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

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Authorship contributions: T.B. and B.S. conceptualized the study, T.B. and D.G. designed experiments, T.B., S.M., J.J., L.A., M.K. and C.L. performed experiments. T.B. analyzed and interpreted the data. T.B. and S.M. performed visualization. T.B. wrote the original draft and, together with B.S., the final manuscript. K.M.S., D.G. and M.M. gave input throughout the study and critically reviewed the manuscript. All authors edited the manuscript.

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In adult mammals, hematopoiesis predominantly occurs in the bone marrow (BM), where hematopoietic stem cells (HSCs) 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 hematopoietic stem cells (HSCs), progress through common lymphoid progenitors (CLPs) to multipotent innate lymphoid progenitors, such as innate lymphocyte progenitors (ILCPs), then commit as NK-cell progenitors (NKPs), 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, accumulating evidence - particularly in humans - indicates that ILCPs 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.

MCMV 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 NKPs in the spleen, accompanied by a diminished capacity of the BM to produce NK cells. Using transplantation experiments, we show that splenic NKPs 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.

To investigate whether viral infection induces extramedullary NK-cell development, we infected mice with MCMV and tested for the presence of NK progenitors (NKPs) in the spleen at different time points post infection (p.i). As a control, we included the analysis of BM cells (Figure 1A). NKPs were defined as lineage-negative, NK1.1$^-$ NKp46$^-$CD11b$^-$cKit$^{-/low}$CD27$^+$CD244$^+$CD122$^+$ cells, which includes both NKPs and refined NKPs[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 NKPs in the spleen, which peaked around day 5 p.i. (Figures 1B, D and 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 NKPs transiently increased with a peak around day 3 p.i. (Figure F).

To test whether the accumulation of NKPs 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 6 days after treatment[11]. CpG-ODN treatment recapitulated our findings with MCMV infection (Figures 1G-I), suggesting that the accumulation of NKPs 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 long-term HSC (LT-HSC) function and reducing multipotent progenitors (MPPs), 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 (Supplementary Figure S1B). BM cells from untreated tdTomato mice showed a comparable replenishment of NK cells as BM from wild-type

mice (Supplementary Figure S1C). In stark contrast, the number of NK cells was significantly reduced when BM from CpG-challenged tdTomato mice was used (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 of splenic NKPs coincides with impaired BM NK-poiesis.

To test whether splenic NKPs can differentiate into mature NK cells, we transplanted splenic NKPs 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 NKPs differentiate into conventional NK cells (Figure 2B and Supplementary Figure 2A). However, consistent with previous reports, the number of mature NK cells generated from transplanted NKPs was very low[14], precluding assessment of NK cell maturation and functionality.

To overcome this limitation, splenic hematopoietic stem and progenitor cells (HSPCs) from CpG-treated tdTomato mice were transplanted into lethally irradiated recipients together with supporting BM cells from wild-type mice (Figure 3A and Supplementary Figure 2B). 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 and Supplementary Figure 3A), BM (Supplementary Figure S3B) and blood (Supplementary Figure 3C), whereas the maturation of classical NK cells was slightly impaired in the liver (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 and Supplementary Figure 3A), indicating that NK cells that develop from splenic HSPCs 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 NKPs in the spleen in response to viral infection and acute inflammation. It remains to be investigated whether NKPs (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 NKPs during viral infection would primarily reflect IL-1β-driven expansion and differentiation of peripheral ILCPs[8], while recent mouse work suggests an IL-18-dependent mobilization of BM-resident sinusoidal ILCPs that may contribute[15]. Notably, a later study identified an NK-lineage-biased progenitor population, referred to as early NK progenitors (ENKPs), which developed into NK cells independently of ILCPs[16]. Transplant experiments demonstrated that Ly49$^+$ NK cells mounting the response to MCMV were predominantly ENKP-derived, implying that ENKP-derived NKPs contribute more than ILCP-derived NKPs to the host defense against MCMV. Whether ENKPs or their progeny egress from the BM at steady state or during infection remains unresolved. Our finding that NKPs 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 ENKPs or downstream NKPs. 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 open question concerns the exact nature of the stress-induced splenic NKPs, 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. Dissecting 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.

Nevertheless, our study provides a key starting point for uncovering of 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.

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.

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Figure legends

Figure 1. Acute MCMV infection and sterile inflammation lead to the accumulation of NKPs in the spleen. (A) Experimental setup: Mice were intraperitoneally (i.p.) infected with 5×10^4 plaque-forming units (PFUs) of MCMV (salivary gland-derived MCMV Smith strain; American Type Culture Collection, ATCC® VR194) or injected with 10 nM CpG-ODN. PBS was used as a 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, 100µg/mL and 100U/mL penicillin-streptomycin, 2% FBS, 0.1 mg/mL collagenase D, 0.02 mg/mL DNAse I for 30 min at 37 °C with 5% CO_2). Linage depletion was performed using Direct Lineage Cell Depletion Kit cocktail according to the manufacturer's instruction (Miltenyi Biotec). Created in BioRender. Strobl, B. (2025) https://BioRender.com/4kpx4ri (B) Flow cytometry plots of splenic NKPs in control mice and three, five, and seven days post-MCMV infection. (C) Number of total splenocytes and (D) splenic NKPs at different time points post-MCMV infection. (E) 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 NKPs (G), splenic cellularity (H) and the abundance of NKPs in spleens (I) six days after CpG-ODN treatment. Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was done with log-transformed data (for cell numbers). Statistical analysis was performed in GraphPad Prism 10 (Student's t-test and one-way ANOVA with Dunnett's multiple comparisons test). Data represent one of two independent experiments (n = 4-5 biological replicates). Asterisks indicate statistical significance between conditions (***p ≤ 0.001, ****p ≤ 0.0001). C, PBS treated controls.

Figure 2. Splenic NKPs give a rise to classical NK cells. (A) Experimental setup for splenic NKP transplantation: Ly5.2 mice were i.p. injected with 10 nM CpG-ODN. Six days later, spleens were digested, splenic NKPs were MACS-enriched by lineage depletion and sorted. Approximately 1×10^4 splenic NKPs were mixed with 5×10^6 $Ly5.1^+$ 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).

Figure 3. Splenic HSPCs differentiate into functional classical NK cells. (A) Experimental setup for splenic HSPCs transplantation. TdTomato transgenic mice were i.p. injected with 10 nM CpG-ODN. Six days later, spleens were digested, HSPCs were MACS enriched by lineage depletion and sorted. 5 × 10^{4} splenic HSPCs (Lin^{-}cKit^{+}SCA$^{+/-}$) were mixed with wild-type (WT) BM supporting cells (depleted from NK and T cells) and intravenously injected into lethally irradiated WT recipient mice. Created in BioRender. Strobl, B. (2025) https://BioRender.com/kj4lx25. (B, C) Splenic NK-cell maturation and corresponding flow cytometry plots six to eight weeks after transplantation (n = 4-5, N = 2) (D, 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) in complete RPMI 1640 medium. (n = 5-6, N = 1). Results are expressed as mean ± SEM. Statistical analysis was done with square root-transformed data (for % of cells). Statistical analysis was performed in GraphPad Prism 10 (Student's t-test). n, biological replicates; N, experimental repetitions.

Figure 1

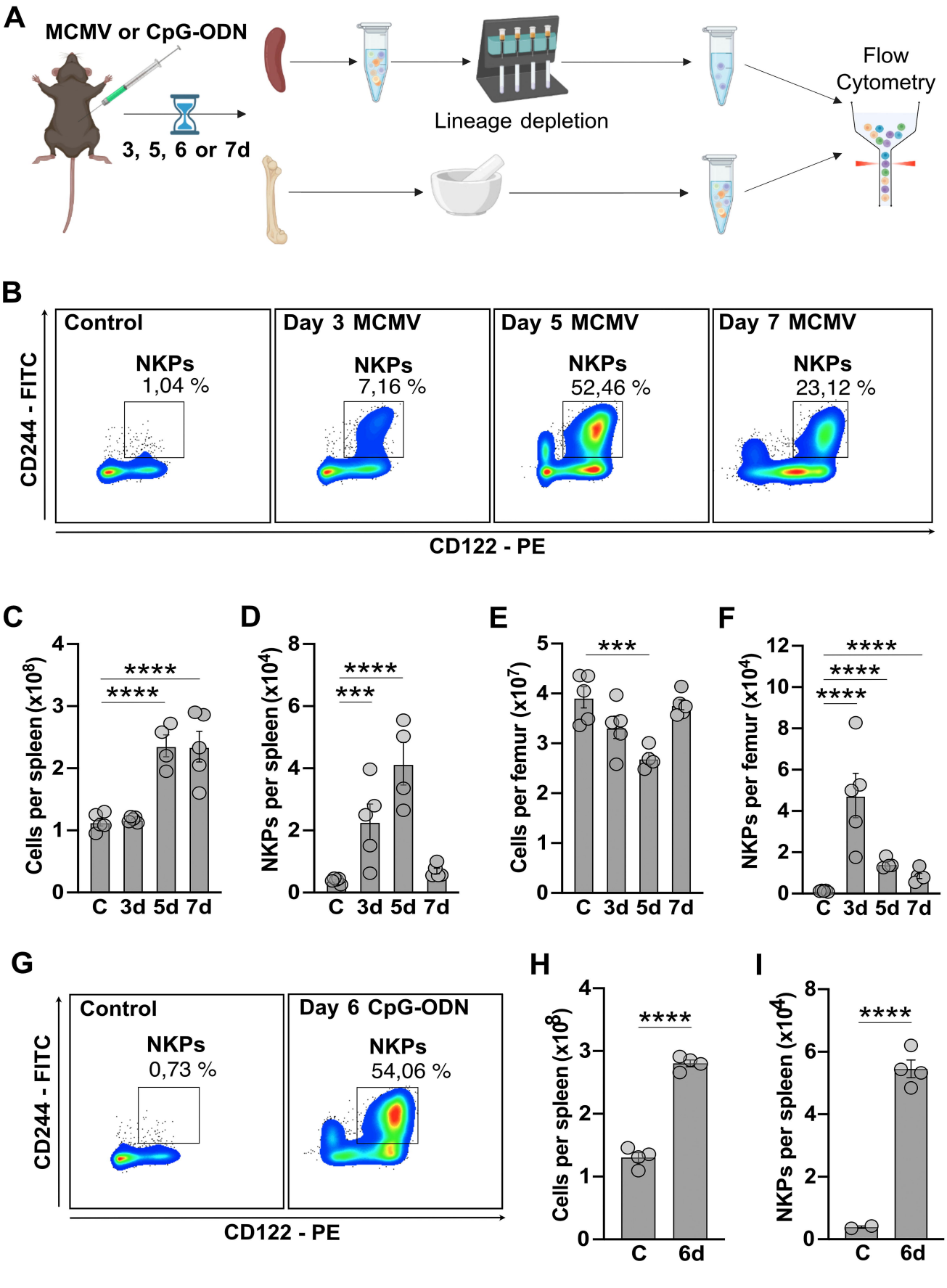
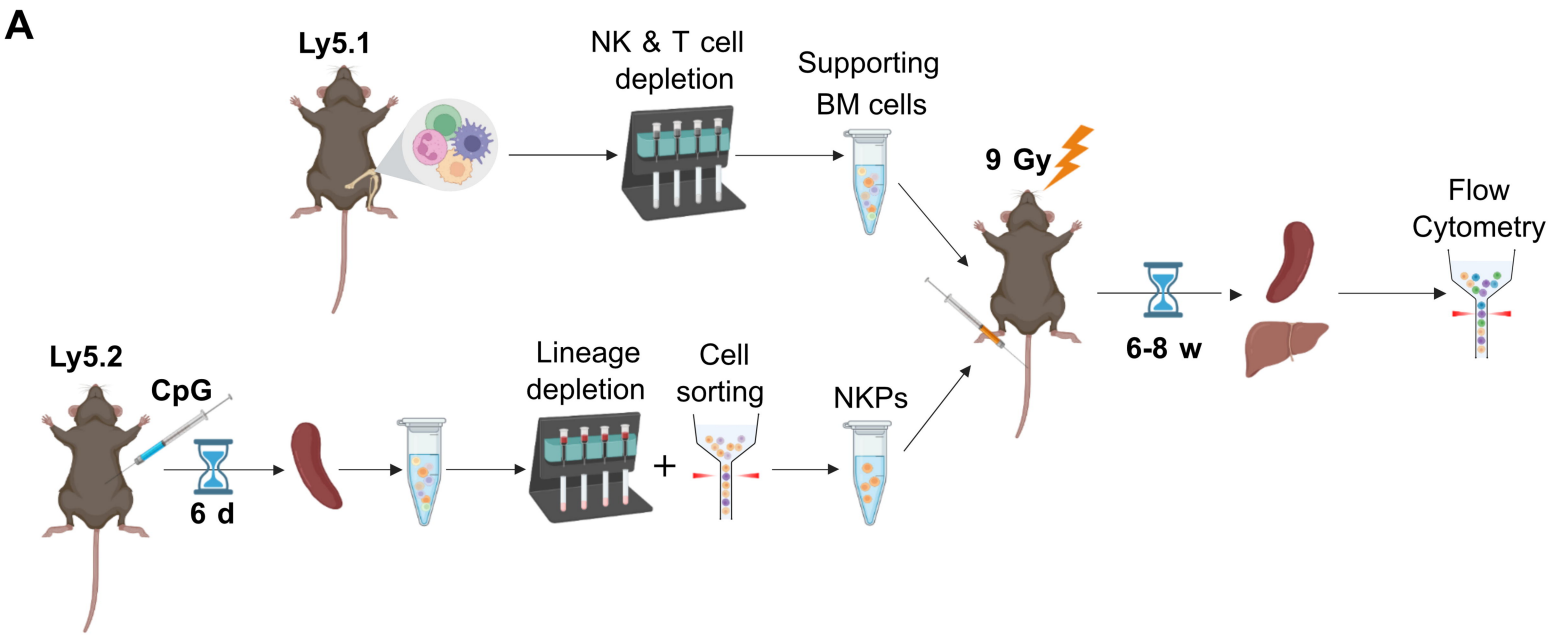

A
MCMV or CpG-ODN
3, 5, 6 or 7d
Lineage depletion
Flow Cytometry
B
Control
NKPs
1,04 %
Day 3 MCMV
NKPs
7,16 %
Day 5 MCMV
NKPs
52,46 %
Day 7 MCMV
NKPs
23,12 %
CD244 - FITC
CD122 - PE
C
Cells per spleen (x10^8)
D
NKPs per spleen (x10^4)
E
Cells per femur (x10^7)
F
NKPs per femur (x10^4)
C 3d 5d 7d
G
Control
NKPs
0,73 %
Day 6 CpG-ODN
NKPs
54,06 %
CD244 - FITC
CD122 - PE
H
Cells per spleen (x10^8)
I
NKPs per spleen (x10^4)
C 6d

Figure 2


A
Ly5.1
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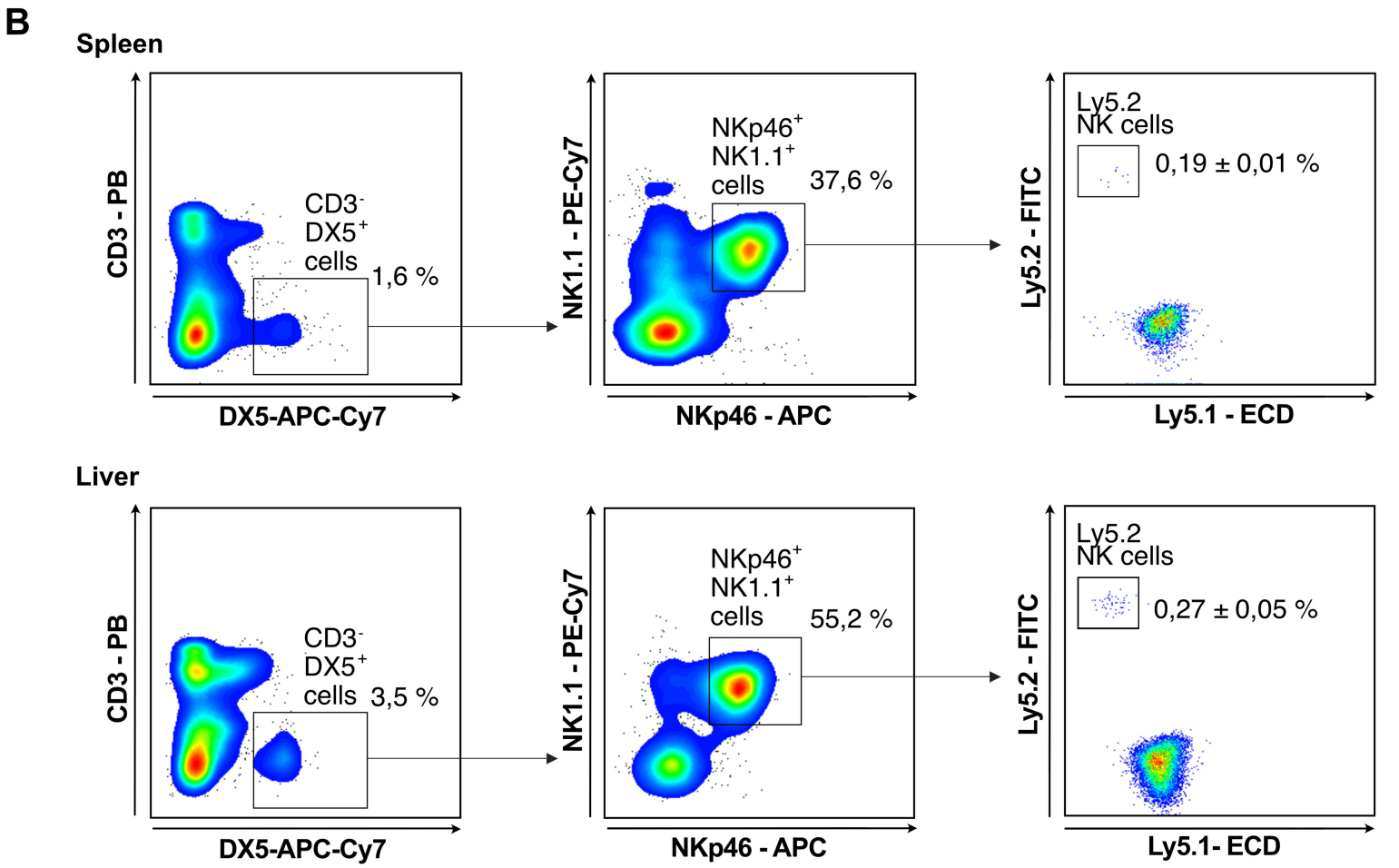
Supporting BM cells
9 Gy
Flow Cytometry
6-8 w
Ly5.2
CpG
6 d
Lineage depletion
Cell sorting
NKPs


B
Spleen
CD3 - PB
CD3- DX5+ cells
1,6 %
DX5-APC-Cy7
NK1.1 - PE-Cy7
NKp46+ NK1.1+ cells
37,6 %
NKp46 - APC
Ly5.2 - FITC
Ly5.2 NK cells
0,19 ± 0,01 %
Ly5.1 - ECD
Liver
CD3 - PB
CD3- DX5+ cells
3,5 %
DX5-APC-Cy7
NK1.1 - PE-Cy7
NKp46+ NK1.1+ cells
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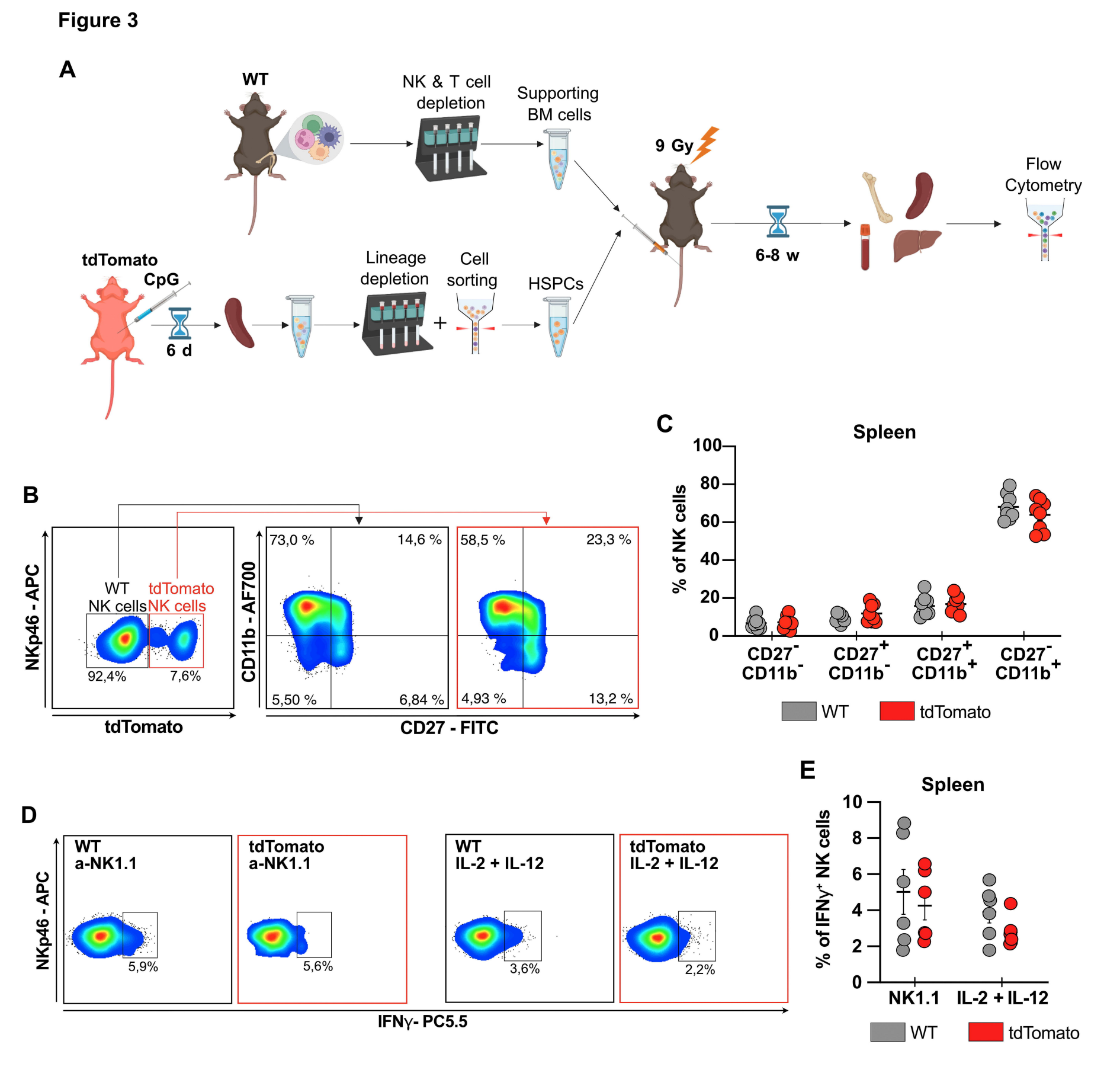
NKp46 - APC
Ly5.2 - FITC
Ly5.2 NK cells
0,27 ± 0,05 %
Ly5.1- ECD

Figure 3


A
WT
NK & T cell depletion
Supporting BM cells
9 Gy
Flow Cytometry
6-8 w
tdTomato
CpG
6 d
Lineage depletion
Cell sorting
HSPCs
B
NKp46 - APC
tdTomato
WT NK cells
tdTomato NK cells
92,4%
7,6%
CD11b - AF700
CD27 - FITC
73,0 %
14,6 %
5,50 %
6,84 %
58,5 %
23,3 %
4,93 %
13,2 %
C
Spleen
% of NK cells
CD27- CD11b-
CD27+ CD11b-
CD27+ CD11b+
CD27- CD11b+
WT
tdTomato
D
WT a-NK1.1
tdTomato a-NK1.1
WT IL-2 + IL-12
tdTomato IL-2 + IL-12
5,9%
5,6%
3,6%
2,2%
IFNγ- PC5.5
E
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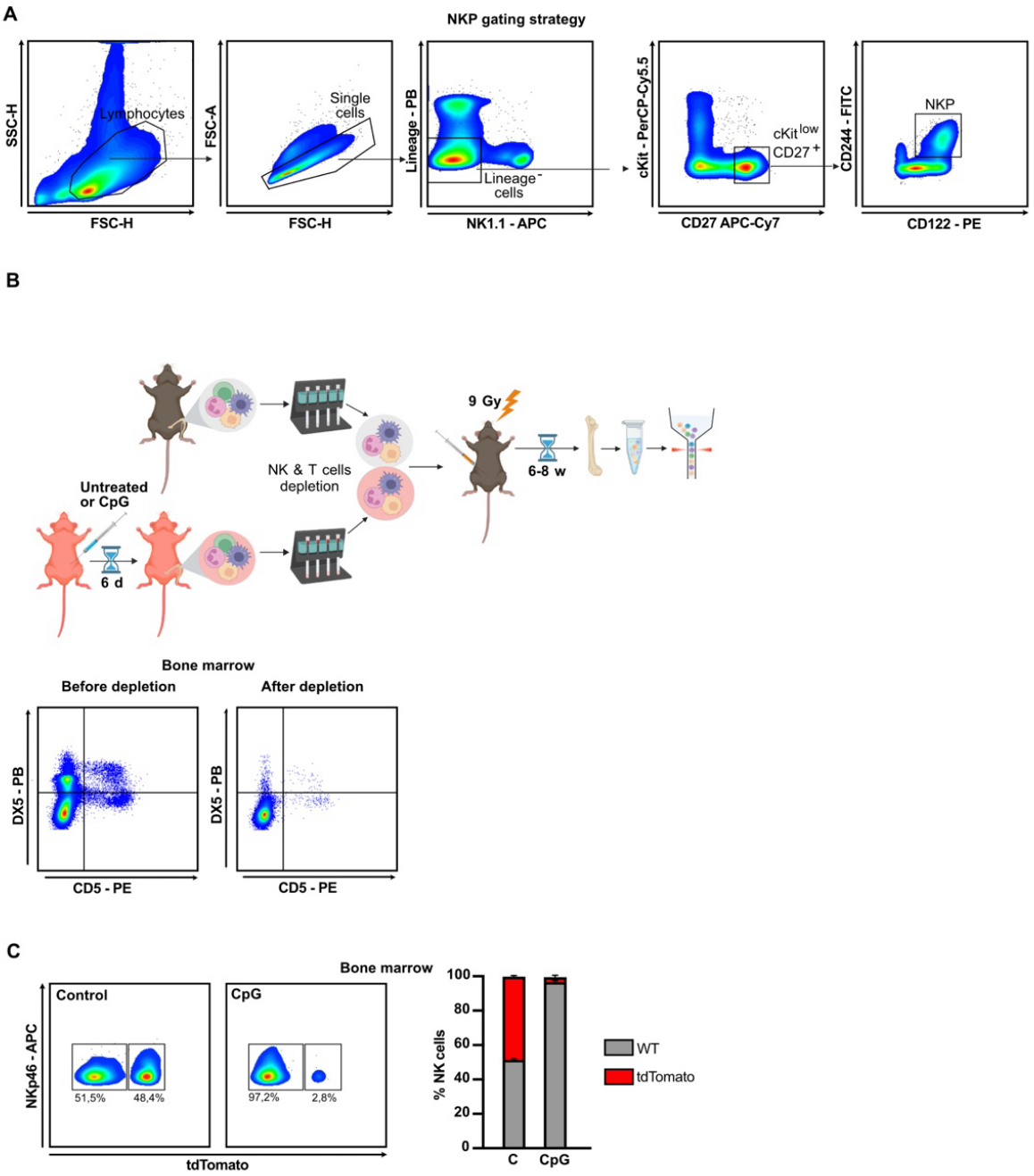
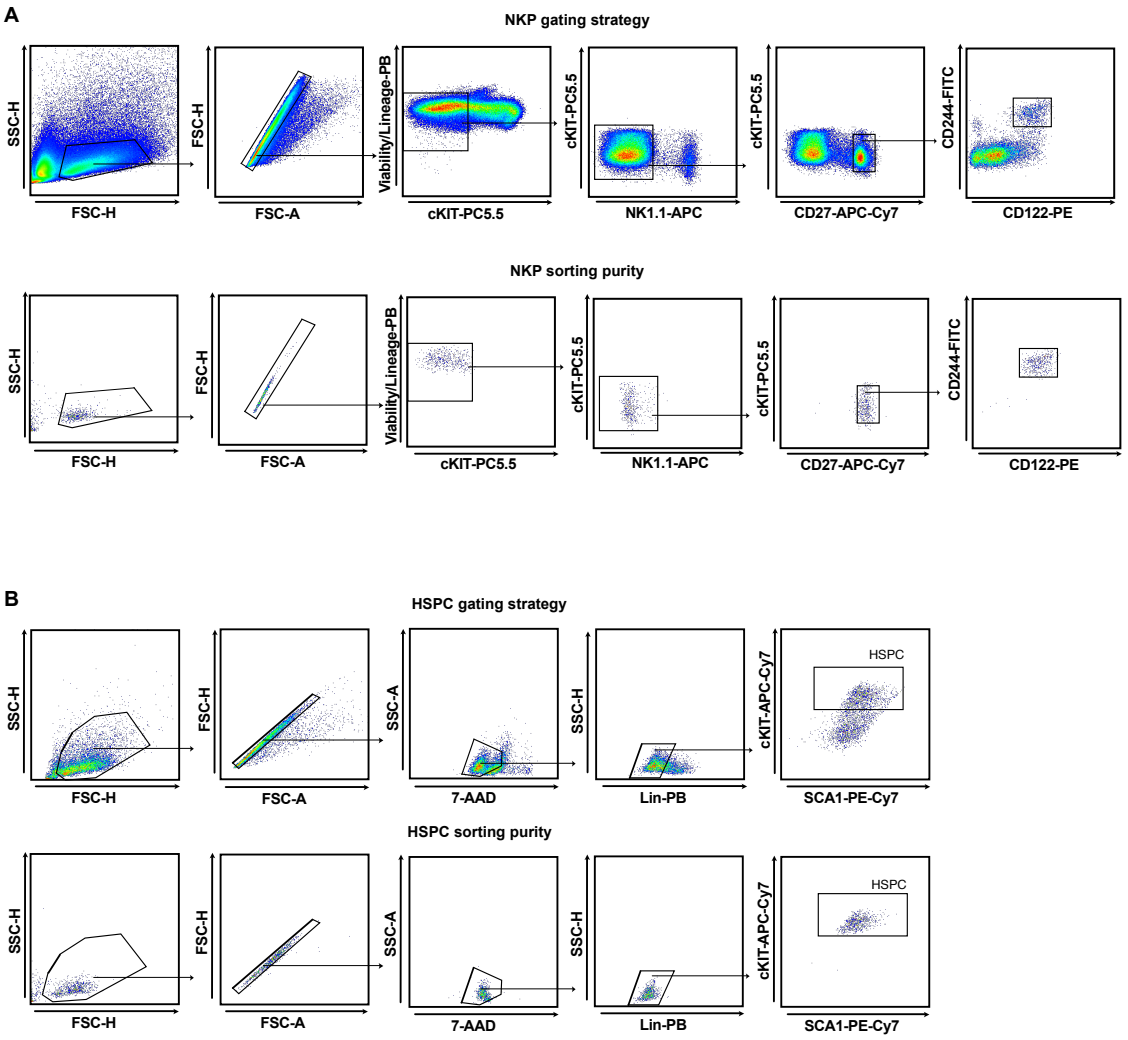
% of IFNγ+ NK cells
NK1.1
IL-2 + IL-12
WT
tdTomato

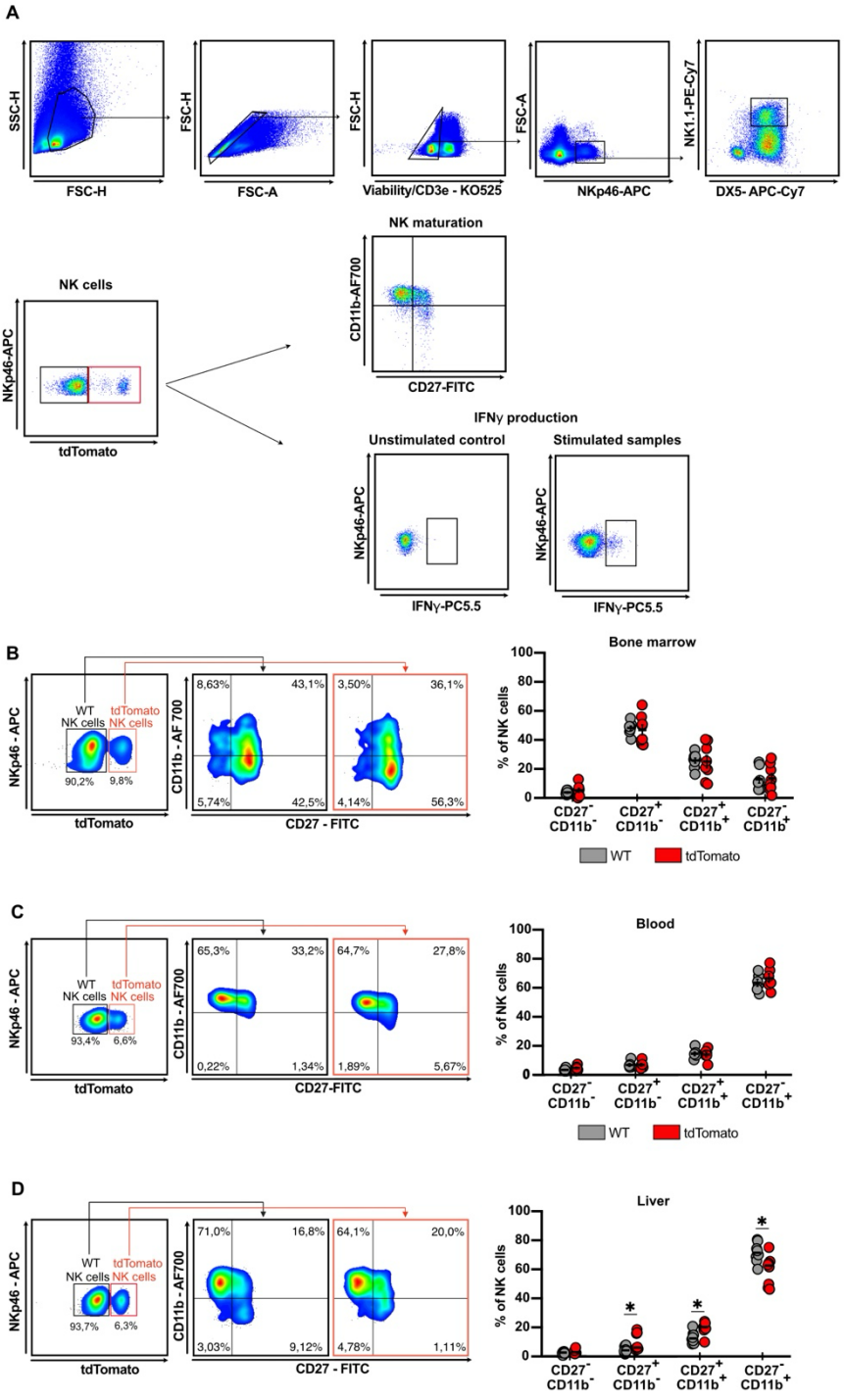


Supplementary Figure 1. **Acute inflammation reduces the BM's capacity to generate NK cells.** (A) Gating strategy for the analysis of NKPs. After exclusion of doublets, dead cells, lineage positive cells (CD19, CD3e, Ter 119, CD11b, Ly6G/Ly6C, F4/80) and NK cells (NK 1.1), cKit$^{low/-}$ CD27^{+} cells were gated. Out of this population, NKPs were defined as CD244^{+} and CD122^{+}. (B) Scheme of the experimental set-up and quality control for BM depletion. TdTomato mice were i.p. injected with 10 nM CpG-ODN. Six days later, femurs were flushed to obtain bone marrow cells. Mature

NK cells and T cells were depleted using anti-CD49b (DX5) and anti-CD5 labelled MACS beads (Miltenyi Biotech), then mixed 1:1 with wild-type (WT) BM cells and intravenously injected into WT recipient mice that had been lethally irradiated (9 Gy, 6 MV photons, Elekta Infinity linear accelerator) 24 h earlier under anaesthesia. Efficient depletion of $DX5^+$ and $CD5^+$ cells (i.e. mature NK cells and T cells) was confirmed by flow cytometry. Six to eight weeks later, NK cells in the BM of recipient mice were analysed. Created in BioRender. Strobl, B. (2025) https://BioRender.com/hf1sswc. (C) Percentage of NK cells in BM six to eight weeks after BM transplantation with representative flow cytometry plots (n=2, N=1). n, biological replicates; N, experimental repetitions. C, PBS treated controls.



Supplementary Figure 2. **Sorting strategy for NKPs and HSPCs.** (A) Gating strategy and post-sorting purity of NKPs used in the transplantation experiments. After exclusion of doublets, dead cells, lineage positive cells, and NK cells (NK 1.1), cKIT$^{low/-}$ CD27^{+} cells were gated. Out of this population, NKPs were sorted as CD244^{+} and CD122^{+}. (B) Gating strategy and post-sorting purity of HSPCs used in the transplantation experiments. After exclusion of doublets, dead cells, and lineage-positive cells, cKIT^{+} SCA1$^{-/+}$ cells were sorted.



Supplementary Figure 3. **Maturation of NK cells from transplanted HSPCs from CpG-ODN-challenged mice is fully intact in the BM and blood, but slightly impaired in the liver.** (A) Gating strategy for NK cell analysis. After exclusion of doublets, dead cells, and CD3$^+$ cells, classical NK cells were defined as NK1.1$^+$, NKp46$^+$, and DX5$^+$. Within this population, we distinguished tdTomato$^-$ and tdTomato$^+$ cells. For each subset, we quantified NK cell maturation using CD27 and CD11b

expression, as well as IFN-γ production. Unstimulated samples were used as controls to determine non-specific antibody binding. (B-D) NK-cell abundance and maturation with representative flow cytometry plots in (B) BM, (C) blood and (D) liver six to eight weeks after transplantation (n=4-5, N=2). Results are given as mean ± standard error of the mean (SEM). Statistical analysis was done with square root-transformed data. Statistical analysis was performed in GraphPad Prism 10 (Student's t-test). For clarity, statistical analysis is only shown for the comparison between WT and tdTomato NK cells. n, biological replicates; N, experimental repetitions.