Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions

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A new langerin⁺ DC subset has recently been identified in murine dermis (langerin⁺ dDC), but the lineage and functional relationships between these cells and langerin⁺ epidermal Langerhans cells (LC) are incompletely characterized. Selective expression of the cell adhesion molecule EpCAM by LC allowed viable LC to be easily distinguished from langerin⁺ dDC in skin and lymphoid tissue and ex vivo as well. Differential expression of EpCAM and langerin revealed the presence of at least 3 distinct skin DC subsets. We determined that LC and langerin⁺ dDC exhibit different migratory capabilities in vitro and repopulate distinct anatomic compartments in skin at different rates after conditional depletion in vivo. Langerin⁺ dDC, in contrast to LC, did not require TGFβ1 for development. Carefully timed gene gun immunization studies designed to take advantage of the distinct repopulation kinetics of langerin⁺ dDC and LC revealed that langerin⁺ dDC were required for optimal production of β-galactosidase-specific IgG2a/c and IgG2b in the acute phase. In contrast, immunization via LC-deficient skin resulted in persistent and strikingly reduced IgG1 and enhanced IgG2a Ab production. Our data support the concepts that LC and langerin⁺ dDC represent distinct DC subsets that have specialized functions and that LC are important immunoregulatory cells. The presence of at least 3 functionally distinct skin DC subsets may have particular relevance for vaccines that are administered epicutaneously.

EpCAM | gene gun | langerin | TGF-beta

The remarkable phenotypic heterogeneity of DC, both between and within certain tissues, has been long recognized. To date, however, it has been possible to clearly relate DC phenotype to DC function in only a few instances, even in mice. For example, plasmacytoid DC are recognized as the primary source of virus-induced type I IFN (1), CD8α⁺ lymph node DC are largely responsible for cross-presentation of cell-associated antigen to CD8 T cells (2–4), and 33D1-reactive (DCIR2⁺) splenic DC (as compared with CD205⁺ DC) preferentially stimulate CD4 T cells (5). Epidermal Langerhans cells (LC) represent perhaps the most striking example of an extensively studied tissue DC subpopulation whose function is incompletely understood.

LC have long been thought to play pivotal roles in initiating immunity by acquiring antigens that are encountered in skin, migrating to draining LN after activation, and stimulating antigen-specific T cells (6). However, recent studies suggested that LC do not function as essential antigen-presenting cells for anti-viral immune responses (2, 7) or for contact hypersensitivity reactions (8–12) in established murine models. Studies of LC have been challenging, in part, because readily detectable cell surface proteins that are constitutively expressed by epidermal LC and LC that have emigrated from epidermis have not been well recognized. The C-type lectin langerin has been regarded as a pathognomonic LC marker, but recent studies suggest that this

protein is present in at least some murine CD8α⁺ DC in LN (12, 13). In addition, a new subset of dermal DC that express langerin (langerin⁺ dDC) was recently identified (10, 14, 15). We previously demonstrated that the putative cell adhesion molecule EpCAM was selectively expressed by LC and their derivatives (16), and Bursch *et al.* have reported that this feature could be used to distinguish LC from langerin⁺ dDC (10).

In the present study, we used EpCAM expression to distinguish LC and langerin⁺ dDC to clarify their lineage and functional relationships. We provide evidence that LC and langerin⁺ dDC develop independently, exhibit distinct repopulation kinetics and migratory properties in vitro and in vivo, and play distinct roles in humoral and cellular responses elicited by gene gun immunization.

Results

Differential Expression of Langerin and EpCAM by Murine Skin Dendritic Cells. We previously reported that EpCAM was expressed on the cell surfaces of viable murine LC and a subset of DC in skin-draining LN, but not by DC in mesenteric LN (16). Thus, it was expected that epidermal LC would express EpCAM in situ in epidermal sheets (Fig. 1A) and in epidermal cell suspensions (Fig. S1A). The latter results contrast with what was found in the case of langerin, where intracellular staining was bright, while cell surface staining was dim to absent (SI Text Fig. S1B). Several recent reports described a new langerin-expressing DC subset in murine dermis (langerin⁺ dDC) (10, 14, 15), and it has been reported that EpCAM expression distinguished LC from langerin⁺ dDC (10). By staining for EpCAM and langerin in dermal sheets, we confirmed the existence of 2 subsets of cells; EpCAM⁺ langerin⁺ DC that correspond to LC that have emigrated from epidermis and EpCAM⁺ langerin⁺ DC that represent the recently identified langerin⁺ dDC (Fig. 1A).

Staining LN and spleen from several strains of mice (C57BL/6, BALB/c, and C3H) for EpCAM revealed that EpCAM^{high} DC were found only in skin-draining LN, that they were similarly

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Fig. 1. Differential EpCAM expression by Langerhans cells and langerin⁺ dDC. (A) Epidermal and dermal sheets stained with anti-langerin and anti-EpCAM mAb. Green to yellow cells (arrows) in the overlay coexpress langerin and EpCAM, whereas red cells (arrowheads) are langerin single positive. (B) Dermal cell suspensions obtained from skin of Langerin-GFP mice (12) assessed for EpCAM expression. Analysis was restricted to CD45⁺ MHC II^{high} cells. (C) Cell suspensions obtained from whole ear skin of C57 BL/6 WT mice. Analysis was restricted to CD45⁺ CD11c⁺ cells. Langerin was detected by anti-langerin Ab ($n = 3$, 2 mice pooled per group). Langerin-DTR mice (9) were injected i.p. with 1 μ g of diphtheria toxin, and epidermal sheets (D) were taken 14 days after. Dermal sheets (E) harvested at indicated time points were stained for langerin and EpCAM. (Scale bars, 20 μ m.) Results are representative of at least 3 similar experiments.

abundant in the 3 mouse strains, and that lymph node langerin⁺ DC comprised 2 populations that differentially expressed EpCAM (Fig. S1 C and D). Thus, EpCAM expression can be used to differentiate LC from langerin⁺ DC in skin and in LN and to discriminate viable LC from all other DC. Although langerin⁺ DC were focally abundant when observed *en face* in dermal sheets (Fig. 1A), they were not uniformly distributed. Assessment of DC frequencies in suspensions of dermal cells from ear skins indicated that LC and langerin⁺ dDC represent minor subpopulations of CD11c⁺ dDC (Fig. 1B). Interestingly, immunophenotyping of CD11c⁺ DC extracted from ear skin that included both epidermis and dermis revealed that LC and langerin⁻ EpCAM⁻ dDC were the predominant cells and were similarly abundant, while langerin⁺ dDC comprised <5% of the total (Fig. 1C).

Distinct Repopulation Kinetics of Langerhans Cells and Langerin⁺ dDC After Conditional Depletion. Langerin-diphtheria toxin receptor knockin (Langerin-DTR) mice represent a powerful tool with

which to investigate the origin and trafficking of langerin-expressing cells (9, 12). We used these mice to study the possible relationships between LC and langerin⁺ dDC in the epidermis and the dermis. Administration of a single 1- μ g dose of diphtheria toxin (DT) led to virtually complete depletion of epidermal and dermal langerin⁺ cells within 24–48 h, as previously reported (9, 12). Careful examination of epidermal sheets revealed rare langerin⁺ cells, present as single cells, as early as 3 days after DT treatment, and langerin⁺ cells present as small clusters of cells by 7 days post-DT treatment. The number of langerin⁺ epidermal “colonies” slowly increased over time, but LC did not fully reconstitute the epidermis within the 6-week observation period (Fig. S2). Repopulating langerin⁺ LC in epidermis uniformly expressed EpCAM (Fig. 1D). Repopulation of conditionally depleted dermis with EpCAM⁻ langerin⁺ dDC occurred much more rapidly and was virtually complete by 7 days (Fig. 1E), consistent with previous results (10, 14). Interestingly, small numbers of LC and langerin⁺ dDC in the dermis expressed Ki-67 in repopulating skin, indicating that both of these populations had proliferative potential (Fig. S3A).

In mice that were lethally irradiated and transplanted with CD45 congenic bone marrow, the majority of epidermal LC were of host origin, as expected (17), and were EpCAM-positive (Fig. S3B). In contrast, the vast majority of the langerin⁺ dDC and CD11c⁺ langerin⁻ DC in the dermis were of donor origin, as reported (17), and were EpCAM-negative, in line with the conditional depletion model.

TGF β 1 Dependence of Langerin Expressing Epidermal and Dermal Dendritic Cells. We previously reported that TGF β 1 is required for development of murine LC *in vivo* (18). To ascertain the dependence of the langerin⁺ cells in the dermis on TGF β 1, we stained epidermal and dermal sheets from adult TGF β 1^{-/-} Rag2^{-/-} mice and appropriate control mice for EpCAM and langerin. The epidermis from TGF β 1^{-/-} Rag2^{-/-} mice was devoid of EpCAM and langerin expressing LC, while LC were appropriately represented in epidermis from TGF β 1^{+/-} mice (Fig. 2A). Dermal sheets from TGF β 1^{+/-} mice contained both EpCAM⁺ langerin⁺ DC and EpCAM⁻ langerin⁺ dDC, whereas dermis from TGF β 1^{-/-} mice contained only EpCAM⁻ langerin⁺ dDC (Fig. 2B). These results indicate that langerin⁺ dDC develop and localize in skin in the absence of TGF β 1, whereas LC are strictly dependent on this cytokine for development, localization, and/or survival. The observation that langerin⁺ DC in the epidermis and the dermis differentially expressed EpCAM, that they repopulated their respective compartments with different kinetics after conditional depletion and in BM chimeras, and that they were differentially dependent on TGF β 1 is consistent with the concept that epidermal LC and langerin⁺ dDC represent distinct subsets.

Distinct Migrating Properties of Cutaneous Dendritic Cell Subsets. To begin to explore the functional characteristics of langerin-expressing skin DC, we studied DC that migrated from skin explants *in vitro*. It has been reported that CD11c^{low} CD205^{high} cells in skin and skin draining LN correspond to LC, whereas CD11c^{high} CD205^{low} cells represent dDC (19). We studied migratory DC from ear skin explants to determine whether EpCAM was expressed by LC and dDC, as defined by CD11c and CD205 expression profiles. We obtained DC that migrated from ear skin explants in the presence or absence of the CCR7 ligand CCL21 (20). The majority of migratory DC, CD11c^{low} CD205^{high} cells, expressed high surface levels of EpCAM in the presence or absence of CCL21 (Fig. 3A).

Interestingly and consistent with previous reports (19), migration of CD11c⁺ EpCAM⁻ (CD11c^{high} CD205^{low}) dDC was markedly increased by CCL21 (Fig. 3A and B). Enhancing effects of CCL21 on CD11c⁺ EpCAM⁺ LC migration from skin

Fig. 2. TGF β 1-dependency of langerin-expressing skin dendritic cells. Epidermal (A) and dermal (B) sheets from ear skin were obtained from 2 TGF β 1^{-/-} Rag2^{-/-} mice and 1 TGF β 1^{+/-} Rag2^{-/-} control littermate and stained for langerin and EpCAM. Scale bars, 10 μ m. Arrowheads indicate migrating LC, and arrows point to langerin⁺ dDC. HF, hair follicles.

explants were less striking (Fig. 3B). The vast majority of EpCAM⁺ DC that emigrated from skin explants expressed high levels of langerin. Surprisingly, EpCAM⁺ DC that emigrated from skin explants in response to CCL21 were also langerin⁺, but

stained less intensely, indicating that this subset corresponded to langerin⁺ dDC (Fig. 3A). It appears that CD11c⁺ langerin[−] “classic” dDC do not efficiently migrate from skin explants, even in the presence of CCL21.

These results prompted us to look for evidence of differential migration of LC and langerin⁺ dDC in vivo. In situ staining for LC and langerin⁺ dDC in skin-draining LN revealed that langerin⁺ DC accumulated predominantly in T cell-rich areas, and most of these cells appeared to be EpCAM-positive (Fig. 3C). Examination of multiple sections from multiple lymph nodes identified langerin⁺ DC in the B cell-rich areas (Fig. 3C) in some, but not all, sections. The majority of these cells were EpCAM-negative, suggesting that the 2 langerin⁺ DC (LC and langerin⁺ dDC) may exhibit different migratory characteristics in vivo as well. Langerin⁺ DC in B cell zones did not express CD8 α , excluding the possibility that they correspond to CD8 α ⁺ lymph node DC (Fig. 3D).

Differential Involvement of Langerin⁺ Dendritic Cells in Humoral Immune Response. To determine if LC and langerin⁺ dDC have different functions, we performed gene gun immunization studies with β -galactosidase as the antigen, taking advantage of the differential repopulation kinetics that the 2 langerin-expressing DC display after conditional depletion in Langerin-DTR mice. Mice that were treated with DT 13 days before gene gun immunization had a normal complement of langerin⁺ dDC (Fig. 1E), but very few LC. In contrast, mice that had been treated with DT 1 day before immunization lacked both LC and langerin⁺ dDC. Langerin⁺ DC-depleted skin is expected to contain abundant classic CD11c⁺ langerin⁻ dDC (see Fig. 1B and C) at all times.

An advantage of using the gene gun as a means of antigen delivery is that small amounts of antigens are produced exclusively in the skin or by cells that emigrate from the skin after gene gun immunization. Because Ab isotype production is influenced by the character of T helper cells that are involved, possible immunoregulatory influences could be assessed by evaluating qualitative changes in IgG isotype concentrations. In previous studies, gene gun immunization has been reported to preferentially elicit Th2-predominant responses (21). Langerin-DTR

Fig. 3. Distinct migratory properties of cutaneous dendritic cell subsets. (A) Migratory DC from skin explants obtained in the presence or the absence of the chemokine CCL21. Shown are dDC and LC populations as defined by CD11c and CD205 expression (*Top*), by CD11c and EpCAM expression (*Middle*), and by langerin and EpCAM (*Bottom*). Analysis was restricted to CD11c⁺ cells. (B) Effects of CCL21 on migratory capacities of CD11c⁺ EpCAM⁺ and EpCAM[−] DC. Lines depict individual experiments. (C) Frozen section from cervical LN stained for langerin and EpCAM. (D) Inguinal LN stained for langerin and CD8 α . (Scale bars, 50 μ m.)

but we do not have data that addresses this issue directly. Kissenpfennig *et al.* (12) used GFP expression in DC from langerin-GFP transgenic mice as a surrogate marker for langerin expression. Henri *et al.* (19) used an anti-langerin mAb (HD24) that is not commercially available. Clone 929F3, the rat anti-langerin mAb used by Poulin *et al.*, was raised against recombinant human langerin and cross-reacts with both mouse and rat langerin. It is certainly possible that L31 [the rat anti-langerin mAb (22) that we used and that was raised using mouse langerin as the immunogen] is a more sensitive reagent than either of these mAbs. The migratory DC that Kissenpfennig *et al.* categorized as langerin[−] based on lack of transgene expression (GFP in langerin-GFP mice) could also correspond to those that we have determined express low, but still detectable, levels of langerin (see Fig. 2D), either because there is not perfect correspondence between GFP and langerin protein expression or because detection of langerin with the L31 mAb is more sensitive than detection of GFP directly. Studies of DC migration in vivo in the setting of contact sensitivity reactions seem to clearly indicate that langerin[−] dDC can move from skin to lymph node (9, 12), suggesting that CD11c⁺ EpCAM[−] langerin[−] dDC migrate in response to stimuli that have yet to be identified.

Although LC have long been regarded as initiators of immune responses to antigens that are acquired in skin, recent studies indicated that LC were not essential for initiating anti-viral immune responses (2, 7) or contact hypersensitivity reactions (9, 11, 12). LC have also been reported to be dispensable for humoral and cellular responses initiated by gene gun immunization (23), but these studies were carried out and interpreted before the existence of langerin⁺ dDC was known. In retrospect, the protocol used in prior gene gun immunization studies involved weekly immunizations and repeated treatments of langerin-DTR mice with DT (23), and it is difficult to interpret the results. We sought to better define the function of LC and langerin⁺ dDC in the context of a response to a strong antigen (β -galactosidase) delivered once into skin via gene gun immunization. Distinct repopulation kinetics of LC and langerin⁺ dDC in Langerin-DTR mice after conditional depletion provided an opportunity to immunize mice when only LC were absent or when both LC and langerin⁺ dDC were absent. This allowed us to make strong inferences about the functional activities of each DC subset. We were interested in measures of Ab formation as well as cellular immunity, because we noted that in skin-draining regional LN, most LC were localized in T-cell areas while some langerin⁺ dDC were found in B cell zones.

In aggregate, the gene gun immunization data suggest that langerin⁺ dDC participate in the initiation of humoral immune responses and are required for optimal Ab formation of IgG2a/c and IgG2b isotypes early in the response. LC, in contrast, appear to regulate Ab-forming responses by facilitating IgG1 formation, a Th2-dependent process. Th1-dependent IgG2a/c formation was augmented in mice immunized via LC-deficient skin. This result could reflect a compensatory shift in the Th1/Th2 balance or, theoretically, a direct inhibitory effect of LC on Th1 response. The only cellular immune responses that could be quantified in mice immunized via this single immunization protocol were IFN γ -forming cells in spleen. IFN γ -producing cells could not be detected in relevant regional LN, and IL-5- and IL-17-forming cells could not be enumerated in LN or spleens. Quantification of IFN γ -producing T cells using MHC class I binding peptide (for CD8 T cells) and whole protein (for CD4 T cells) did not reveal a role for LC and suggested that langerin⁺ dDC acted as negative regulators of Th1 cellular responses.

Our Ab formation data are, on a superficial level, consistent with recent data obtained with langerin-DTR mice that indicates that when application of contact sensitizer is carefully timed in relation to DT administration, langerin⁺ dDC appear to be required for optimal contact hypersensitivity reactions measured

at 5 days (24), and previous data indicating that hypersensitivity reactions are actually enhanced in mice that are selectively deficient in LC (11). Although one could speculate the Ab results are in some way related to our data that suggest that langerin⁺ dDC may localize in B-cell areas while LC are found in T-cell zones, significant mechanistic insights will require additional experiments. Our results raise the possibility that targeting or depleting skin DC subsets during epicutaneous vaccination will be a useful strategy with which to augment or shape humoral immune responses. Anti-CD205 and anti-DCIR2 (33D1) have previously been used to preferentially deliver antigens to murine DC subpopulations (5, 25), and very recent results indicate that anti-langerin Ab (L31) will also be useful (26). Given that most langerin that is expressed by LC and langerin⁺ dDC is sequestered inside cells and is therefore not readily accessible for Ab binding, langerin may not be an optimal target for antigen delivery. In addition, it is now clear that langerin is expressed by at least 3 DC subsets in mice. Because EpCAM is expressed by LC constitutively and at high levels, anti-EpCAM Ab may be an alternative and perhaps more effective vehicle for preferentially directing physically associated antigens to LC. Selective targeting of antigen to LC using Ab-based methodology should also provide additional insights into the physiology of these enigmatic cells.

Materials and Methods

Mice. Adult female C57BL/6, C3H/HeN, and BALB/c mice were obtained from Charles River Laboratories. Heterozygous C57BL/6 Langerin-DTR mice (9, 12) were obtained by breeding homozygous transgenic males with wild-type females. TGF β 1^{+/−} Rag2^{−/−} mice were obtained from the Mouse Models of Human Cancer Repository (National Cancer Institute, MD) and experimental animals were obtained by interbreeding. All mice were bred and housed in a pathogen-free environment and used in experiments in accordance with institutional guidelines. Results depicted in this article were obtained with C57BL/6 mice unless otherwise indicated.

Preparation of Epidermal and Dermal Sheets. Ears were split into dorsal and ventral halves, cartilage and s.c. tissue were removed, and skin was floated on 3.8% ammonium thiocyanate (Sigma-Aldrich) in 100 mM sodium phosphate/100 mM potassium phosphate for 20 min at 37 °C. Epidermal and dermal sheets were separated and fixed in acetone at −20 °C for 15 min.

Preparation of Cell Suspensions for Flow Cytometry. Epidermal cells were prepared as described previously with slight modifications (27). Briefly, ears were split into dorsal and ventral halves and incubated in HBSS containing 0.5% trypsin and 1 mM EDTA for 45 min at 37 °C. Epidermis was peeled from the dermis and dissociated into single cells using vigorous trituration. Leukocytes were enriched by flotation on Lympholyte M (Cedarlane). To prepare single cell suspensions from dermis, dermal sheets were minced and incubated for 2.5 h in collagenase D (Worthington or Roche). Whole skin cell suspensions were prepared by incubating split ears with Liberase CI (Roche) for 45 min at 37 °C after removal of cartilage and s.c. fat. After digestion, residual tissue was minced and disrupted in a Medimachine (BD Biosciences) in complete RPMI containing 0.05% DNase. Single-cell suspensions were prepared from fragments of lymphoid organs after incubation in collagenase D for 30 min at 37 °C. Low density cells were enriched from lymph node cell and splenocyte preparations using metrizamide density gradients (Sigma).

Preparation of Migratory Dendritic Cells. DC that migrated from skin explants were prepared as previously described (19). Mouse ears were split into dorsal and ventral halves and floated on complete RPMI media with or without 0.1 μ g/mL CCL21 (R&D Systems) for 24 h. Migratory DC were collected, and wells were replenished with fresh culture media. Collected migratory DC were stored at 4 °C, pooled with emigrants harvested after an additional 24-h incubation period, and analyzed via flow cytometry.

Antibodies. Rat IgG2a κ anti-mouse EpCAM mAb (clone G8.8) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) and labeled with either Alexa Fluor 488 or 647 (Invitrogen). Purified rat IgG2a κ anti-mouse langerin mAb (clone L31) was provided by Dr. Ralph Steinman, Rockefeller University, New York, NY) and labeled with Alexa Fluor 488 and 647 or PE (Dojindo). Two independent rat IgG2a κ clones were used for

blocking (BD PharMingen) or as isotype controls (Serotec). Additional mAb were used for immunofluorescence microscopy and flow cytometry to detect the following: CD11c (N418, eBioscience), MHC class II (M5/114.15.2, BD PharMingen), CD205 (NLDC-145, Serotec), CD45 (30F-11, BD PharMingen), Ki-67 (Novacastra), and CD8 α (53-6.7, Biolegend). Anti-mouse CD16/32 (10 μ g/mL) (2.4G2, BD PharMingen) was routinely used to block Fc γ receptors before staining.

Immunofluorescence Microscopy. Vertical sections of skin and epidermal sheets were fixed in cold acetone and blocked in 3% dry milk-PBS (Bio-Rad Laboratories) and anti-CD16/32 mAb before incubation with fluorochrome-labeled mAb for 1 h at room temperature or overnight at 4 °C. Rat IgG2a κ mAb (10 μ g/mL) (BD PharMingen) was added to blocking buffer to minimize isotype-related nonspecific staining when directly labeled L31 and G8.8 mAb were used. Goat anti-rabbit Ab conjugated with AlexaFluor 568 was used for secondary detection of Ki-67 Ab. All immunofluorescence images were collected and visualized with an AxioImager A1 immunofluorescence microscope (Zeiss) or DeltaVision (Applied Precision). Intensity levels of digital images in experimental and control specimens were adjusted linearly either in Axiovision or Photoshop CS2 (Adobe).

Flow Cytometry. Data were collected with FACSCalibur (BD Biosciences) or LSR II (BD Biosciences) devices and analyzed with FlowJo software (Treestar). Nonviable cells were excluded after 7-AAD staining, unless cells had been fixed and permeabilized before analysis.

Conditional Depletion of Langerin⁺ Cells and Gene Gun Immunizations. Heterozygous Langerin-DTR mice (9) received i.p. injections of 1 μ g of diphtheria toxin (Biomol) in 200 μ L of PBS. Gene gun immunization was performed by delivering gold particles coated with plasmid DNA encoding β -galactosidase from *Escherichia coli* (pSport- β Gal) or empty plasmid (pcDNA3.1) after DT at indicated time points with Gene Gun Helios (BD) on the shaved abdomen of mice at 3 nonoverlapping sites.

Generation of Bone Marrow Chimeric Mice. Eight-week-old recipient CD45.1⁺ mice were lethally irradiated (1,200 rads delivered in 2 doses of 600 rads each

at 3-h intervals), and injected i.v. with 1×10^6 BM cells obtained from congenic CD45.2⁺ mice. Complete chimerism ($\geq 95\%$) was assured by measuring the percentages of donor cells among total B220⁺ B cells, Ly6C/G⁺ CD115⁺ granulocytes, and CD115⁺ monocytes in the blood 3 weeks after transplantation. Chimerized mice were analyzed 8 weeks after BM reconstitution.

ELISA. β -Galactosidase from *E. coli* (Sigma) was coated (50 ng per well) onto Maxisorp ELISA plates (Nunc) via incubation at 4 °C overnight. After blocking, sera from experimental animals were serially diluted in PBS with 1% BSA and incubated for 90 min at room temperature. After washing, plates were incubated with HRP-conjugated goat anti-mouse IgG or isotype-specific Ab (Jackson) for 90 min, developed with TMB substrate solution (Pierce), and enzymatic reactions were halted with 1 N H₂SO₄ before determining the OD at 450 nm. Statistical differences were confirmed by 1-way ANOVA, and then individual groups using Turkey's post test (Minitab software package ver.14, Penn State University).

ELISPOT Assays. Splenocytes (5×10^5 per well in 96-well plates) were cultured with β -galactosidase (10 μ g/mL) or incubated with β -gal peptide (20 μ g/mL)-pulsed MC38 cells (1×10^4 per well) for 24 h, and IFN γ -producing cells were enumerated by ELISPOT assay as described in ref. 28 using matched Ab pairs (R&D Systems). Plates were developed using streptavidin-alkaline phosphatase (100 μ L per well of 1/1,500 dilution; Mabtech) and 100 μ L per well of BCIP-NBT phosphatase substrate (Vector Laboratories). Statistical analysis was performed using Student *t* test using Prism software (GraphPad).

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