adjusted with PBS for proper concentration and individual titers were determined after each experiment by serial dilution. Mice were infected with 0.5×10^9–2×10^9 CFU. Postinfection, mice were weighed daily, and body weight was calculated as a percentage of initial weight on day 0. Mice were sacrificed at the indicated time points post-infection. For CFU assays, fecal pellets were weighed, homogenized, serially diluted, and plated on chloramphenicol-containing MacConkey agar plates (Teknova).

LCMV clone 13 and Armstrong infection and determination of viral titer

For LCMV clone 13 infections, mice were infected i.v. with LCMV clone 13 at 4×10^6 PFU. Postinfection, mice were weighed daily, and body weight was calculated as a percentage of initial weight on day 0. To determine viral load, serum and kidneys were collected at indicated time points, and viral titers were determined by plating diluted serum/tissue samples on Vero cells with an agarose overlay. Four days later, the Vero cells were stained with neutral red dye, and 14 h later, plaques were quantified. For LCMV Armstrong infections, mice were infected i.p. with LCMV Armstrong at 2×10^5 PFU.

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Adoptive transfer experiments

For studies with *L. monocytogenes*, naive $CD8^+$ T cells were isolated using the MACS Naive CD8 Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Then, 3×10^6 WT or $CD160^{-/-}$ naive $CD8^+$ T cells were adoptively transferred into $RAG^{-/-}$ recipients. $RAG^{-/-}$ recipients were infected with 2×10^9 *L. monocytogenes* the next day.

For studies with LCMV Armstrong, congenically marked WT and $CD160^{-/-}$ P14 T cells were isolated using the MACS Naive CD8 Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Five hundred WT and 500 $CD160^{-/-}$ P14 T cells were mixed at 1:1 ratio and adoptively transferred into WT recipients. WT recipients were infected the next day with 2×10^5 PFU LCMV Armstrong.

Isolation of IELs

For cellular analysis of colonic IELs, large intestines were removed from mice. Peyer patches were removed and fat was trimmed off the intestines. Intestines were then flipped inside out and shaken for 30 min in RPMI 1640 supplemented with 1 mM DTT and 10% FBS. IELs were collected from the interface of a 44%/67% Percoll gradient, washed, and resuspended in culture medium for analysis.

Abs and flow cytometry analysis

Conjugated Abs specific to CD4 (RM4-5), CD8β (YTS156.7.7), CD8α (53-6.7), TCRβ (H57-597), TNF-α (MP6-X722), and IFN-γ (XMG1.2), granzyme B (GB11), CD44 (IM7), CD62L (MEL-14), and CD107a (1D4B) were purchased from BioLegend (San Diego, CA). Anti-CD160 (CNX46-3), anti-TCRγδ (GL3), and anti-Ki67 (B56) were from BD Biosciences (San Jose, CA). SIINFEKL dextramers (JD2163) were purchased from Immudex. Single-cell suspensions from spleen or lymph nodes were prepared and resuspended in staining buffer (PBS containing 1% FBS and 2 mM EDTA) and stained with the indicated Abs. For intracellular cytokine staining, cells were activated with PMA (Sigma-Aldrich) and Ionomycin (Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences) for 4 h, followed by intracellular staining using the eBioscience Foxp3 Transcription Factor Staining Buffer Set (ThermoFisher Scientific) according to manufacturer's protocol. For peptide restimulation, splenocytes were restimulated with 1 μg/ml SIINFEKL peptide (GenScript) or gp33 peptide (GenScript) for 5 h in the presence of GolgiPlug (BD Biosciences). Data were acquired on a LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Analysis of cell proliferation by BrdU incorporation

All fixation and permeabilization reagents were obtained from BD Biosciences. Mice were injected i.p. with 1.8 mg of BrdU (BD Biosciences) and given water containing 0.8 μg/ml BrdU (Sigma-Aldrich) for 5 d. Cells were stained according to the manufacturer's protocol (BD Biosciences).

T cell cytokine assays

Splenocytes were plated at 1×10^5 and cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1% penicillin/streptomycin, and 50 μM 2-ME. One microgram per milliliter of anti-CD3 (145-2C11; Bio X Cell) was used to activate splenocytes in the presence of 5 ng/ml different cytokines: TGF-β (R&D Systems), IL-10 (BioLegend), and TNF-α (BioLegend).

Antibiotics treatment

Mice were given antibiotics in drinking water as described (17). In brief, mice were provided 1 g/l ampicillin (Sigma-Aldrich), 500 mg/l vancomycin (Sigma-Aldrich), 1 g/l neomycin sulfate (Sigma-Aldrich), and 1 g/l metronidazole (Sigma-Aldrich) in drinking water for 4 wk.

Statistical analysis

All of the statistical analyses were performed using Prism Software, version 6 (GraphPad). Results are presented as mean ± SEM, and significance was determined using an unpaired two-tailed Student *t* test, Mann–Whitney nonparametric test, one-way ANOVA, χ^2 or log-rank (Mantel–Cox) test, as indicated in the figure legends. Asterisks denote level of statistical significance ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, and $^{****}p < 0.001$).

RESULTS

The gut mucosal environment regulates CD160 expression

CD160 is predominately expressed on IELs in the colon at steady state (18, 19). To understand the role of CD160 in regulating $CD8^+$ T cell functions in the gut mucosal environment, we first sought to understand what regulates CD160 expression in this microenvironment. We hypothesized that the mucosal environment drives CD160 expression on colonic IELs. To test this hypothesis, we isolated naive $CD8^+$ T cells from the spleen and adoptively transferred them into $RAG^{-/-}$ mice. After 10 d, we isolated $CD8^+$ T cells from the spleen, mesenteric lymph nodes (mLN), and colonic IEL compartment and analyzed them for CD160 expression. $CD8^+$ T cells in the colonic IEL compartment expressed higher levels of CD160 compared with mLN and spleen (Fig. 1A), and the frequency of CD160-expressing $CD8^+$ T cells in the colonic IEL compartment was increased compared with $CD8^+$ T cells in the spleen and mLN (Fig. 1B), suggesting that the intestinal environment promotes CD160 expression on $CD8^+$ T cells.

We next investigated factors in the intestinal environment that might induce CD160 expression. Because intestinal T cells are described as activated (20), we tested whether TCR engagement induces CD160 expression by using P14 TCR transgenic mice in which the TCR recognizes LCMV peptide gp33. We hypothesized that IELs from unmanipulated P14 transgenic mice might express lower CD160 levels because of the absence of cognate Ag gp33 in the gut environment as compared with IELs isolated from non-P14 transgenic mice. Consistent with this hypothesis, the frequency of $CD160^+$ IELs (Fig. 1C) in P14 transgenic mice was reduced compared with IELs isolated from non-P14 transgenic mice. These results suggest that TCR engagement may be one factor that promotes CD160 expression.

One of the unique features of the intestinal environment is the cytokine milieu. The intestinal environment has high levels of

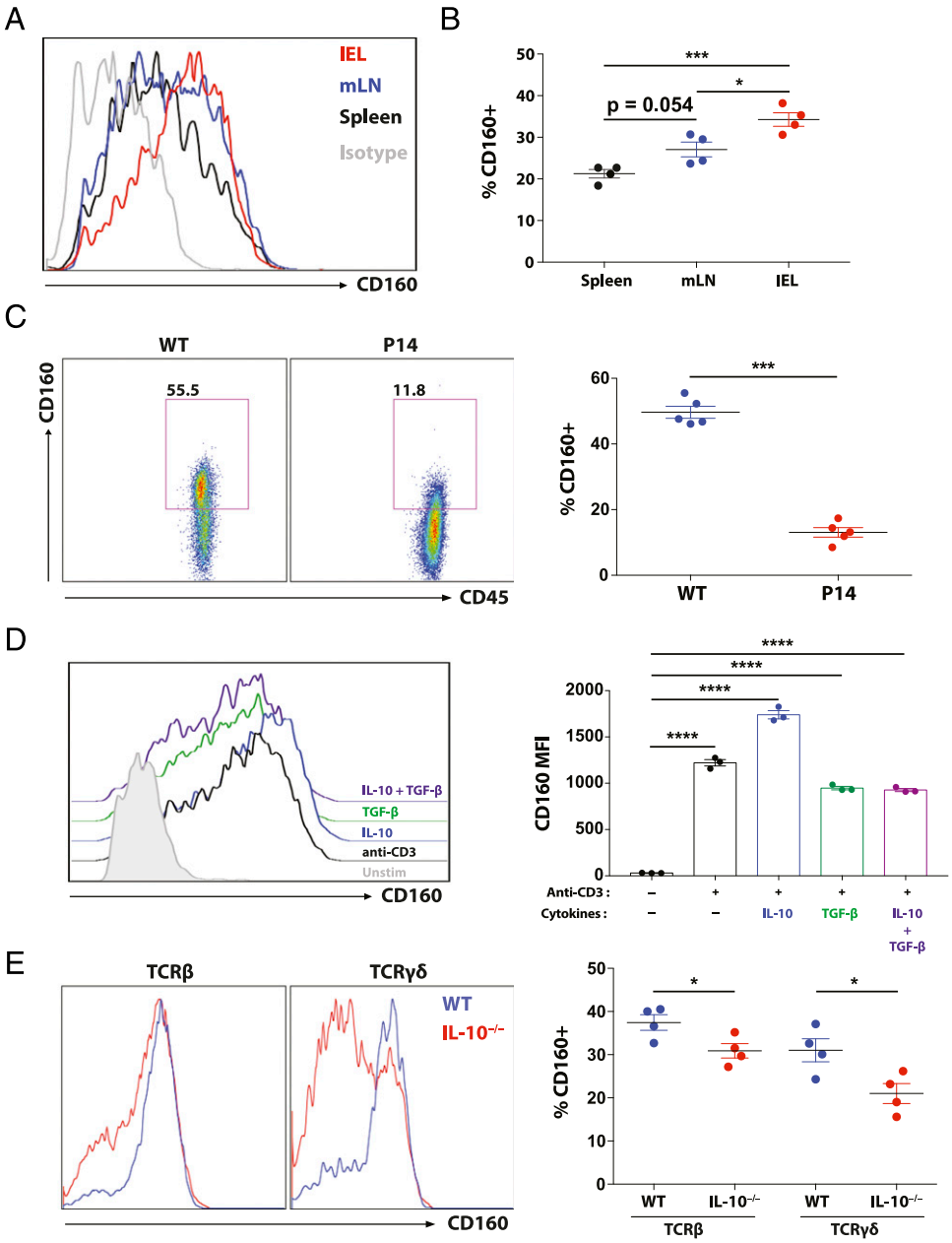

FIGURE 1. The mucosal environment regulates CD160 expression on IELs.

Naive $CD8^+$ T cells were transferred into $RAG^{-/-}$ recipients and isolated 10 d later from mLN (blue), spleen (black), and colonic IELs (red) and analyzed for (**A**) CD160 expression levels and (**B**) percentages of cells expressing CD160. (**C**) Percentages of colonic IELs expressing CD160 from age- and sex-matched WT (blue) and P14 TCR transgenic mice (red). Representative plots are shown to the left and summary of CD160-expressing IELs to the right. (**D**) CD160 expression levels on $CD8^+$ T cells that were unstimulated (gray) or activated with anti-CD3 in the presence of IL-10 (blue), TGF-β (green), IL-10 plus TGF-β (purple), and anti-CD3 alone (black). Representative plots are shown to the left and summary of CD160 mean fluorescence intensity (MFI) to the right. (**E**) CD160 expression levels and percentages of $TCR\beta^+$ and $TCR\gamma\delta^+$ colonic IELs expressing CD160 from age- and sex-matched WT (blue) and $IL\text{-}10^{-/-}$ mice (red). Representative plots are shown to the left and summary of CD160-expressing $TCR\beta^+$ and $TCR\gamma\delta^+$ colonic IELs to the right. Data are represented as means ± SEM. The results are representative of at least two independent experiments. Significance was assessed using one-way ANOVA in (B) and (D) and Student *t* test in (C) and (E). $*p < 0.05$, $***p < 0.005$, $****p < 0.001$.

IL-10 and TGF-β (21, 22). To test whether IL-10 and TGF-β can induce CD160 expression on $CD8^+$ T cells, we stimulated $CD8^+$ T cells with anti-CD3 in vitro in the presence of IL-10 and/or TGF-β. Activated $CD8^+$ T cells expressed higher levels of CD160 than unstimulated $CD8^+$ T cells. $CD8^+$ T cells activated in the presence of IL-10 had even higher levels of CD160, whereas

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$CD8^+$ T cells activated in the presence of TGF-β expressed less CD160 (Fig. 1D). It is likely that $CD8^+$ T cells stimulated in the presence of TGF-β were not maximally activated because of the immunosuppressive effects of TGF-β (23). To determine whether IL-10 can regulate CD160 expression on IELs, we compared CD160 expression on $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ colonic IELs from WT and $IL\text{-}10^{-/-}$ mice ex vivo. $IL\text{-}10^{-/-}$ mice had a reduced frequency of CD160-expressing $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ colonic IELs (Fig. 1E), suggesting that IL-10 can augment CD160 expression on colonic IELs and $CD8^+$ T cells.

The microbiota can regulate CD160 expression on IELs

Another unique feature of the intestinal environment is the microbiota. Therefore, we also investigated whether CD160 expression on colonic IELs is regulated by the microbiota. First, we treated mice with a mixture of antibiotics in drinking water for 4 wk (17) and then analyzed colonic IELs for CD160 expression. Colonic IELs from the antibiotic-treated mice expressed higher levels of CD160 compared with mice given regular drinking water (Fig. 2A). We next validated these findings using age-matched mice that were GF versus SPF (SPF but containing normal microbiota). CD160 expression was elevated on colonic IELs from GF mice compared with SPF mice (Fig. 2B). Collectively, our results suggest that the normal microbiota may downregulate CD160 expression on IELs.

Immune characterization of IELs in $CD160^{-/-}$ mice at steady state

To study the role of CD160 on $CD8^+$ T cells and colonic IELs, we used CD160-deficient ($CD160^{-/-}$) mice. We generated $CD160^{-/-}$ mice by gene targeting (Supplemental Fig. 1A). The CD160 signal and Ig-V–like exons are deleted in $CD160^{-/-}$ mice (Supplemental Fig. 1A), eliminating CD160 binding to HVEM. We verified the absence of CD160 protein expression in $CD160^{-/-}$ mice (Supplemental Fig. 1B) by evaluating CD160 expression on colonic IELs by flow cytometry ex vivo. $CD160^{-/-}$ mice had comparable thymic development and peripheral lymphoid compartment at steady state as WT littermates. There were no differences in the frequencies and numbers of $CD4^+$ and $CD8^+$ T cells, $Foxp3^+$ T regulatory cells, double positive and double negative T cells in $CD160^{-/-}$, and age-matched WT littermates (Supplemental Fig. 1C). Similarly, $CD160^{-/-}$ mice and WT littermates had comparable frequencies and numbers of $CD8^+$, $CD4^+$, $Foxp3^+$ T regulatory cells, $CD11b^+$, $CD11c^+$, and $NK1.1^+$ immune cells in the spleen (Supplemental Fig. 1D).

When we compared WT and $CD160^{-/-}$ IELs from the colon, there was a modest reduction in colonic IEL cellularity in $CD160^{-/-}$ mice (Supplemental Fig. 2A), and the numbers of both $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ colonic IELs were reduced (Supplemental Fig. 2B). To determine whether the reduction in colonic IEL numbers in $CD160^{-/-}$ mice is due to impaired proliferation, we compared proliferation of WT and $CD160^{-/-}$ colonic IELs using BrdU. We administered BrdU in the drinking water for 5 d and measured BrdU incorporation. Colonic IELs in WT and $CD160^{-/-}$ mice incorporated similar levels of BrdU, suggesting that WT and $CD160^{-/-}$ colonic IELs proliferate at a similar rate (Supplemental Fig. 2C). We also investigated whether CD160 contributes to colonic IEL functions by comparing granzyme B, IFN-γ, and TNF-α production in WT and $CD160^{-/-}$ colonic IELs. There were similar frequencies of granzyme B–expressing $CD160^{-/-}$ and WT

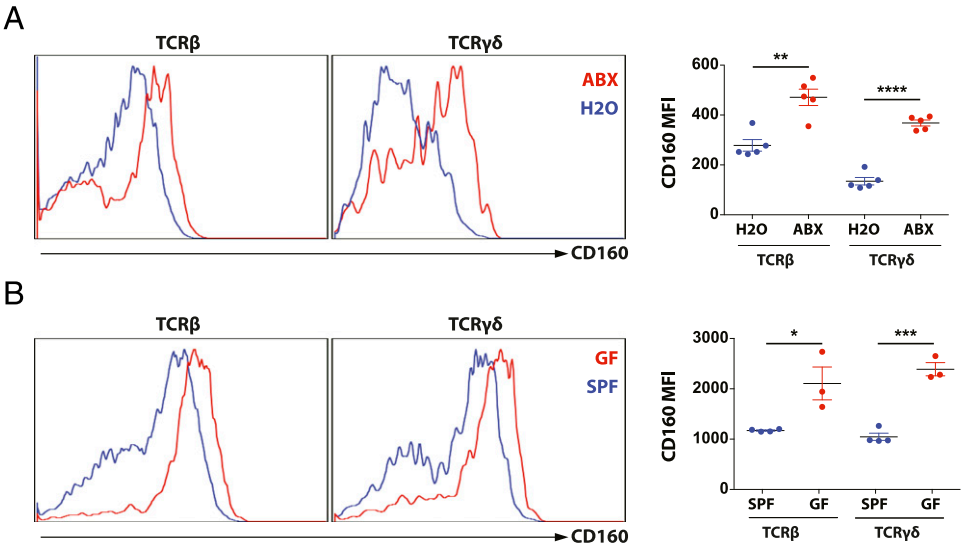

FIGURE 2. The microbiota downregulate CD160 expression on IELs.

(**A**) CD160 expression levels on $TCR\beta^+$ and $TCR\gamma\delta^+$ colonic IELs from mice given regular drinking water (H_2O, blue) and mice treated with mixture of antibiotics for 4 wk mice (ABX, red). Representative plots are shown to the left and summary of CD160 mean fluorescence intensity (MFI) to the right. (**B**) CD160 expression levels on $TCR\beta^+$ and $TCR\gamma\delta^+$ colonic IELs from age- and sex-matched SPF mice (blue) and GF mice (red). Representative plots are shown to the left and summary of CD160 MFI to the right. The results are representative of at least three independent experiments. Significance was assessed using Student *t* test. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, $****p < 0.001$.

colonic IELs (Supplemental Fig. 2D). Additionally, similar frequencies of IFN-γ– and TNF-α–producing colonic IELs (Supplemental Fig. 2E) were found in CD160$^{-/-}$ and WT littermates. These findings indicate that CD160$^{-/-}$ colonic IELs do not exhibit immune defects at steady state.

CD160$^{-/-}$ and WT mice exhibit comparable immune responses during LCMV clone 13 and Armstrong infection

Because CD160 is expressed on exhausted CD8$^+$ T cells (15, 24, 25) and in vitro studies support an inhibitory role for CD160 in exhausted CD8$^+$ T cells, we investigated CD160 function during LCMV clone 13 infection, a chronic viral infection. We infected WT and CD160$^{-/-}$ mice with LCMV clone 13 and found comparable viral titers in the serum (Supplemental Fig. 3A) and in the kidney (Supplemental Fig. 3B). There were similar frequencies (Supplemental Fig. 3C) and numbers (Supplemental Fig. 3D) of IFN-γ– and TNF-α–producing splenic CD8$^+$ T cells on day 65 after LCMV clone 13 infection as determined by stimulation of LCMV Armstrong–infected splenocytes with gp33 peptide and subsequent evaluation of intracellular IFN-γ and TNF-α expression, suggesting that there are no functional differences between WT and CD160$^{-/-}$ splenic CD8$^+$ T cells during LCMV clone 13 infection.

We also investigated whether CD160 has a cell intrinsic role in regulating CD8$^+$ T cell functions during acute LCMV infection by adoptively transferring equal numbers of congenically marked WT and CD160$^{-/-}$ P14 T cells into WT recipient mice that were infected with LCMV Armstrong the following day (Supplemental Fig. 3E). On day 7 after LCMV Armstrong infection, similar numbers (Supplemental Fig. 3F) and similar frequencies of CD44^{hi} CD62L^{lo} activated (Supplemental Fig. 3G), and granzyme B–expressing (Supplemental Fig. 4H) WT and CD160$^{-/-}$ P14 T cells were observed. Likewise, there were similar frequencies of IFN-γ– and TNF-α–producing (Supplemental Fig. 3I, 3J) WT and CD160$^{-/-}$ P14 T cells as determined by stimulation of LCMV Armstrong–infected splenocytes with gp33 peptide and subsequent evaluation of intracellular IFN-γ and TNF-α expression. These results suggest there are no functional differences between WT and CD160$^{-/-}$ P14 during acute LCMV Armstrong infection. It may be that other inhibitory pathways are redundant with and compensate for CD160 deficiency during acute and chronic LCMV infection in vivo.

CD160$^{-/-}$ and WT mice respond similarly to C. rodentium *infection*

Because recent studies have pointed to an important role for CD160 during enteric infections with *C. rodentium* and *Clostridium difficile* (8, 26), we investigated CD160 function during *C. rodentium* infection. We infected WT and CD160$^{-/-}$ mice with *C. rodentium* and measured bacterial burden as well as weight loss. WT and CD160$^{-/-}$ mice exhibited similar *C. rodentium* burden in the stool on days 8, 11, and 19 postinfection (Supplemental Fig. 4A) and displayed similar weight loss in the first 11 d postinfection (Supplemental Fig. 4B). These findings indicate that CD160$^{-/-}$ mice do not exhibit defects in clearance of *C. rodentium* infection. Because the clearance of *C. rodentium* infection is dependent on CD4$^+$ T cells, and not CD8$^+$ T cells (27, 28), it is likely that CD160$^{-/-}$ and WT mice had similar CD4$^+$ T cell responses to *C. rodentium*.

CD160$^{-/-}$ mice have impaired clearance of oral L. monocytogenes *infection*

Because CD160 is predominately expressed on IELs in the colon at steady state and induced on CD8$^+$ T cells upon activation, we next examined the functions of CD160 in regulating CD8$^+$ T cells and IELs functions during an oral *L. monocytogenes* infection using an OVA-expressing *L. monocytogenes* strain (16). We determined whether CD160 expression changes during the course of oral *L. monocytogenes* infection. CD160 expression on both TCRαβ$^+$ and TCRγδ$^+$ colonic IELs was upregulated on day 3, when *L. monocytogenes* burden in vivo is at the peak, and began to decline by day 7 after *L. monocytogenes* infection (Fig. 3A, 3B). The numbers of CD160-expressing, TCRαβ$^+$ colonic IELs (Fig. 3B) also were dramatically increased on day 7 postinfection. Similarly, CD160 expression was higher on CD8$^+$ T cells in the spleen and mLN (Fig. 3C) on day 7 postinfection compared with uninfected controls. These findings suggest a potential role for CD160 in regulating colonic IELs and CD8$^+$ T cells during *L. monocytogenes* infection.

To examine the functional significance of CD160 during *L. monocytogenes* infection, we challenged WT and CD160$^{-/-}$ littermates with oral *L. monocytogenes* infection and measured bacterial burden at the peak of infection 3 d later. WT and CD160$^{-/-}$ mice exhibited similar *L. monocytogenes* burden in the spleen, mLN, small intestine, and colon at peak disease (Fig. 4A). In contrast, on day 7 postinfection, CD160$^{-/-}$ mice had a higher *L. monocytogenes* burden in the colon compared with WT littermates (Fig. 4B), and markedly fewer CD160$^{-/-}$ mice (~18%) cleared *L. monocytogenes* in the colon compared with WT littermates (~70%) (Fig. 4C). However, CD160$^{-/-}$ mice exhibited similar *L. monocytogenes* burden in the spleen and mLN on day 7 postinfection (Fig. 4D). These results indicate defective clearance of *L. monocytogenes* in the colon of CD160$^{-/-}$ mice.

Although CD160 is predominately expressed on colonic IELs at steady state, CD160 also is expressed on intraepithelial ILC1 and NK cells, cells of the innate immune system (3, 4, 29). Therefore, impaired clearance of *L. monocytogenes* in CD160$^{-/-}$ mice may reflect altered functions of CD160-deficient colonic IELs, CD8$^+$ T cells, intraepithelial ILC1, and/or NK cells. To determine whether CD160 on intraepithelial ILC1 and NK cells contributes to optimal clearance of *L. monocytogenes*, we examined *L. monocytogenes* infection in RAG$^{-/-}$ mice. We orally infected WT RAG$^{-/-}$ and CD160$^{-/-}$ RAG$^{-/-}$ littermates with *L. monocytogenes* and measured bacterial burden at 3 and 7 d postinfection. WT RAG$^{-/-}$ and CD160$^{-/-}$ RAG$^{-/-}$ mice had similar *L. monocytogenes* burdens in the spleen, mLN small intestine, and colon on day 3 (Fig. 4E) and day 7 postinfection (Fig. 4F). The similar *L. monocytogenes* clearance in WT RAG$^{-/-}$ and CD160$^{-/-}$ RAG$^{-/-}$ mice suggests that CD160 on intraepithelial ILC1 and NK cells does not contribute to *L. monocytogenes* clearance. These studies indicate that impaired *L. monocytogenes* clearance in CD160$^{-/-}$ mice reflects alterations in colonic CD160$^{-/-}$ IELs and CD8$^+$ T cells.

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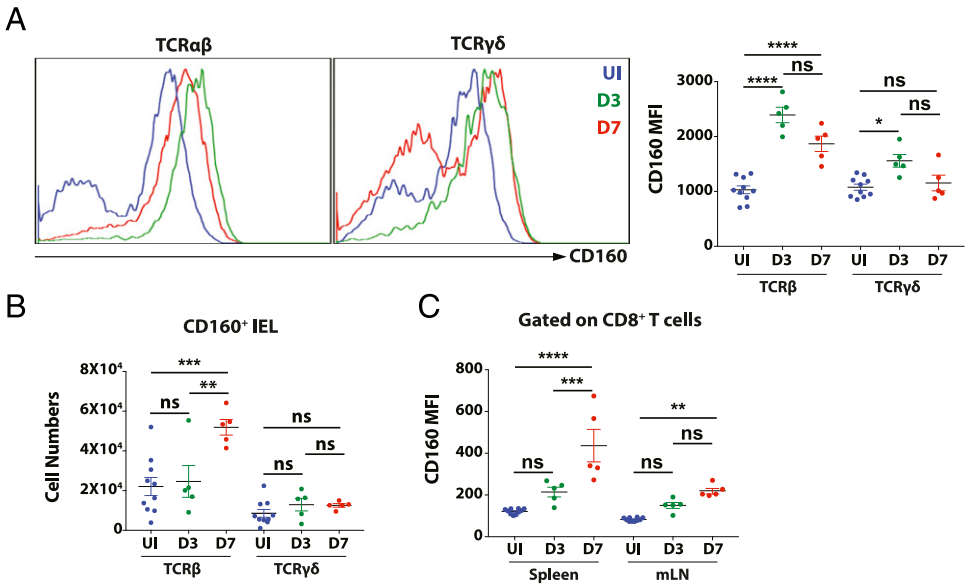

FIGURE 3. ***L. monocytogenes*** **infection promotes CD160 expression on IELs.**

(**A**) CD160 expression levels on TCRβ$^+$ and TCRγδ$^+$ colonic IELs from uninfected (UI) mice (blue) and at 3 (green) or 7 (red) d after oral *L. monocytogenes* infection with 2×10^9 *L. monocytogenes*. Representative plots are shown to the left and summary of CD160 mean fluorescence intensity (MFI) to the right. (**B**) Numbers of CD160-expressing TCRβ$^+$ and TCRγδ$^+$ colonic IELs from UI mice (blue) and at 3 (green) or 7 (red) d after oral *L. monocytogenes* infection with 2×10^9 *L. monocytogenes*. (**C**) CD160 expression levels on CD8$^+$ T cells isolated from spleen and mLN of UI mice (blue) or from mice at 3 (green) or 7 (red) d after oral *L. monocytogenes* infection with 2×10^9 *L. monocytogenes*. Data are represented as means ± SEM. The results are representative of at least two independent experiments. Significance was assessed using one-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$.

CD160 promotes CD8$^+$ T cell effector functions during L. monocytogenes *infection*

Because clearance of *L. monocytogenes* is dependent on an effective CD8$^+$ T cell response (30–34), we next examined if CD160 on CD8$^+$ T cells contributes to optimal clearance of *L. monocytogenes*. We first evaluated colonic IEL cellularity in WT and CD160$^{-/-}$ littermates on day 7 after *L. monocytogenes* infection. Colonic IELs from *L. monocytogenes*–infected WT and CD160$^{-/-}$ littermates were similar in number (Fig. 5A) and Ki67 expression (Fig. 5B), suggesting that impaired clearance of *L. monocytogenes* in CD160$^{-/-}$ mice is not due to defects in colonic IEL numbers or proliferation in CD160$^{-/-}$ mice. Next, we analyzed WT and CD160$^{-/-}$ colonic IEL effector functions on day 7 after *L. monocytogenes* infection. There were similar frequencies of WT and CD160$^{-/-}$ colonic IELs producing IFN-γ and TNF-α (Fig. 5C). However, the frequency of granzyme B–expressing colonic IELs in CD160$^{-/-}$ mice was reduced compared with WT littermates (Fig. 5D), pointing to a role for CD160 in regulating cytolytic function in IELs.

Because *L. monocytogenes* can spread systemically during infection (35, 36), we also characterized CD8$^+$ T cell responses in the spleen on day 7 after *L. monocytogenes* infection. The frequencies of OVA-specific (Fig. 6A) and Ki67-expressing (Fig. 6B) CD8$^+$ T cells were reduced, suggesting a proliferation defect in CD160$^{-/-}$ CD8$^+$ T cells. In addition, the frequency of granzyme B–expressing CD8$^+$ T cells was decreased significantly in CD160$^{-/-}$ mice compared with WT littermates (Fig. 6C). Likewise, the frequency of IFN-γ and TNF-α CD8$^+$ T cell double producers in the spleen in CD160$^{-/-}$ mice was less than WT littermates (Fig. 6D), as determined by stimulation of *L. monocytogenes*–ova-infected splenocytes with SIINFEKL peptide and subsequent evaluation of intracellular IFN-γ and TNF-α expression. These findings demonstrate that CD160 promotes CD8$^+$ T cell effector functions (including granzyme B, IFN-γ, and TNF-α production).

CD160 on CD8$^+$ T cells is required for optimal clearance of L. monocytogenes

Because CD160 is absent on a variety of cell types in CD160$^{-/-}$ mice, we further examined the role of CD160 on CD8$^+$ T cells for *L. monocytogenes* clearance using an adoptive transfer approach. We transferred naive WT or CD160$^{-/-}$ CD8$^+$ T cells into RAG$^{-/-}$ mice, infected them with *L. monocytogenes* the following day (Fig. 7A), and compared the course of infection. RAG$^{-/-}$ recipients receiving CD160$^{-/-}$ CD8$^+$ T cells had a higher mortality (Fig. 7B) and exhibited more weight loss (Fig. 7C) as well as a higher bacteria burden compared with RAG$^{-/-}$ recipients receiving WT CD8$^+$ T cells (Fig. 7D). These data demonstrate that CD160 is required on CD8$^+$ T cells for optimal clearance of *L. monocytogenes*.

DISCUSSION

There are data to support both coinhibitory and costimulatory functions for CD160. CD160 can promote NK cell cytotoxicity and

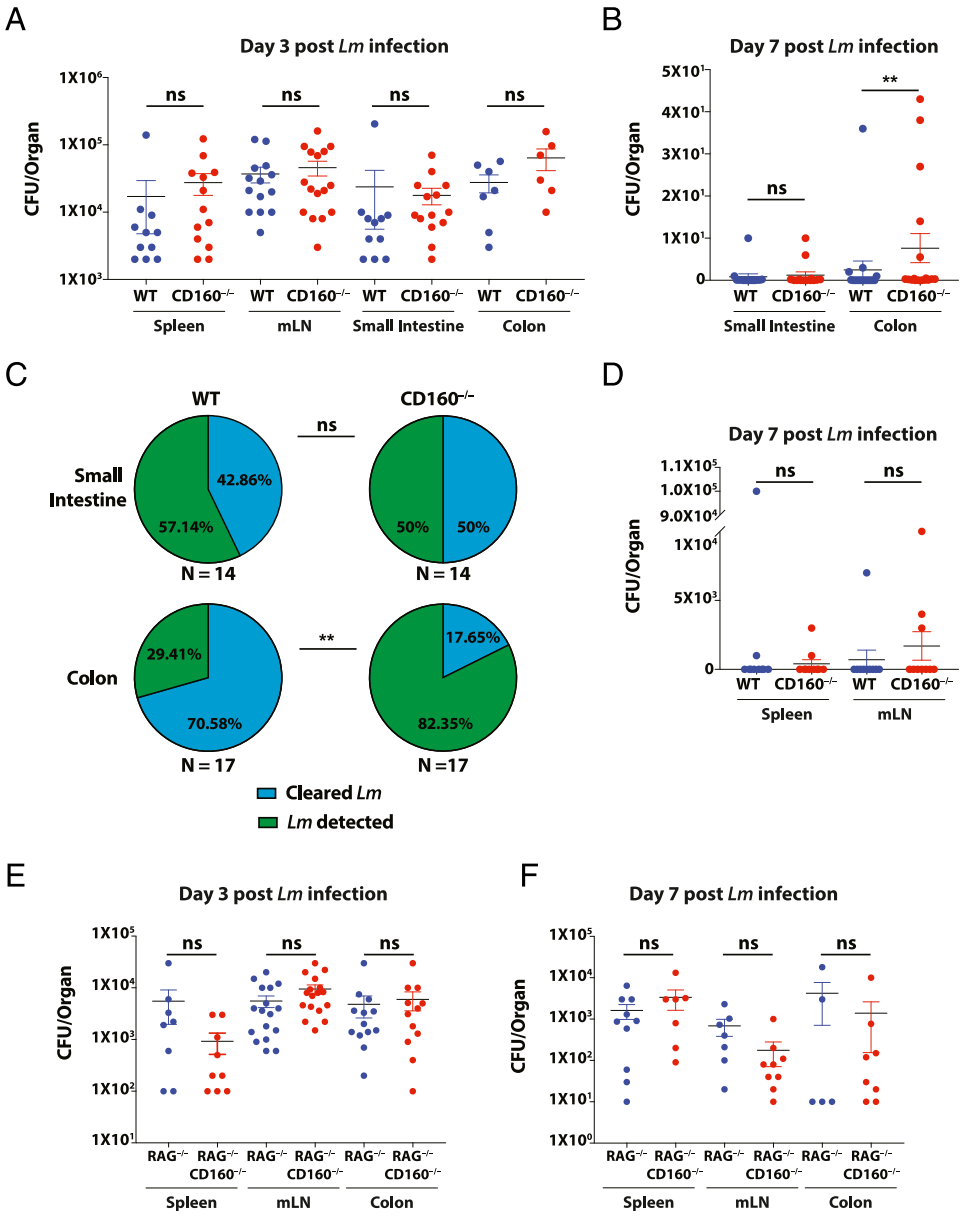

FIGURE 4. CD160$^{-/-}$ mice have impaired clearance of oral *L. monocytogenes* infection.

(**A**) Age-matched WT (blue circles, n = 7–16) and CD160$^{-/-}$ (red circles, n = 7–16) littermates were infected with 2 × 10^9 *L. monocytogenes* orally, and bacterial burden from spleen, mLN, small intestine, and colon was assessed on day 3 postinfection. (**B**) Bacterial burden from small intestine and colon on day 7 after oral *L. monocytogenes* infection with 2 × 10^9 *L. monocytogenes* from age-matched WT (blue circles, n = 14–17) and CD160$^{-/-}$ (red circles, n = 14–17) littermates. (**C**) Percentages of WT (n = 14–17) and CD160$^{-/-}$ (n = 14–17) littermates that cleared (cyan) versus those with detectable *L. monocytogenes* (green) in the small intestine and colon on day 7 after *L. monocytogenes* oral infection with 2 × 10^9 *L. monocytogenes*. (**D**) Bacterial burden from spleen and mLN on day 7 after oral *L. monocytogenes* infection with 2 × 10^9 *L. monocytogenes* from age-matched WT (blue circles, n = 10) and CD160$^{-/-}$ (red circles, n = 10) littermates. Age-matched RAG$^{-/-}$ (blue circles, n = 5–18) and CD160$^{-/-}$ RAG$^{-/-}$ (red circles, n = 7–18) littermates were infected with 2 × 10^9 *L. monocytogenes* orally, and bacterial burden from spleen, mLN, and colon was assessed on (**E**) day 3 and (**F**) day 7 postinfection. Data are pooled from four independent experiments, (A), (C), and (D). Data are pooled from three independent experiments, (B), (E), and (F). Significance was assessed using Mann–Whitney nonparametric test in (A), (B), (D), and (E) and χ^2 test in (C). Data are represented means ± SEM. **$p < 0.01$.

IFN-γ production in vitro and in vivo (4, 10, 13, 29). In vitro binding of HVEM to CD160 on human CD4$^+$ T cells delivered an inhibitory signal to the T cell (5), whereas anti-CD160 mAb enhanced the function of exhausted CD8$^+$ T cells from LCMV clone 13–infected mice in vitro (15). Less is known about the function of CD160 on T cells in vivo. Recent reports indicating that CD160 may regulate immune responses during enteric infections (8, 26) led us to investigate CD160 expression and function in the gut

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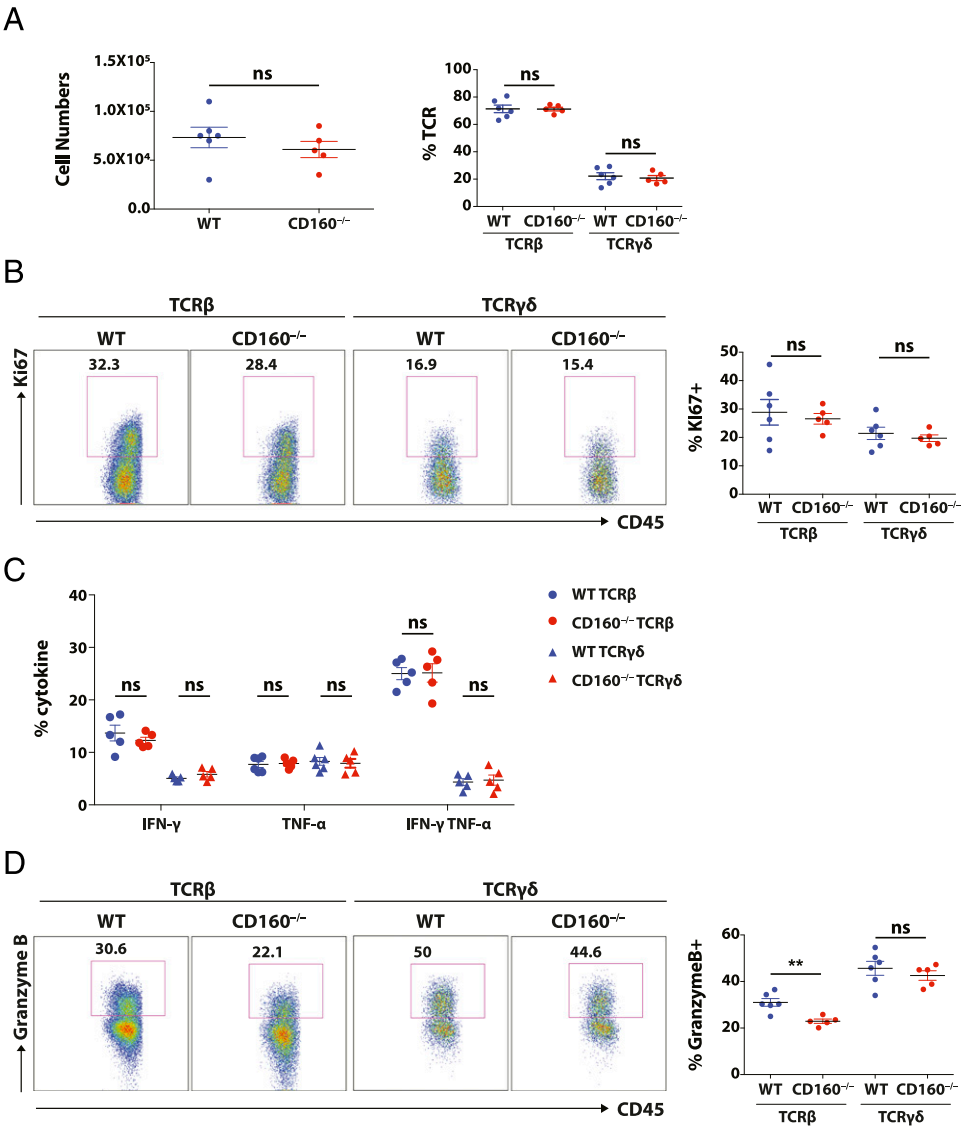

FIGURE 5. CD160$^{-/-}$ IELs have a defect in granzyme B production during *L. monocytogenes* infection.
(**A**) Numbers (left) and percentages (right) of IELs isolated from the colon of WT (blue circles) and CD160$^{-/-}$ (red circles) littermates on day 7 after oral infection with 2×10^9 *L. monocytogenes*. (**B**) Representative flow cytometry plots (left) and summary of percentages (right) of Ki67$^+$ TCRβ$^+$ and TCRγδ$^+$ IELs from the colon of WT (blue circles) and CD160$^{-/-}$ (red circles) littermates on day 7 after oral infection with 2×10^9 *L. monocytogenes*. (**C**) Percentages of WT TCRβ$^+$ (blue circles), CD160$^{-/-}$ TCRβ$^+$ (red circles), WT TCRγδ$^+$ (blue triangles), and CD160$^{-/-}$ TCRγδ$^+$ (red triangles) colonic IELs expressing IFN-γ, TNF-α, or both cytokines on day 7 after oral infection with 2×10^9 *L. monocytogenes*. (**D**) Representative flow cytometry plots (left) and summary of percentages (right) of granzyme B$^+$ TCRβ$^+$ and TCRγδ$^+$ IELs from the colon of WT (blue circles) and CD160$^{-/-}$ (red circles) littermates on day 7 after oral infection with 2×10^9 *L. monocytogenes*. Data are represented as means ± SEM. The results are representative of three independent experiments. Significance was assessed using Student *t* test. $^{**}p < 0.01$.

microenvironment as well as during acute and chronic LCMV infection. Our studies reveal that CD160 promotes CD8$^+$ T cell effector functions during oral infection with *L. monocytogenes* and is critical for optimal clearance of this acute bacterial infection.

We identified several factors that regulate CD160 expression in IELs in the intestinal microenvironment. We determined that TCR signals as well as cytokines drive CD160 expression on colonic IELs. Unexpectedly, we found that CD160 expression is elevated on colonic IELs from GF mice and antibiotic-treated mice, suggesting that the microbiota may downregulate CD160 expression on colonic IELs. A recent study elegantly demonstrated that interactions between CD160 on IELs and HVEM on intestinal epithelial cells result in HVEM signaling within intestinal epithelial cells that induces production of antimicrobial peptides and inflammatory cytokines critical for protection against *C. rodentium* infection (8). Our findings suggest that the microbiota

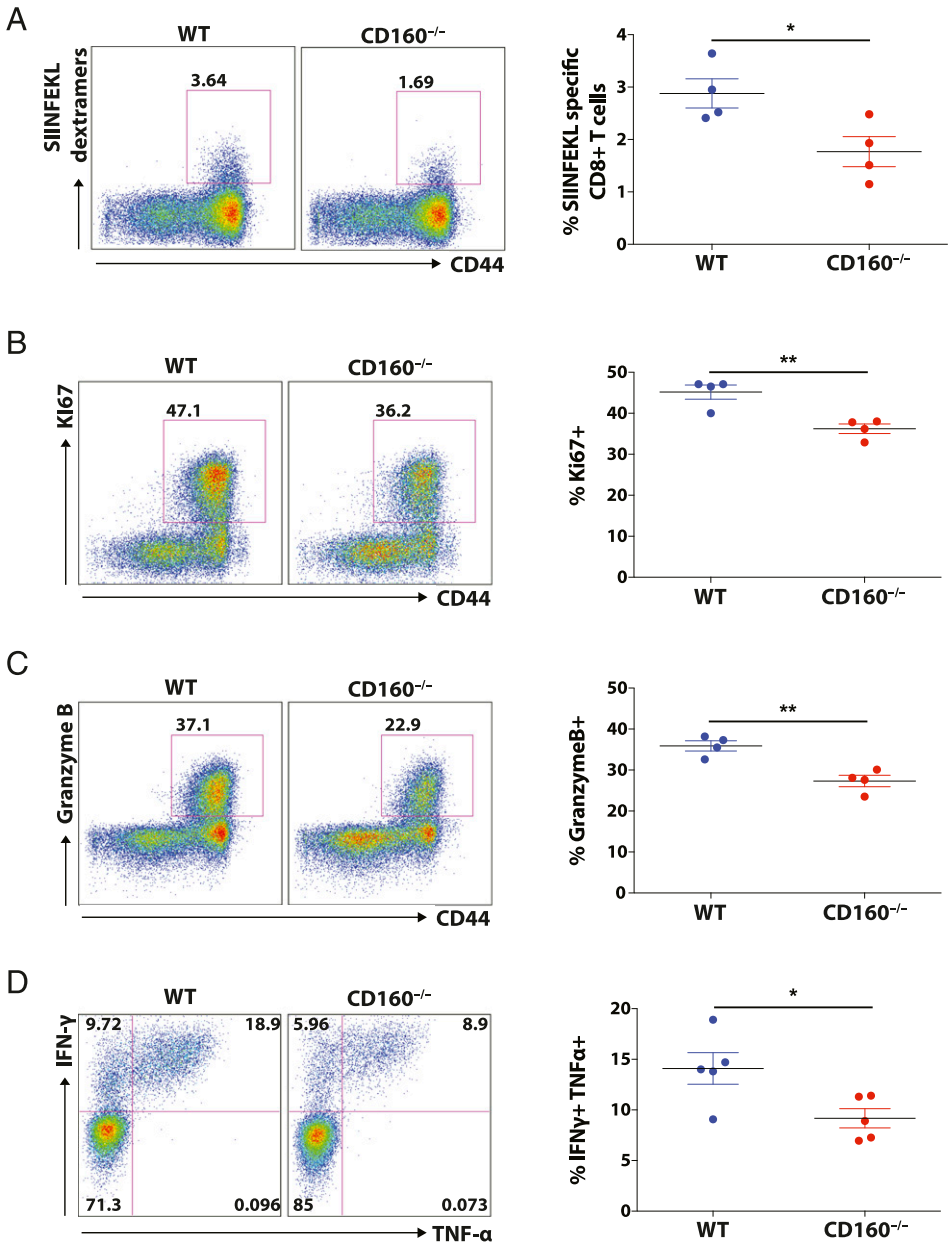

FIGURE 6. CD160 promotes CD8$^+$ T cells effector functions during *L. monocytogenes* infection.
Representative flow cytometry plots (left) and summary of percentages (right) of SIINFEKL-specific CD8$^+$ T cells (**A**), Ki67$^+$ CD44$^+$ CD8$^+$ T cells (**B**), granzyme B$^+$ CD44$^+$ CD8$^+$ T cells (**C**), and CD8$^+$ T cells producing IFN-γ, TNF-α, or both cytokines (**D**) in the spleens of WT (blue circles) and CD160$^{-/-}$ (red circles) littermates on day 7 after oral infection with 2×10^9 *L. monocytogenes*. Data are represented as means ± SEM. The results are representative of three independent experiments. Significance was assessed using Student *t* test. *$p < 0.05$, **$p < 0.01$.

may attenuate CD160 expression on colonic IELs as a mechanism to enable their optimal survival in the intestinal microenvironment. Downregulation of CD160 on IELs is partially dependent on TLR signaling based on analysis of colonic IELs from MyD88$^{-/-}$ mice in which we found that CD160 expression is reduced on MyD88$^{-/-}$ colonic IELs as compared with WT colonic IELs (C. Tan and A. H. Sharpe, unpublished observations). How the microbiota downregulate CD160 expression on colonic IELs and whether the downregulation of CD160 expression on colonic IELs is dependent on certain microbial species warrant future investigation.

Although recent studies revealed that interactions between CD160-expressing IELs and HVEM on intestinal epithelial cells were critical for mediating intestinal immunity during enteric infections (8, 26), the biological significance of CD160 signaling during an enteric infection is unknown. To address this issue, we compared oral infection of WT and CD160$^{-/-}$ mice with

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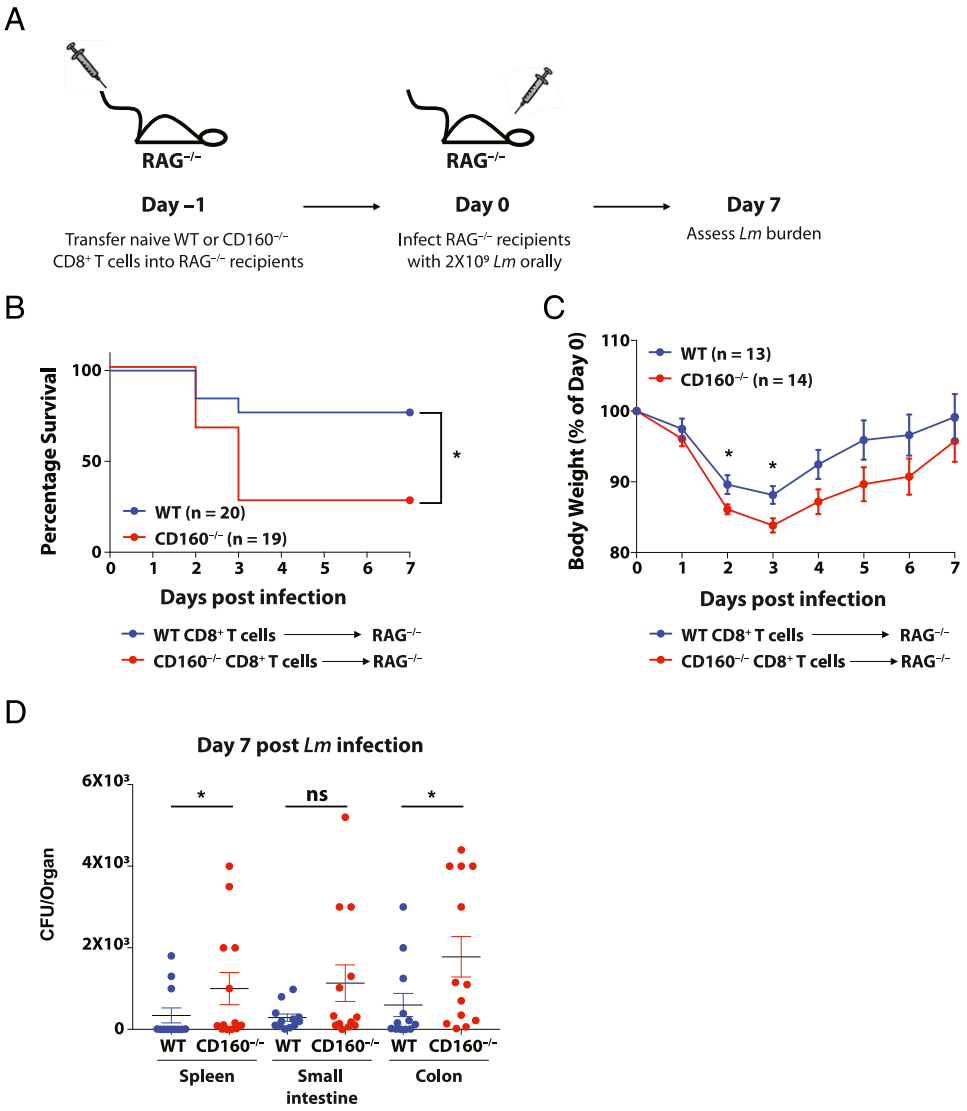

FIGURE 7. CD160 on CD8$^+$ T cells is required for optimal clearance of *L. monocytogenes*.

(**A**) Schematic of experiment designed to assess function of CD160 on CD8$^+$ T cells during oral *L. monocytogenes* infection. WT or CD160$^{-/-}$ naive CD8$^+$ CD8$^+$ T cell were transferred into RAG$^{-/-}$ recipients, infected with 2 × 10^9 *L. monocytogenes* orally on the following day, and analyzed for bacterial burden and morbidity. (**B**) Survival of RAG$^{-/-}$ recipients receiving 3 × 10^6 WT (blue, n = 20) or CD160$^{-/-}$ (red, n = 19) naive CD8$^+$ T cells and infected with 2 × 10^9 *L. monocytogenes* orally. (**C**) Weight curve of WT (blue, n = 13) and CD160$^{-/-}$ (red, n = 14) littermates infected with 2 × 10^9 *L. monocytogenes* orally. (**D**) Bacterial burden in spleen, small intestine, and colon from WT (blue circles, n = 11–12) and CD160$^{-/-}$ (red circles, n = 12) littermates on day 7 after oral infection with 2 × 10^9 *L. monocytogenes*. Data are pooled from four independent experiments in (B)–(D). Data are represented as means ± SEM. Significance was assessed using log-rank (Mantel–Cox) test in (B), Student t test in (C), and Mann–Whitney nonparametric test in (D). *$p < 0.05$.

C. rodentium and found comparable clearance. *C. rodentium* infection induces robust Th1 and Th17 responses, and the clearance of *C. rodentium* infection depends on CD4$^+$ T cells and not CD8$^+$ T cells (27, 28). Th17 responses are important for the control of *C. rodentium* infection (37, 38). We did not assess Th17 responses in CD160$^{-/-}$ mice infected with *C. rodentium*. It is possible that CD160$^{-/-}$ and WT mice were able to clear *C. rodentium* similarly because of comparable Th17 responses. Further work is needed to determine whether CD160 regulates CD4$^+$ T cells functions and Th17 responses during intestinal inflammation and enteric infections.

We also examined the role of CD160 during oral *Listeria* infection. We determined that CD160 was necessary in CD8$^+$ T cells (independent of CD160 functions on ILC1 and NK cells) for clearance of *L. monocytogenes* in the colon. The role of IFN-γ production by NK cells in early *L. monocytogenes* infection is controversial (39). Some studies suggest that IFN-γ production by NK cells is critical for resistance to *L. monocytogenes*, whereas

others suggest that NK cells do not take part in early host defense during *L. monocytogenes* infection (40–42) and that IFN-γ production by NK cells has no effect on control of *L. monocytogenes* infection (43). These conflicting results regarding the role of NK cells during *L. monocytogenes* infection are most likely due to differences in experimental setup such as Ab-mediated depletion of NK cells versus genetic knockout mouse models, route of *L. monocytogenes* infections (i.p., i.v., or oral gavage), and the background of the mouse strains (BALB/c versus C57BL/6). Hence, the role of NK cells during *L. monocytogenes* infection is still poorly understood. CD160-mediated IFN-γ production in NK cells plays a critical role in several tumor models (29), and CD160 on intraepithelial ILC1 cells may contribute to intestinal inflammation in an anti-CD40 colitis model (3). Although we did not assess IFN-γ production in CD160$^{-/-}$ NK cells or intraepithelial ILC1 cells during *L. monocytogenes* infection, our studies show that CD160 on CD8$^+$ T cells was required for optimal clearance of *L. monocytogenes*. Further work is needed to understand how CD160 regulates NK cell and intraepithelial ILC1 cell functions during intestinal inflammation and enteric infections.

Our studies support a costimulatory function in CD8$^+$ T cells during acute *L. monocytogenes* infection. We demonstrated that CD160 promotes CD8$^+$ T cell effector functions needed for optimal clearance of *L. monocytogenes*. The frequency of granzyme B–expressing colonic IELs was decreased in *L. monocytogenes*–infected CD160$^{-/-}$ mice, pointing to a role for CD160 in promoting colonic IEL functions during *L. monocytogenes* infection. Likewise, the frequencies of splenic CD8$^+$ T cells that produced granzyme B and IFN-γ and TNF-α double producers were reduced in CD160$^{-/-}$ mice. The reduced frequencies of granzyme B and IFN-γ may also indicate a role for CD160 in regulating CD8$^+$ T cell migration or survival. The regulation of colonic IEL functions is still poorly understood, and CD160$^{-/-}$ mice provide a useful means to study how the functions of IEL are regulated.

CD160 is a GPI-anchored protein that is localized in lipid rafts. Therefore, it is possible that CD160 may regulate the activation of TCR proximal signaling. Studies have suggested that CD160 may interact with protein tyrosine kinase p56lck and promote the PI3 kinase/AKT pathway (11, 44, 45). The reduced T cell effector functions in CD160$^{-/-}$ mice are consistent with a role for CD160 in modulating TCR signals and encourage investigation of mechanisms by which CD160 provides costimulatory signals.

Although CD160 is expressed on exhausted CD8$^+$ T cells (15, 24, 25) and in vitro studies support an inhibitory role for CD160 in exhausted CD8$^+$ T cells, we found no differences in viral clearance and CD8$^+$ T cell functions in CD160$^{-/-}$ and WT mice infected with LCMV clone 13. It may be that other inhibitory pathways compensate for CD160 during chronic LCMV in vivo. We also did not find any differences in responses of CD160$^{-/-}$ and WT P14 T cells to acute LCMV infection. Given the predominant expression of CD160 in the intestinal microenvironment and our findings, we speculate that CD160 may have a major role in mucosal immunity.

In summary, our work reveals a previously unappreciated stimulatory role for CD160 in promoting CD8$^+$ T cell effector functions during oral *L. monocytogenes* infection. Using CD160$^{-/-}$ mice, we demonstrated that CD160 is required for optimal granzyme B, IFN-γ, and TNF-α production by CD8$^+$ T cells following *L. monocytogenes* infection. Our work adds to the growing literature supporting a special immunoregulatory role for CD160 in the intestinal microenvironment. Further understanding of how CD160 regulates mucosal immunity is needed to determine how CD160 modulation could be used effectively to treat intestinal inflammatory diseases, infections, and cancer.

DISCLOSURES

The authors have no financial conflicts of interest.

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