Extramedullary hematopoiesis in the spleen contributes to natural killer cell development during infection and inflammation

In adult mammals, hematopoiesis predominantly occurs in the bone marrow (BM), where hematopoietic stem cells (HSC) generate all major blood cell lineages through intermediate progenitor stages.[1] Under pathological conditions, including acute infection and systemic inflammation, hematopoiesis can shift to extramedullary sites, a process known as extramedullary hematopoiesis (EMH). EMH has been observed in a wide range of species, including humans, rodents and other mammals, and is most commonly associated with the spleen and liver.[2] It is typically characterized by an increase in erythroid progenitors, granulocyte-macrophage progenitors and megakaryocytes at these sites.[3] During acute viral infection, murine cytomegalovirus (MCMV) triggers rapid, robust splenic EMH in erythroid and myeloid compartments.[4,5] Whether lymphoid lineages undergo EMH during infectious diseases remains poorly understood.

Natural killer (NK) cells have a crucial role in the innate immune response against viral infections by producing interferon-gamma (IFNγ) and exhibiting cytotoxic functions.[6] NK cells arise from HSC, progress through common lymphoid progenitors (CLP) to multipotent innate lymphoid progenitors, such as innate lymphocyte progenitors (ILCP), then commit as NK-cell progenitors (NKP) and differentiate into immature NK (iNK) and mature NK (mNK) cells.[7] Although the BM has long been considered the principal site of NK-cell development in adults, there is growing evidence - particularly in humans – to indicate that ILCP primarily reside in blood and secondary lymphoid organs and can expand and differentiate within peripheral tissues in response to inflammatory cues.[8] However, direct evidence for peripheral NK-cell development during infectious diseases remains limited.

Murine cytomegalovirus elicits a robust NK-cell-dependent immunity and represents a tractable model to test whether systemic infection directs NK-cell generation at extramedullary tissues, such as the spleen, rather than merely mobilizing existing NK cells. In this study, we demonstrate that MCMV infection and sterile inflammation are associated with marked accumulation of NKP in the spleen, accompanied by a diminished capacity of the BM to produce NK cells. Using transplantation experiments, we show that splenic NKP can differentiate into mature classical NK cells capable of producing IFNγ upon activation, establishing functional competence. These findings provide direct evidence that EMH extends to a lymphoid lineage under inflammatory conditions and identify the spleen as a dynamic site for NK-lineage production during systemic stress. These insights refine the concept of infection-induced EMH beyond the classical boundaries and have implications for understanding antiviral immunity and the targeting of hematopoietic niches in infectious and inflammatory conditions.

Wild-type (C57BL/6N, Ly5.2), tdTomato transgenic mice (B6.129(Cg)-Gt(ROSA)26Sor$^{<tm4(ACTB-tdTomato,-EGFP)Luo>}$/J) and Ly5.1 (B6.SJL-Ptprca Pepcb/BoyJ) mice were bred under specific pathogen-free conditions according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines at the University of Veterinary Medicine Vienna. Animal experiments were conducted by trained personnel and were approved by the ethics and animal welfare committee of the University of Veterinary Medicine Vienna and the Austrian Federal Ministry of Science and Research according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012—TVG 2012 (BMBWF-68.205/0173-V/3b/2019) and conform to the guidelines of FELASA and ARRIVE (Animal Research: Reporting of In Vivo Experiments). Age- and sex-matched mice (9-12 weeks) were used in all experiments. To investigate whether viral infection induces extramedullary NK-cell development, we infected mice with MCMV and tested for the presence of NKP in the spleen at different time points post infection (p.i). As a control, we included the analysis of BM cells (Figure 1A). NKP were defined as lineage-negative, NK1.1^{-}NKp46^{-}CD11b^{-}cKit$^{-/low}$CD27^{+}CD244^{+}CD122^{+} cells, which includes both NKP and refined NKP.[9] In line with previous reports by us and others,[4,5] spleen cellularity was increased at day 5 p.i., which is indicative of EMH (Figure 1C). MCMV infection resulted in a profound increase in the total number and frequency of NKP in the spleen, which peaked around day 5 p.i. (Figure 1B, D, *Online Supplementary Figure S1A*). As expected from previous reports in mice and humans,[10] BM cellularity decreased in response to MCMV infection (Figure 1E). The abundance of NKP transiently increased with a peak around day 3 p.i. (Figure F).

To test whether the accumulation of NKP in the spleen is specific for MCMV infection, we challenged mice with the TLR9 ligand CpG-oligodeoxynucleotides (CpG-ODN), which is a frequently used model for sterile inflammation that induces EMH with a peak six days after treatment.[11] CpG-ODN treatment recapitulated our findings with MCMV infection (Figure 1G-I), suggesting that the accumulation of NKP in the spleen is not limited to viral infection but also occurs in response to bacterial DNA that is recognized by TLR9.

Prior work showed that MCMV infection or treatment with TLR3 agonists impair BM hematopoiesis by diminishing

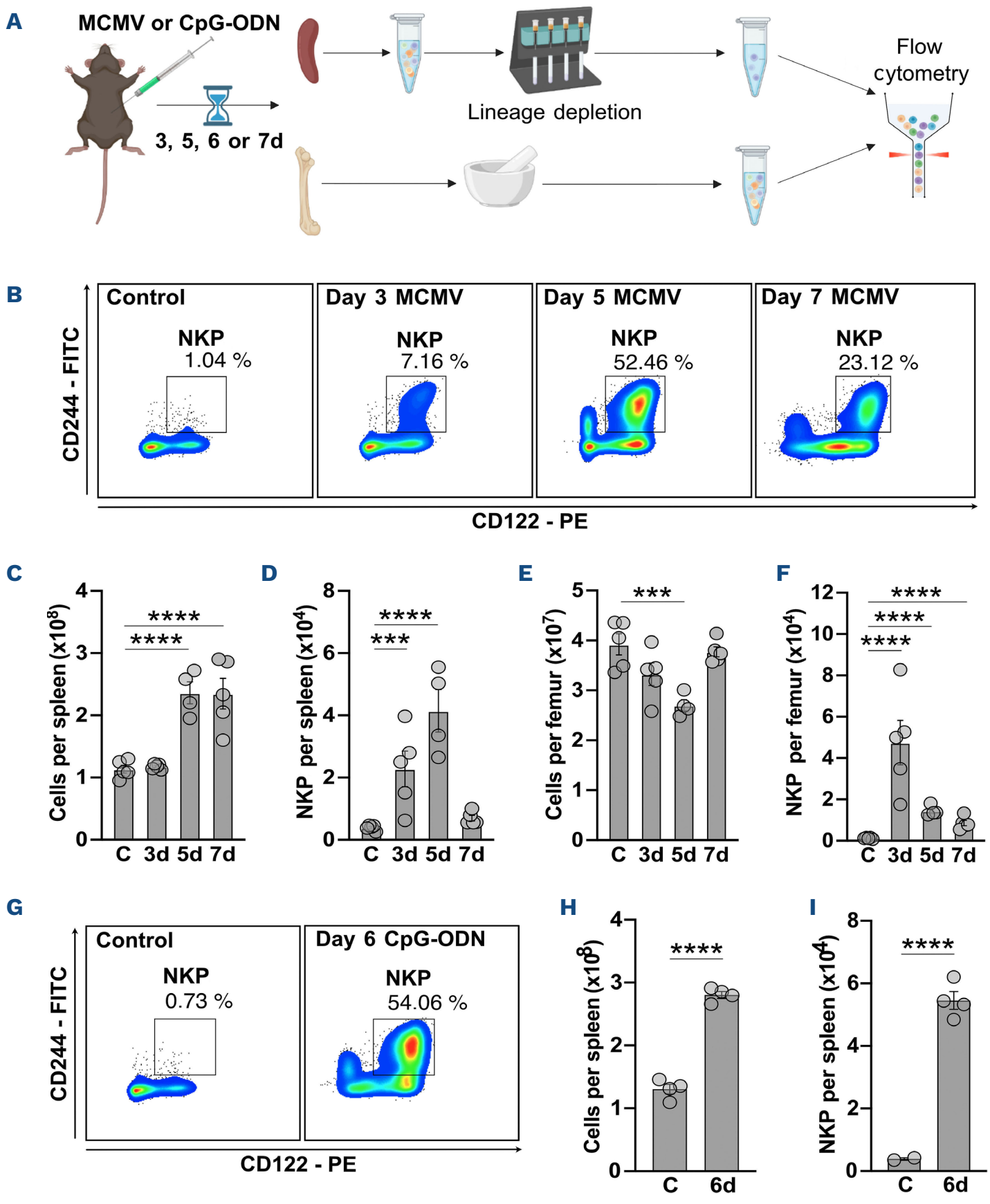

Figure 1. Acute murine cytomegalovirus infection and sterile inflammation lead to the accumulation of natural killer-cell progenitors in the spleen. (A) Experimental setup: mice were intraperitoneally (i.p.) infected with $5x10^4$ plaque-forming units (PFU) of murine cytomegalovirus (MCMV) (salivary gland-derived MCMV Smith strain; American Type Culture Collection, ATCC® VR194) or injected with 10 nM CpG-oligodeoxynucleotides (CpG-ODN). PBS was used as control. In all cases, the injected volume was 200 μL. Spleens and femurs were collected three, five, and seven days (3d, 5d, and 7d) after MCMV infection, or six days (6d) after CpG-ODN injection. Spleens were enzymatically digested (RPMI1640 medium, 100 μg/mL and 100 U/mL penicillin-streptomycin, 2% FBS, 0.1 mg/mL collagenase D, 0.02 mg/mL DNAse I for 30 minutes at 37°C with 5% CO_2). Linage depletion was performed using Direct Lineage Cell Depletion Kit cocktail according to the manufacturer's instructions (Miltenyi Biotec). Created in BioRender. Strobl, B. (2025) https://BioRender.com/4kpx4ri. (B) Flow cytometry plots of splenic natural killer-cell progenitors (NKP) in control mice and three, five, and seven days post-MCMV infection. (C) Number of total splenocytes and (D) splenic NKP at different time points post-MCMV infection. (E) Bone marrow (BM) cellularity and (F) NKP abundance per femur. (G-I) Mice were i.p. injected with 10 nM CpG-ODN (InvivoGen). Representative flow cytometry plots of splenic NKP (G), splenic cellularity (H), and the abundance of NKP in spleens (I) six days after CpG-ODN treatment. Results are expressed as mean ± standard error of the mean. Statistical analysis was performed in GraphPad Prism 10 (Student *t* test and one-way ANOVA with Dunnett's multiple comparisons test). Data represent one of two independent experiments (N=4-5 biological replicates). ***$P \leq 0.001$, ****$P \leq 0.0001$. C: PBS-treated controls.

long-term HSC (LT-HSC) function and reducing multipotent progenitors (MPP), respectively.[12,13] Because this should also constrain NK-cell production, we tested the *in vivo* capacity of BM from CpG-treated mice to generate NK cells. To capture the integrated impact of the BM compartment on NK-lineage output, we used unfractionated whole BM in mixed competitive chimeras and as support cells. BM cells from untreated or CpG-treated tdTomato transgenic mice were transplanted together with wild-type BM cells at a 1:1 ratio. Six to eight weeks post transplantation, the proportion of tdTomato-positive *versus* wild-type NK cells was analyzed in the BM (*Online Supplementary Figure S1B*). BM cells from untreated tdTomato mice showed a comparable replenishment of NK cells as BM from wild-type mice (*Online Supplementary Figure S1C*). In stark contrast, the number of NK cells was significantly reduced when BM from CpG-challenged tdTomato mice was used (*Online Supplementary Figure S1C*). Together, these findings indicate that acute inflammation diminishes the net *in vivo* contribution of BM to NK-lineage output and that the emergence

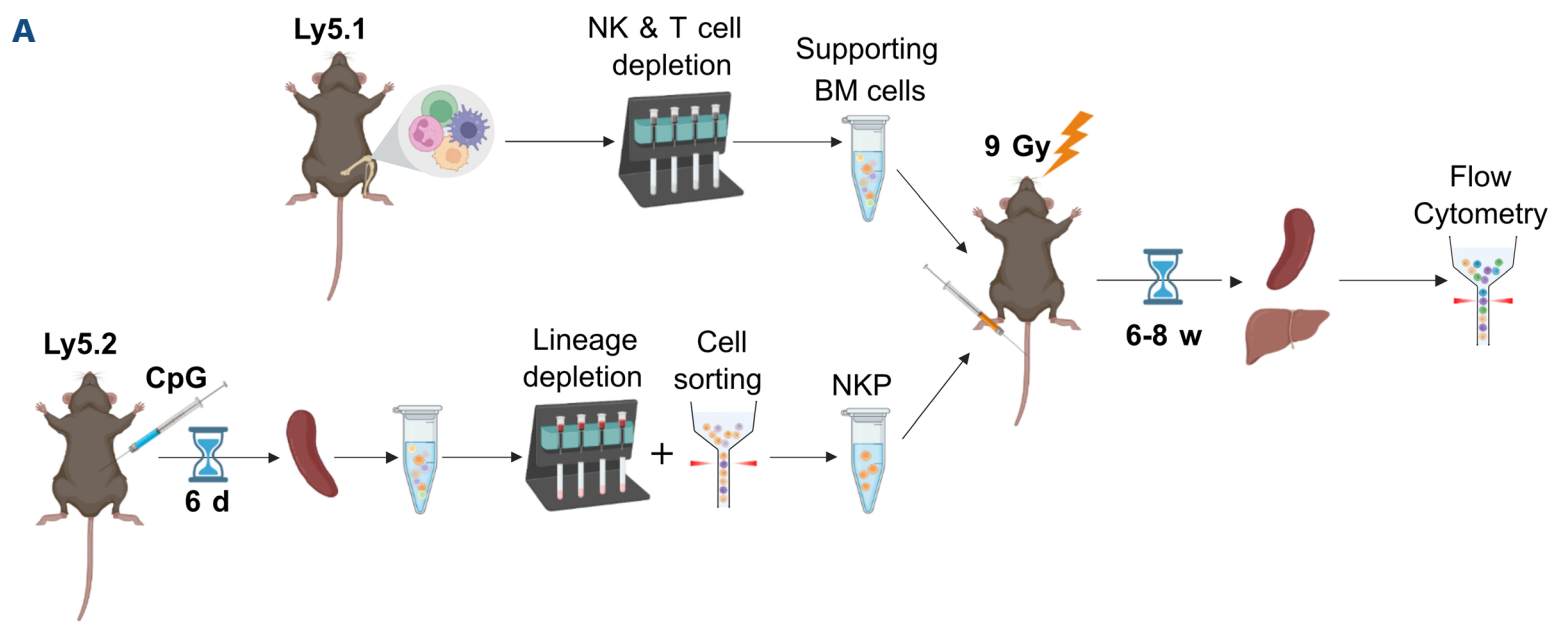

Figure 2. Splenic natural killer-cell progenitors give a rise to classical natural killer cells. (A) Experimental setup for splenic natural killer-cell progenitor (NKP) transplantation: Ly5.2 mice were intraperitoneally (i.p.) injected with 10 nM CpG-oligodeoxynucleotides (CpG-ODN). Six days later, spleens were digested, splenic NKP were MACS-enriched by lineage depletion and sorted. Approximately 1x10^4 splenic NKP were mixed with 5x10^6 Ly5.1$^+$ bone marrow (BM) supporting cells (depleted of NK and T cells) and intravenously transplanted into lethally irradiated Ly5.1 recipient mice. Created in BioRender. Strobl, B. (2025) https://BioRender.com/ee9bll4. (B) Representative flow cytometry plots for Ly5.1$^+$ and Ly5.2$^+$ NK cells in spleen and liver from recipient mice. Data represent one of two independent experiments (N=3-4 biological replicates).

of splenic NKP coincides with impaired BM NK-poiesis.

To test whether splenic NKP can differentiate into mature NK cells, we transplanted splenic NKP from CpG-ODN-treated wild-type (Ly5.2$^+$) mice together with supporting BM cells into lethally irradiated Ly5.1$^+$ recipient mice (Figure 2A). Six to eight weeks after transplantation, Ly5.2$^+$ NK cells (CD3$^-$DX5$^+$NKp46$^+$NK1.1$^+$) were detected in the spleen and liver, demonstrating that inflammation-induced splenic NKP differentiate into conventional NK cells (Figure 2B, *Online Supplementary Figure S2A*). However, consistent with previous reports, the number of mature NK cells generated from transplanted NKP was very low,[14] precluding assessment of NK-cell maturation and functionality.

To overcome this limitation, splenic hematopoietic stem and progenitor cells (HSPC) from CpG-treated tdTomato mice were transplanted into lethally irradiated recipients

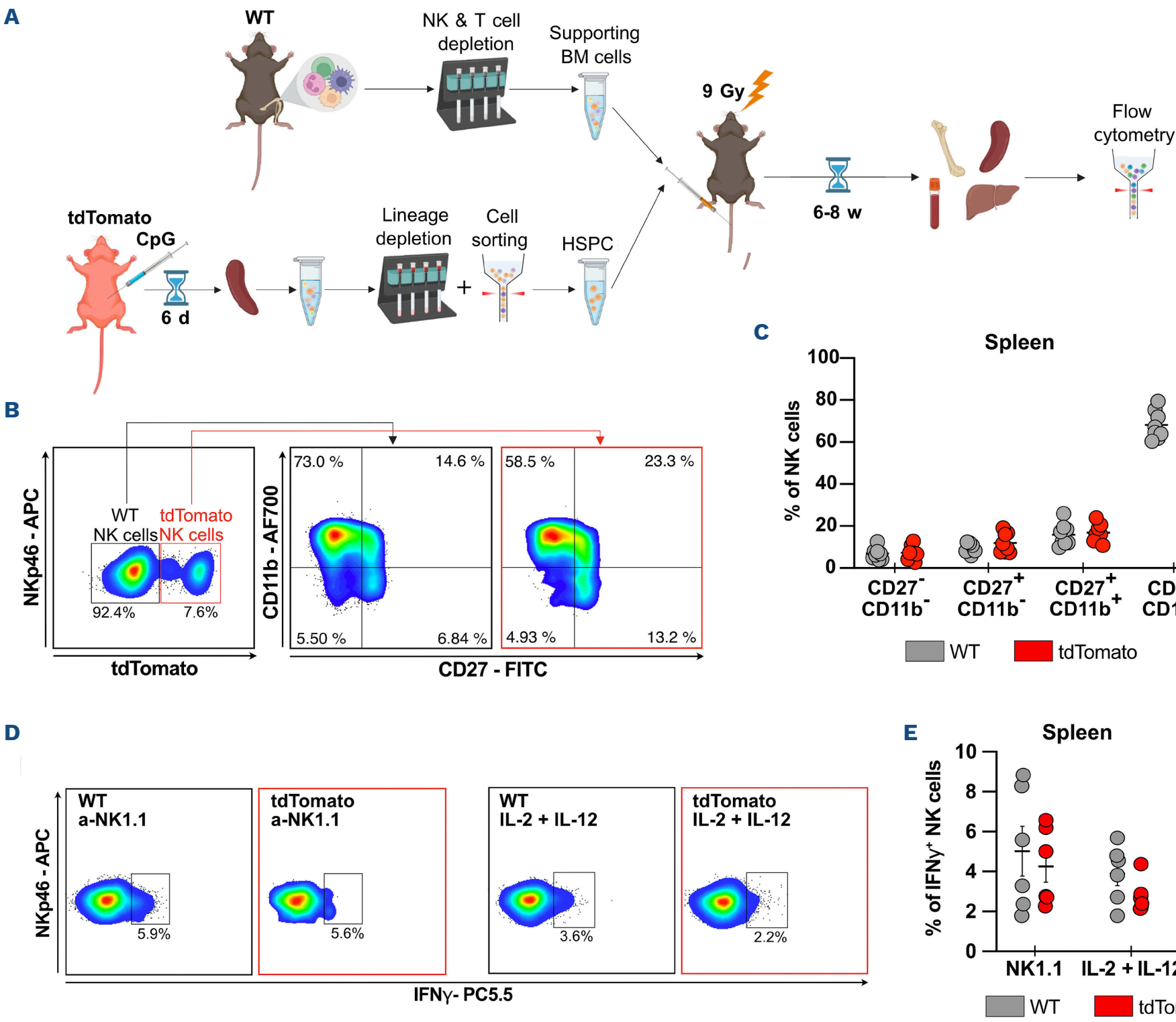

Figure 3. Splenic hematopoietic stem and progenitor cells differentiate into functional classical natural killer cells. (A) Experimental setup for splenic hematopoietic stem and progenitor cell (HSPC) transplantation. TdTomato transgenic mice were intraperitoneally (i.p.) injected with 10 nM CpG-oligodeoxynucleotides (CpG-ODN). Six days later, spleens were digested, HSPC were MACS enriched by lineage depletion and sorted. 5x10^4 splenic HSPC (Lin$^-$cKit$^+$SCA$^{+/-}$) were mixed with wild-type (WT) bone marrow (BM) supporting cells (depleted from natural killer [NK] and T cells) and intravenously injected into lethally irradiated WT recipient mice. Created in BioRender. Strobl, B. (2025) https://BioRender.com/kj4lx25. (B and C) Splenic NK-cell maturation and corresponding flow cytometry plots six to eight weeks (w) after transplantation. N=4-5 biological replicates, N=2 experimental repetitions. (D and E) Percentage of IFNγ-producing NK cells stimulated *ex vivo* with tube-bound anti NK1.1 antibody (10 μg/mL, clone PK136, BioLegend) after NK1.1 or IL-2 (5 ng/mL) and IL-12 (5 ng/mL) (PeproTech) stimulation in complete RPMI 1640 medium. N=5-6 biological replicates, N=1 experimental repetition. Results are expressed as mean ± standard error of the mean. Statistical analysis was carried out with square root-transformed data (for % of cells). Statistical analysis was performed in GraphPad Prism 10 (Student *t* test).

together with supporting BM cells from wild-type mice (Figure 3A, *Online Supplementary Figure S2B*). Six to eight weeks post transplantation, the maturation of NK cells was analyzed in the spleen, BM, blood, and liver. NK-cell maturation stages are defined by the cell surface markers CD27 and CD11b. They mature from $CD27^-CD11b^-$, through $CD27^+CD11b^-$ and $CD27^+CD11b^+$ stages, to the most mature $CD27^-CD11b^+$ NK cells. TdTomato-positive NK cells matured comparably to recipient wild-type NK cells in the spleen (Figure 3B, C, *Online Supplementary Figure S3A*), BM (*Online Supplementary Figure S3B*), and blood (*Online Supplementary Figure S3C*), whereas the maturation of classical NK cells was slightly impaired in the liver (*Online Supplementary Figure S3D*). To assess NK-cell functionality, we stimulated NK cells *ex vivo* with anti-NK1.1 antibody or IL-2 and IL-12 and analyzed IFNγ production by flow cytometry. IFNγ production by splenic tdTomato-positive NK cells was comparable to that of wild-type NK cells (Figure 3D, E, *Online Supplementary Figure S3A*), indicating that NK cells that develop from splenic HSPC are fully capable of producing IFNγ in response to activating receptor stimulation or cytokine exposure.

To our knowledge, this is the first study that shows a massive accumulation of NKP in the spleen in response to viral infection and acute inflammation. It remains to be investigated whether NKP (or earlier progenitors) are mobilized from the BM or if they arise locally from multipotent progenitors. According to the ILC-poiesis model, the rapid splenic increase in NKP during viral infection would primarily reflect IL-1β-driven expansion and differentiation of peripheral ILCP,[8] while recent work on mice suggests an IL-18-dependent mobilization of BM-resident sinusoidal ILCP that may contribute.[15] Notably, a later study identified an NK-lineage-biased progenitor population, referred to as early NK progenitors (ENKP), which developed into NK cells independently of ILCP.[16] Transplant experiments demonstrated that $Ly49^+$ NK cells mounting the response to MCMV were predominantly ENKP-derived, implying that ENKP-derived NKP contribute more than ILCP-derived NKP to the host defense against MCMV. Whether ENKP or their progeny egress from the BM at steady state or during infection remains unresolved. Our finding that NKP transiently increase in the BM before rising in the spleen suggests a redistribution of NK development from the BM to peripheral tissue during MCMV infection, consistent with egress of ENKP or downstream NKP. Definitive resolution will require systematic mapping of the splenic hematopoietic progenitor pool and the cues that drive NK-lineage generation in this niche.

Another important question that remains to be answered concerns the exact nature of the stress-induced splenic NKP, including their epigenetic and transcriptional programs. Comprehensive profiling is particularly important given the high plasticity of hematopoietic paths during stress conditions,[17] and to distinguish NKP subsets committed to classical NK cells from various precursors with broader ILC potential. Future work on the roles of cytokines (e.g., IL-1β, IL-18), chemokine axes (e.g., CXCL12/CXCR4), and stromal interactions will clarify how the splenic microenvironment supports NK-cell production during MCMV infection and systemic inflammation. In the meantime, our study provides a key starting point for uncovering how increased NK-cell demand during viral infection can be met when BM NK-cell output is constrained. Given the evolutionary conservation of EMH across mammals,[2] our results may have broader implications for understanding immune responses in humans and other species, particularly in contexts where splenic EMH plays a critical role in host defense or may be therapeutically used as an alternative site for hematopoiesis.[18] Collectively, our finding broadens the understanding of the spleen's hematopoietic role during infectious and inflammatory diseases, and opens new avenues for investigating NK-cell developmental paths under stress conditions.

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https://doi.org/10.3324/haematol.2025.289028

Received: August 21, 2025.
Accepted: February 5, 2026.
Early view: February 12, 2026.

Disclosures

No conflicts of interest to disclose.

Contributions

TB conceptualized the study, designed and performed experiments, analyzed and interpreted the data, performed visualization, and wrote the original draft and the final manuscript; SM performed experiments and performed visualization; JJ, LA, MK and CL performed experiments; DG designed experiments, gave input throughout the study, and critically reviewed the manuscript; KMS and MM gave input throughout the study and critically reviewed the manuscript; BS conceptualized the study and wrote the final

manuscript. All authors edited the manuscript.

Acknowledgments

We are grateful to Claus Vogl for help with the statistical analysis.

Funding

This work was supported by the Austrian Science Fund (FWF; Grant number P-34286, to BS). The work was, furthermore, supported in part by the FWF doc.funds doctoral program TissueHome (DOC-32B28, to BS), the FWF SFB-F6101 (to MM), and the FWF project P-36664 (to KMS).

Data-sharing statement

For original data, please contact birgit.strobl@vetmeduni.ac.at

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