Flt3 permits survival during infection by rendering dendritic cells competent to activate NK cells

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A previously unappreciated signal necessary for dendritic cell (DC)-mediated activation of natural killer (NK) cells during viral infection was revealed by a recessive *N*-ethyl-*N*-nitrosourea-induced mutation called *warmflash* (*wmfl*). *Wmfl* homozygotes displayed increased susceptibility to mouse cytomegalovirus (MCMV) infection. In response to MCMV infection in vivo, delayed NK cell activation was observed, but no intrinsic defects in NK cell activation or function were identified. Rather, coculture experiments demonstrated that NK cells are suboptimally activated by *wmfl* DCs, which showed impaired cytokine production in response to MCMV or synthetic TLR7 and TLR9 ligands. The *wmfl* mutation was identified in the gene encoding the Fms-like tyrosine kinase 3 (Flt3). Flt3 ligand (Flt3L) is transiently induced in the serum upon infection or TLR activation. However, antibody blockade reveals no acute requirement for Flt3L, suggesting that the Flt3L → Flt3 axis programs the development of DCs, making them competent to support NK effector function. In the absence of Flt3 signaling, NK cell activation is delayed and survival during MCMV infection is markedly compromised.

Fms-like tyrosine kinase 3 | natural killer–dendritic cell crosstalk | viral infection | natural killer cell activation | dendritic cell activation

Host defense against mouse cytomegalovirus (MCMV) depends on the function of natural killer (NK) cells that respond within the first 2 days of infection by expanding, producing IFNγ, and mediating cytotoxicity (1, 2). NK cell activation is controlled by signaling from activating and inhibitory receptors and also by signals specifically from dendritic cells (DCs) (3). In particular, DCs secrete soluble IL-12 and IL-18 that elicit IFNγ production and type I IFN that promotes cytotoxicity and proliferation by NK cells (4–6). Additionally, direct DC-NK contact contributes to NK cell survival, IFNγ production, and cytotoxicity by enabling DCs to present IL-15 in complex with IL-15Rα to NK cells in secondary lymphoid organs (6–9).

Mature DCs sense microbial products via Toll-like, NOD-like, and RIG-I-like helicase receptors, and mice deficient in Toll-like receptor (TLR) 9 are particularly susceptible to MCMV (10, 11). However, comprehensive understanding of the events that render DCs competent to support NK cell activation remains elusive. Through analysis of the *warmflash* mutant phenotype, we found that although the Fms-like tyrosine kinase 3 ligand (Flt3L) → Flt3 axis has no acute role during MCMV infection, Flt3 signaling programs DCs to support NK cell effector function. Without this developmental programming, mice cannot survive MCMV infection.

Results

Susceptibility to MCMV Infection Due to an *Flt3* Mutation. The recessive *warmflash* (*wmfl*) phenotype was isolated in a screen for *N*-ethyl-*N*-nitrosourea-induced mutations that confer susceptibility to MCMV infection (12). One hundred percent of *wmfl* homozygotes died when infected with 2×10^5 pfu of MCMV, a normally sublethal inoculum (Fig. 1*A*). Five days after infection with 10^5 pfu of MCMV *wmfl* homozygotes showed increased viral titers in the spleen and liver comparable to those observed in BALB/c mice (Fig. 1*B* and Fig. S1*A*). *Wmfl* homozygotes also failed to control lymphocytic choriomeningitis virus (LCMV) (clone 13) infection (Fig. S1*B*).

The spleens and lymph nodes, but not thymi, of *wmfl* homozygotes were consistently smaller and showed reduced cellularity compared to those of control mice (Fig. S2*A*). *Wmfl* homozygotes were slightly smaller than control mice of equivalent age and sex (Fig. S2*B*). However, we considered that these differences alone were not likely to explain enhanced viral susceptibility of *wmfl* mice and sought to identify qualitative defects in immune performance.

Thirty-six hours post-MCMV infection, exaggerated production of tumor necrosis factor (TNF)-α (Fig. 2*A*) and IL-6 was noticeable in the serum of *wmfl* homozygotes, possibly driven by an increased viral load. In contrast, the level of IFN-γ was significantly reduced in *wmfl* homozygotes relative to that in WT control mice (Fig. 2*B*), and levels of IL-12p70 and type I IFN were moderately but not significantly reduced in the serum of *wmfl* mice (Fig. 2 *C* and *D*). The *wmfl* mutation does not impair virus recognition (Fig. S3*A*) or alter TLR-mediated signaling (Fig. S3*B*) in macrophages in vitro.

We mapped the *wmfl* mutation on the basis of MCMV-induced lethality and identified a G to A transition in the splice donor site of intron 9 of the Fms-like tyrosine kinase 3 encoding gene (*Flt3*), resulting in a translation product lacking amino acids Tyr402 and Ser403, encoded by exon 9 (Fig. S4 *A–D*). The $Flt3^{wmfl}$ allele encodes a nonfunctional protein (Fig. S4 *E* and *F*).

The *warmflash* Defect Is Not Intrinsic to NK Cells. NK cells are the main producers of IFN-γ in response to MCMV (13). We therefore examined NK cell numbers and functions in homozygous $Flt3^{wmfl/wmfl}$ mice. We found that relative to controls, splenic $NK1.1^+CD3\varepsilon^-$ cells were reduced in both percentage and number in $Flt3^{wmfl/wmfl}$ homozygotes (Fig. 3*A*), whereas bone marrow NK cells were slightly but not significantly reduced in percentage. The disproportionate scarcity of $Flt3^{wmfl/wmfl}$ NK cells in the spleen suggested a defect in NK cell development that may contribute to MCMV susceptibility.

NK cells are activated within 48 h of MCMV infection. The earliest inducible NK cell activation marker, CD69, acts as a costimulatory molecule for activation and proliferation. We found that NK cells from $Flt3^{wmfl/wmfl}$ mice underexpressed CD69 at 6 h post-MCMV infection, but up-regulated expression to WT levels by 24 h after infection (Fig. 3*B*). In addition, a reduced percentage

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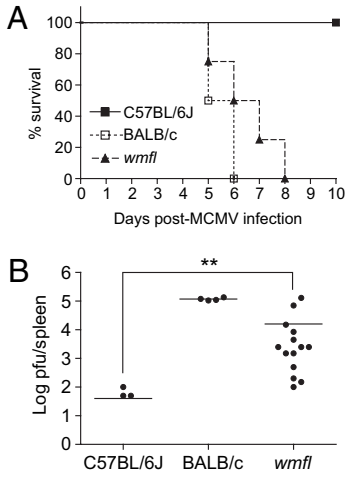

Fig. 1. Homozygous *wmfl* mice show increased susceptibility to MCMV infection. (*A*) Survival curve of C57BL/6J, BALB/c, and *wmfl* mice after infection with 2×10^5 pfu of MCMV ($n = 5$ mice per genotype). Results are representative of three experiments. (*B*) Viral titer in spleen of C57BL/6J, BALB/c, and *wmfl* mice at day 5 postinfection with 10^5 pfu of MCMV. **, $P = 0.0046$; Student's *t* test.

of $Flt3^{wmfl/wmfl}$ NK cells produced IFN-γ 24 h following MCMV infection, but by 36 h postinfection, comparable percentages of WT and $Flt3^{wmfl/wmfl}$ NK cells were IFN-γ^+ (Fig. 3*C*). Thus, we considered that the increased MCMV susceptibility of $Flt3^{wmfl/wmfl}$ mice might potentially result in part from a functional defect in $Flt3^{wmfl/wmfl}$ NK cells in which both activation marker expression and IFN-γ production in response to MCMV infection are delayed.

We examined the intrinsic function of $Flt3^{wmfl/wmfl}$ NK cells. When stimulated in vitro by plate-bound antibodies against activating NK cell receptors such as NK1.1, NKp46, or Ly49D or exposed to IL-12 or IL-12 plus IL-18, $Flt3^{wmfl/wmfl}$ NK cells produced IFN-γ (Fig. S5*A*) and degranulated (Fig. S5*B*) as well as WT NK cells. In vivo, $Flt3^{wmfl/wmfl}$ NK cells were competent to kill $Tap1^{-/-}$ splenocytes as efficiently as WT NK cells (Fig. 3*D*). Moreover, when NK cells from $Flt3^{wmfl/wmfl}$ homozygotes or WT

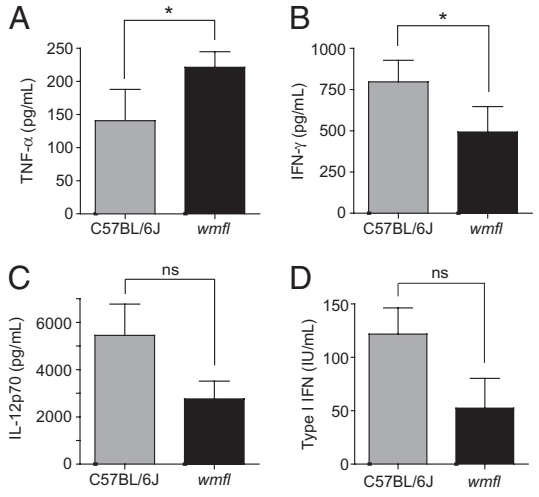

Fig. 2. MCMV-induced cytokine production in *wmfl* homozygotes. C57BL/6J and *wmfl* mice were infected with 2×10^5 pfu of MCMV ($n = 3$ mice per genotype). Production of (*A*) TNF-α, (*B*) IFN-γ, (*C*) IL-12p70, and (*D*) type I IFN was measured in the serum at 36 h postinfection. *, $P < 0.05$; Student's *t* test. Results are representative of three experiments. ns, not significant.

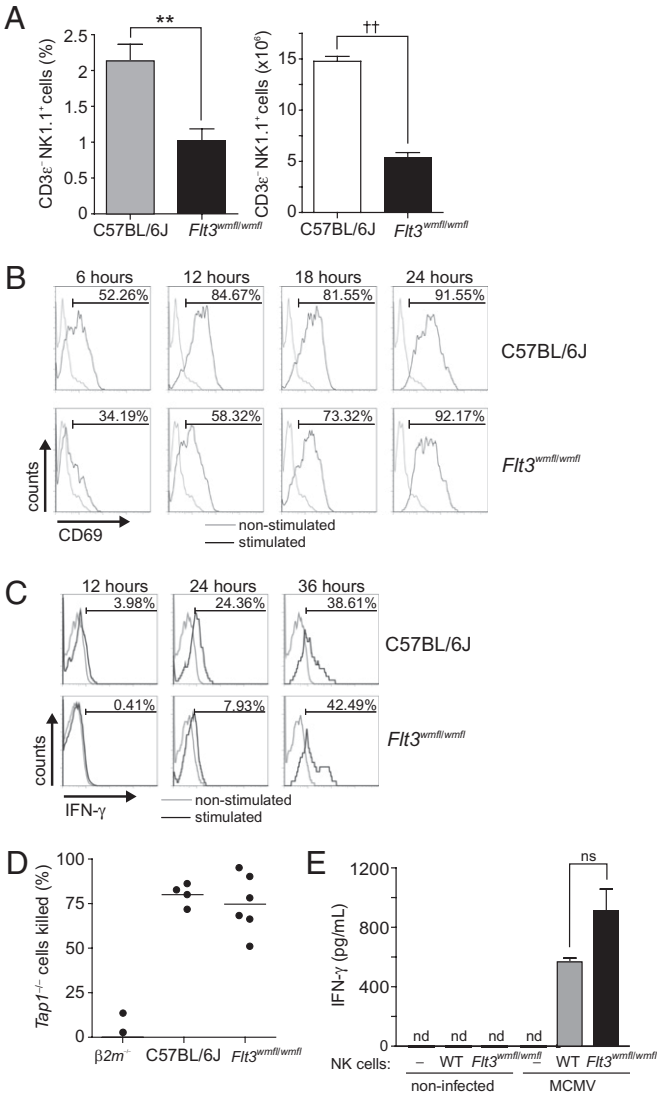

Fig. 3. Intact intrinsic function of homozygous $Flt3^{wmfl/wmfl}$ NK cells. (*A*) Percentage and absolute number of NK cells in the spleen of C57BL/6J ($n = 3$) and $Flt3^{wmfl/wmfl}$ mice ($n = 3$). **, $P = 0.0023$; ††, $P = 0.0022$; Student's *t* test. (*B* and *C*) Percentage of NK cells from the spleens of C57BL/6J and $Flt3^{wmfl/wmfl}$ mice expressing CD69 (*B*) or producing IFN-γ (*C*) at the indicated time points after infection with 2×10^5 pfu of MCMV ($n = 3$ mice per genotype per time point). Results are representative of three experiments. (*D*) Percentage of CFSE-labeled $Tap1^{-/-}$ cells remaining in the blood of $\beta 2m^{-/-}$, C57BL/6J, and $Flt3^{wmfl/wmfl}$ mice 24 h after injection of a mixture of CFSE-labeled $Tap1^{-/-}$ and WT splenocytes. (*E*) Serum concentration of IFN-γ 36 h after MCMV infection of $Rag^{-/-}IL2R\gamma^{-/-}$ mice reconstituted with WT or *wmfl* NK cells ($n = 4$ recipient mice per condition). nd, not detected; ns, not significant. –, $Rag^{-/-}IL2R\gamma^{-/-}$ mice reconstituted with medium only.

mice were transferred into $Rag1^{-/-}Il2rg^{-/-}$ recipient mice (lacking B and T lymphocytes as well as NK cells), $Flt3^{wmfl/wmfl}$ NK cells induced serum levels of IFN-γ equivalent to those induced by WT NK cells 36 h after MCMV infection (Fig. 3*E*). When provided with either $Flt3^{wmfl/wmfl}$ or WT NK cells, recipient mice survived for >10 days after MCMV infection, demonstrating that $Flt3^{wmfl/wmfl}$ NK cells are as competent as WT NK cells in protecting mice from MCMV-induced death. Thus, despite their diminished number in mice, $Flt3^{wmfl/wmfl}$ NK cells are intrinsically capable of acquiring full functionality in response to activating stimuli in vitro and in vivo. These observations suggested that MCMV susceptibility in $Flt3^{wmfl/wmfl}$ mice is not due to an in-

trinsic defect in the function of NK cells, but to a reduced number of NK cells and an extrinsic defect affecting NK cell performance during MCMV infection.

Inadequate *Flt3*$^{wmfl/wmfl}$ DC Response to MCMV Infection and TLR Stimulation. Flt3 signaling is known to stimulate DC development and proliferation. We hypothesized that impaired DC development and/or function might result in impaired NK cell activation leading to MCMV susceptibility in *Flt3*$^{wmfl/wmfl}$ mice. Therefore, we examined *Flt3*$^{wmfl/wmfl}$ DC populations and their functionality. In both bone marrow and spleen, the numbers of *Flt3*$^{wmfl/wmfl}$ CD11c$^+$ cells were significantly lower than those of WT mice, but percentages of *Flt3*$^{wmfl/wmfl}$ CD11c$^+$ cells were similar to those of WT mice (Fig. 4*A*). Among conventional DCs (cDCs) (CD8α$^-$CD11b$^+$CD11c$^+$ plus CD8α$^+$CD11b$^-$CD11c$^+$ cells) and plasmacytoid DCs (pDCs) (CD11c$^+$B220$^+$PDCA-1$^+$), the number of *Flt3*$^{wmfl/wmfl}$ cDCs was reduced in bone marrow and spleen, and both the number and the percentage of pDCs were significantly reduced in bone marrow and spleen, compared to those in WT mice (Fig. 4 *B* and *C*). However, mice depleted of pDCs do not recapitulate the MCMV susceptibility of *Flt3*$^{wmfl/wmfl}$ mice (11), demonstrating that the reduction in pDCs cannot by itself account for the MCMV susceptibility of *Flt3*$^{wmfl/wmfl}$ mice.

Conventional and CD8α$^+$ DCs are infected by MCMV (14, 15) and respond by producing type I IFN, IL-12 (16, 17), and surface IL-15Rα (6–9) that drive the NK response against MCMV. We tested the cytokine responses of splenic DC populations (CD11c$^+$ cells including CD11b$^+$ DCs, CD8α$^+$CD11b$^-$ DCs, and pDCs) to MCMV infection or stimulation by TLR ligands. *Flt3*$^{wmfl/wmfl}$ DCs produced reduced amounts of IL-12p40, type I IFN, and surface IL-15Rα relative to WT DCs infected with MCMV (Fig. 5 *A*–*C*). Similarly, *Flt3*$^{wmfl/wmfl}$ DCs produced reduced surface IL-12p40, type I IFN, and surface IL-15 in response to stimulation with either TLR7 or TLR9 ligands (Fig. 5 *A*–*C*). Notably, poly(I:C), a TLR3 ligand, induced similar concentrations of type I IFN in cultures of *Flt3*$^{wmfl/wmfl}$ and WT DCs. TLR4 stimulation with LPS elicited no cytokine responses from either *Flt3*$^{wmfl/wmfl}$ or WT DCs.

Finally, we measured up-regulation of costimulatory molecules and class II MHC protein by DCs in response to a panel of TLR ligands. We observed no differences between DCs from *wmfl* homozygotes and WT mice in the up-regulation of CD40 or CD86 (Fig. S6*A*). However, *Flt3*$^{wmfl/wmfl}$ DCs showed a moderate defect, similar in magnitude to that of DCs from *Unc93b1*$^{3d/3d}$ mice (18), in an in vivo assay for T cell proliferation dependent on cross-presentation by DCs (Fig. S6*B*).

Our observation of reduced numbers of DCs, particularly pDCs, in *Flt3*$^{wmfl/wmfl}$ mice is consistent with the reported role of the Flt3L → Flt3 axis in DC development. In addition, we observed that Flt3 signaling is required for DCs to produce cytokines, but not costimulatory molecules, in response to activation by MCMV, TLR7, or TLR9 stimuli.

Defective DC-Dependent NK Cell Activation in *Flt3*$^{wmfl/wmfl}$ Mice. We then tested the ability of TLR ligands and MCMV to drive DC-mediated NK cell activation when WT or *Flt3*$^{wmfl/wmfl}$ NK cells were cocultured with mixed DCs obtained from either homozygous *wmfl* or WT mice. Up-regulation of CD69 expression and IFN-γ production were both determined strictly by the DC genotype rather than the NK cell genotype: Whereas DCs from WT mice supported normal CD69 expression and IFN-γ production by either *Flt3*$^{wmfl/wmfl}$ or WT NK cells, DCs from *Flt3*$^{wmfl/wmfl}$ mice were unable to do so (Fig. 6 and Fig. S7). These effects were observed in response to MCMV infection or TLR7 or TLR9 stimulation. Poly(I:C) induced normal levels of IFN-γ in NK cells cultured with *Flt3*$^{wmfl/wmfl}$ DCs, consistent with the finding that poly(I:C) elicited WT levels of type I IFN and IL-15Rα expression in *Flt3*$^{wmfl/wmfl}$ DCs. Importantly, the same number of mixed DCs, with identical expression of immunophenotypic markers

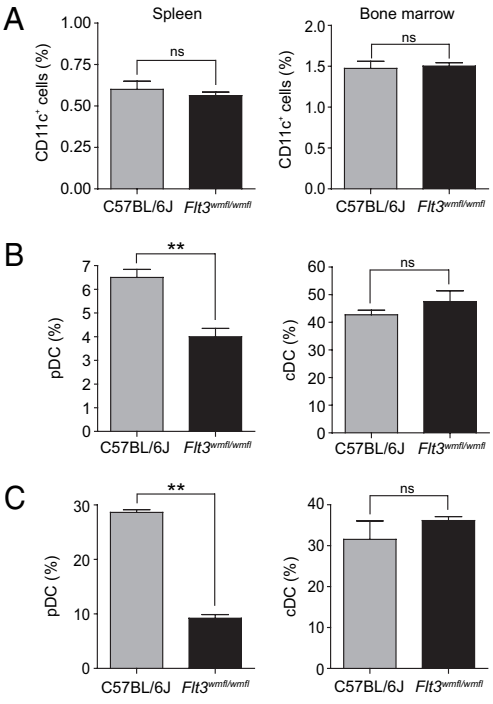

Fig. 4. pDCs are reduced in *Flt3*$^{wmfl/wmfl}$ mice. (*A*) Percentage of DCs (CD19$^-$CD3ε$^-$NK1.1$^-$CD11c$^+$ cells) from spleen and bone marrow of C57BL/6J and *Flt3*$^{wmfl/wmfl}$ mice. (*B* and *C*) Percentages of pDCs and cDCs from spleen (*B*) and bone marrow (*C*) of C57BL/6J and *Flt3*$^{wmfl/wmfl}$ mice. (*A*–*C*) $n = 3$ mice per genotype; results are representative of three experiments. (*B* and *C*) **, $P = 0.0022$; Student's *t* test. ns, not significant.

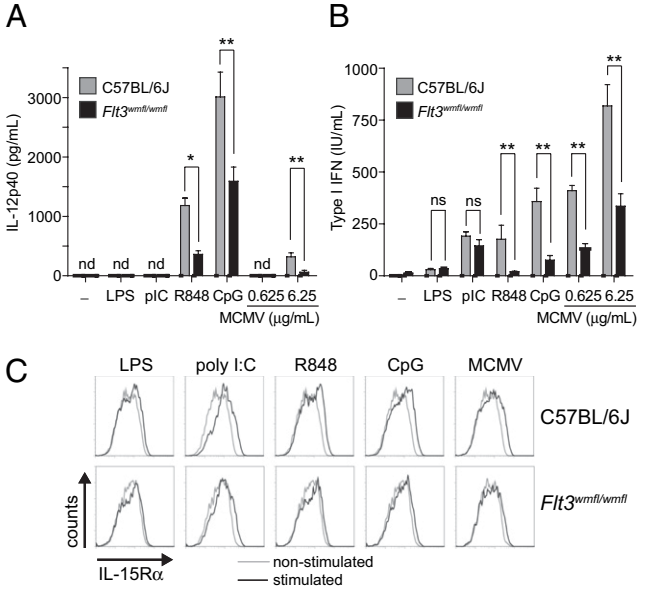

Fig. 5. Reduced cytokine production by *Flt3*$^{wmfl/wmfl}$ DCs in response to TLR stimulation or MCMV infection. Shown are concentrations of (*A*) IL-12p40 or (*B*) type I IFN in the culture medium or (*C*) cell surface expression of IL-15Rα on enriched CD11c$^+$ splenocytes of the indicated genotype 20 h following stimulation with ligands for TLR3, TLR4, TLR7, or TLR9 or after infection with MCMV. **, $P < 0.005$, Student's *t* test. nd, not detected; ns, not significant. Cells were isolated from $n = 3$ mice per genotype. Results are representative of two experiments.

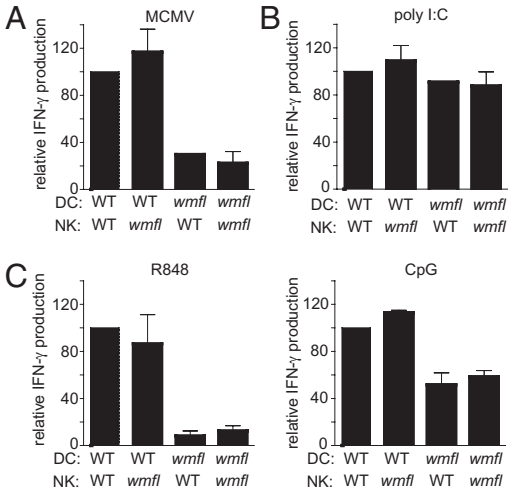

Fig. 6. *Flt3*$^{wmfl/wmfl}$ DCs do not support NK cell activation. Shown is relative IFN-γ production of NK cells isolated from C57BL/6J or *Flt3*$^{wmfl/wmfl}$ (*wmfl*) spleens and cocultured with C57BL/6J or *Flt3*$^{wmfl/wmfl}$ (*wmfl*) CD11c^{+} splenocytes that were infected with MCMV or activated with ligands for TLR3, TLR7, or TLR9. IFN-γ production by WT NK cells cocultured with WT DCs was used as the reference. Results are representative of three experiments.

(CD11c^{+}, CD11b^{+}, PDCA1^{+}, and CD8^{+}), were obtained from control and *Flt3*$^{wmfl/wmfl}$ donors, ruling out a difference in DC numbers as the cause of impaired NK cell activation in these coculture experiments.

The principal defect underlying MCMV susceptibility caused by the *Flt3*^{wmfl} mutation thus appears to be an impaired ability of DCs to assist in the activation of NK cells, which exist in diminished numbers in *Flt3*$^{wmfl/wmfl}$ mice. The most dramatic difference in performance of *Flt3*$^{wmfl/wmfl}$ DCs was seen in the failure of these cells to respond to TLR7 and TLR9 stimuli by producing IL-12, type I IFN, and IL-15Rα and in the failure of cocultured NK cells to produce IFN-γ and up-regulate CD69.

Flt3L Production Is Induced upon MCMV Infection or TLR Stimulation in Vivo. Whereas Flt3L is commonly regarded as a tonic stimulus for the differentiation and expansion of specific hematopoietic lineages, our data suggested a conditional role for its receptor (Flt3) in the response to infection. We examined the kinetics of Flt3L in the course of MCMV infection and investigated its cellular origins. Flt3L is up-regulated in the serum of MCMV-infected C57BL/6J mice as early as 36 h and peaks at 48 h postinoculation (Fig. 7*A*). Flt3L is also induced by i.p. injection of TLR3, TLR4, or TLR9 ligands, with serum levels peaking at 2 h and decreasing to baseline by 12 h after injection (Fig. 7*A*). We performed reciprocal bone marrow transplantation using WT and *Flt3L*$^{-/-}$ mice and measured serum Flt3L levels in response to either LPS or MCMV injection. When *Flt3L*$^{-/-}$ bone marrow was transferred to WT recipient mice, the level of Flt3L production was comparable to that observed in control mice (WT → WT) after MCMV infection (Fig. 7*B*) or LPS stimulation (Fig. S8*A*). However, greatly reduced levels of Flt3L were detected in the serum of *Flt3L*$^{-/-}$ recipient mice transplanted with WT bone marrow. Thus, cells of the nonhematopoietic compartment are required for induction of Flt3L in response to microbial stimulation.

Flt3 Signaling Induced by MCMV Is Not Essential for Control of Viral Infection. We sought to determine whether the acute increase of Flt3 signaling triggered by viral infection is necessary for survival. WT mice were treated with a neutralizing antibody against Flt3L 24 h before and 24 h after infection with 2×10^5 pfu of MCMV. Complete depletion of Flt3L in the serum of the mice was observed by ELISA at the time of the peak of Flt3L production

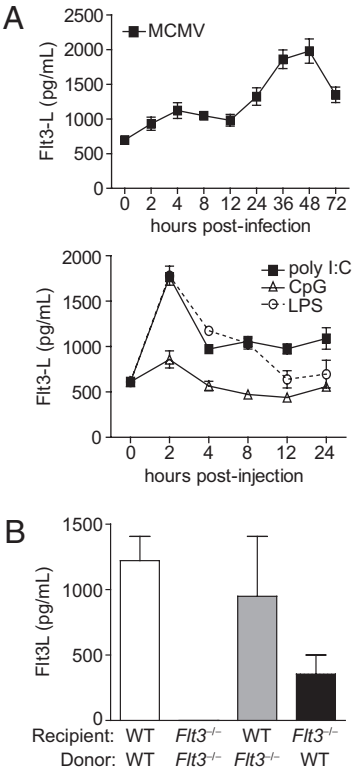

Fig. 7. Flt3L is induced in the nonhematopoietic compartment after MCMV infection. (*A*) Concentration of Flt3L in the serum of C57BL/6J mice at the indicated times after infection with MCMV or injection i.p. with ligands for TLR3, TLR4, or TLR9 (*n* = 3 mice per condition). (*B*) Concentration of Flt3L in the serum of bone marrow transplant recipient mice of the indicated genotypes 36 h after MCMV infection (*n* = 3 mice per condition).

(44 h postinfection). Viral titers in the spleen and liver of treated mice 5 days postinfection were no different from those of control mice that received a nonspecific antibody (Fig. S8*B*). These data indicate that the MCMV-induced burst of Flt3L is not necessary for the control of MCMV infection in vivo.

We also tested whether a burst of exogenous Flt3L before infection is sufficient to protect WT mice against a lethal inoculum of MCMV. Injections of 20 μg of Flt3L 6 days, 3 days, and 1 h before MCMV infection failed to protect mice from infection with 10^6 pfu of MCMV. Hence a burst of Flt3 signaling before virus inoculation is not by itself sufficient to promote survival during MCMV infection.

Discussion

Flt3 signaling is well known for its role in hematopoiesis and particularly in DC ontogeny (19–23). Flt3 signaling is thought to contribute predominantly to DC differentiation, although other factors such as GM-CSF drive DC function during inflammation and infection (24, 25). Consistent with previous studies, *Flt3*$^{wmfl/wmfl}$ mice had reduced numbers of DCs. We also found, however, that the function of Flt3 is not restricted to the differentiation of precursors to the DC identity per se. DCs that survived to maturity in a Flt3-deficient background, although expressing a variety of DC markers, lacked the full functionality of DCs that developed in the presence of Flt3. Flt3-deficient DCs produced reduced amounts of cytokines and thus failed to activate NK cells in response to viral infection. Recent data that Flt3L-induced expansion of NK cells in the absence of infection requires a CD11c^{hi} DC population expressing IL-15 (26) further support the idea that Flt3 signaling, by an unknown mechanism, renders DCs competent to stimulate NK cell activation and expansion.

We believe that the reduction in NK cell numbers is not likely, by itself, to account for the death of *Flt3*$^{wmfl/wmfl}$ homozygotes following sublethal inoculation with MCMV. NK cells from *Flt3*$^{wmfl/wmfl}$ mice exhibited full intrinsic functionality, and delays in *Flt3*$^{wmfl/wmfl}$ NK cell activation and function were observed only under conditions in which NK cells required activation by DCs, e.g., during TLR stimulation or MCMV infection in vivo, and in NK-DC cocultures. Thus, our findings suggest that impaired activation of NK cells by DCs is largely responsible for the MCMV susceptibility of *Flt3*$^{wmfl/wmfl}$ mice.

DCs are specialized to capture and present antigenic peptides in conjunction with MHC proteins for recognition by and activation of T cells in a process known as cross-presentation. The T cell response is essential for control of LCMV, but dispensable for early control of MCMV. Impaired cross-presentation by *Flt3*$^{wmfl/wmfl}$ DCs is consistent with the reduced ability of *Flt3*$^{wmfl/wmfl}$ mice to control LCMV and suggests that CD8$^+$ T cell expansion or cross-presentation itself may require signals from DC-derived cytokines, including type I IFN, IL-12, and IL-15, which are produced at reduced levels by *Flt3*$^{wmfl/wmfl}$ DCs.

In summary, we have revealed Flt3 and its ligand as key, nonredundant factors in surviving viral infection. We initially hypothesized that Flt3 signaling during viral infection is necessary for DCs to fully respond to TLR stimuli. However, Flt3 depletion during infection did not affect control of viral titers, indicating that Flt3 signaling as it occurs acutely during MCMV infection is neither necessary nor sufficient to promote the DC functions that activate NK cells and permit survival. We conclude that Flt3 signaling during DC development preconditions these cells to become competent to produce an adequate cytokine response and thus activate NK cells to full cytolytic function during viral infection. We cannot exclude the possibility that the burst of Flt3L production induced by TLR signaling in extrahematopoietic cells serves an important function in other types of infection, yet to be identified.

Materials and Methods

Mice. All mice were maintained under specific pathogen-free conditions in The Scripps Research Institute vivarium, and all studies involving mice were performed in accordance with institutional regulations governing animal care and use. BALB/c, C57BL/6-*Flt3L*$^{-/-}$, and *Rag1*$^{-/-}$*Il2rc*$^{-/-}$ mice were purchased from The Jackson Laboratory. C3H/HeN mice were purchased from Taconic Laboratories. *IFN*γ*R*$^{-/-}$ mice were a gift from C.D. Surh (The Scripps Research Institute). The *Flt3*$^{wmfl/wmfl}$ and *Unc93b1*^{3d} strains are described at http://mutagenetix.scripps.edu and may be obtained from the Mutant Mouse Regional Resource Center.

Viruses and Viral Load. The Smith strain was used for in vivo infection with MCMV. Preparation of the MCMV stock was described previously (12). C57BL/6J mice, which are naturally resistant to MCMV, were used for mutagenesis as previously described (27). The conditions of the in vivo MCMV susceptibility screen and the determination of the viral load were described previously (12). MCMV-GFP was used for in vitro experiments. MCMV-GFP was generated by insertion of the GFP gene into the MCMV bacterial artificial chromosome plasmid pSM3fr and provided by Chris Benedict (La Jolla Institute of Allergy and Immunology, La Jolla, CA).

Measurement of Cytokine Production. For serum cytokine detection, mice were bled from the retro-orbital sinus at different times poststimulation as indicated. Concentrations of IL-12p40, IL-12p70, IL-6, TNF-α, and IFN-γ were assayed by ELISA (eBioscience). The Flt3L ELISA Kit was purchased from R&D Systems (MFK00). The bioactivity of serum type I IFN was measured by luciferase assay using L929-ISRE cells as described previously (12).

Measurements of NK Cell and DC Populations and Isolation of NK Cells and DCs. Bone marrow cells were extracted from femurs and tibias. Bone marrow cells and splenocytes were stained for CD3ε^-NK1.1$^+$ cells to determine the percentage of NK cells. Cells were stained and gated on DCs determined by CD3ε^-CD19$^-$NK1.1$^-$CD11c$^+$ cells. To quantify the percentage of different DC populations, DCs were stained with anti-PDCA-1 and anti-B220 or anti-CD11b and anti-CD8α antibodies.

NK cells and DCs were isolated from spleens using the DX5 (CD49b) (130-052-501) or CD11c (130-052-001) magnetic affinity cell sorting (MACS) cell separation kit, respectively, from Miltenyi Biotech according to the manufacturer's instructions.

Ex Vivo NK Cell Activation After MCMV Infection and Adoptive Transfer of NK Cells. C57BL/6J and *Flt3*$^{wmfl/wmfl}$ mice were infected with 2×10^5 pfu of MCMV. Spleens were harvested after 6, 12, 18, and 24 h of infection and splenocytes were stained with CD3, NK1.1, and CD69 antibodies. Thirty-six hours postinfection, splenocytes were stained for intracellular IFN-γ production and 5 days postinfection, the expression of Ly49H was quantified by staining splenocytes with CD3, NK1.1, and Ly49H antibodies. Splenocytes harvested from noninfected mice were used as controls.

NK cells were isolated from C57BL/6J or *Flt3*$^{wmfl/wmfl}$ mice as described and 2×10^6 cells resuspended in PBS were i.v. injected per *Rag1*$^{-/-}$*Il2rc*$^{-/-}$ mouse. Mice were infected 24 h later with 2×10^5 pfu of MCMV and bled 36 h postinfection. IFN-γ production in the serum was quantified by ELISA.

In Vitro NK Cell Activity Assays. NK cells were plated at a concentration of 10^6 cells/mL in DMEM and 10% FCS in Immunol 2HB plates (Thermo Lab System) and stimulated with IL-2 (3,000 IU/mL), IL-12 (10 ng/mL), IL-18 (20 ng/mL), anti-NKp46 (10 μg/mL) (AF2225; R&D Systems), anti-NK1.1 (25 μg/mL) (clone PK136; eBioscience), or anti-Ly49D (5 μg/mL) (clone 4E5; BD Pharmingen). Cytokines were purchased from R&D Systems. To determine the percentage of cells degranulating, anti-CD107α antibody (clone 1D4B; BD Pharmingen) was added at the same time as the stimulus. After 6 h, cells were stained for NK cell markers (CD3ε and NK1.1). To measure the production of IFN-γ, Golgi STOP (BD Biosciences) was added simultaneously with the stimulus. Six hours later, cells were stained for surface expression of CD3ε and NK1.1. Intracellular staining for IFN-γ production was performed using Cytofix/Cytoperm and Perm/Wash buffers (BD Biosciences) according to the manufacturer's instructions.

In Vivo NK Cell Cytotoxicity Assay. A total of 10^7 control C57BL/6J splenocytes or TAP-deficient splenocytes suspended in 1 mL PBS were labeled with low (0.5 μM) and high (5 μM) concentrations of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma-Aldrich), respectively, at room temperature for 10 min. The labeling was stopped by addition of cold FCS. Cells were washed twice, counted, and resuspended at a concentration of 5×10^7 cells/mL. The two populations were mixed at a 1:1 ratio and injected i.v. into recipient mice. Recipients were bled the next day, and PBMCs were analyzed for CFSE staining by flow cytometry.

In Vitro Assay for DC Activation. The enriched CD11c$^+$ population was plated at a concentration of 1.25×10^6 cells/mL in DMEM, 10% FCS, 2% penicillin–streptomycin, and 5% β-mercaptoethanol and stimulated with LPS (800 pg/mL), poly(I:C) (50 μg/mL), R848 (30 ng/mL), CpG (15 μg/mL), or MCMV (0.625 or 6.25 μg/mL). Supernatant was harvested 20 h poststimulation and cytokine production was measured by ELISA. Cells were stained with CD11c, CD40, CD80, CD86, MHC class II, or IL-15Rα antibodies to measure the induction of these markers.

NK Cells/DCs Coculture. The enriched CD11c$^+$ population was plated at a concentration of 1.25×10^6 cells/mL in DMEM, 10% FCS, 2% penicillin–streptomycin, and 5% β-mercaptoethanol and stimulated with LPS (4 ng/mL), poly(I:C) (250 μg/mL), R848 (150 ng/mL), CpG (75 μg/mL), or MCMV (6.25 μg/mL). A total of 2×10^6/mL of NK cells were added 30 min later. After 24 h of coculture, expression of CD69 was determined by flow cytometry and IFN-γ production was measured in the supernatant by ELISA. Cells were pooled from four mice for CD11c$^+$ enrichment and from eight mice for NK cell purification, from each genotype.

Bone Marrow Transplantation. Bone marrow cells were extracted from femurs and tibias and were placed in PBS/0.1% BSA (vol/vol). Cell suspensions were filtered through 40-μm cell strainers (BD Falcon). Bone marrow cells (2×10^6) were injected i.v. into the orbital sinus of recipient mice that had been irradiated (1,000 rad) 24 h earlier. Eight weeks later, mice were injected with 50 μg of LPS or 2×10^5 pfu of MCMV. Flt3L production was quantified by ELISA in the serum of the mice 2 h post-LPS injection or 36 h post-MCMV infection.

Experiments on Peritoneal Macrophages. Peritoneal macrophages were isolated as previously described (12). Peritoneal macrophages were infected with MCMV-GFP at a multiplicity of infection (MOI) of 1. Twenty-four hours later, cells were trypsinized and the percentage of GFP-positive cells was determined by flow cytometry. Alternatively, peritoneal macrophages were

stimulated with TLR ligands as previously described (12). Cytokine production was measured in the supernatant 24 h after stimulation.

Assessment of cross-presentation by DCs. Spleens were isolated from OT-I Thy1.1$^+$ mice and homogenized into a single cell suspension. Splenocytes were then labeled with CFSE, and a total of 10^6 MACS-purified CD8$^+$ transgenic T cells were injected i.v. into recipient mice. Recipients were immunized i.v. one day later with 10^7 irradiated act-mOVA.Kb$^{-/-}$ splenocytes (28), and spleens were isolated 3 days after the immunization. OT-I CD8$^+$ T cell proliferation was recorded gating on Thy1.1$^+$ cells.

Flt3L Neutralization in Vivo. Twenty-four hours pre- and postinfection with 2×10^5 pfu MCMV, mice were injected i.p. with 10 μg of anti-mouse Flt3L (ref AF427; R&D Systems) or 10 μg of normal goat IgG (refAB-108-C; R&D Systems) as a control. Forty-four hours post-MCMV infection, mice were bled and sera were tested for the presence of Flt3L by ELISA. Viral titers were determined at day 5 postinfection by RT-PCR as described (29). The following PCR primers were used: forward primer 5′-AGAAAGTCTCCACGCAAGTGTCC-3′ and reverse primer 5′-TATCTCATCTTCACGAGGCAGCGG-3′.

Flow Cytometry and Antibodies. Single-cell suspensions from spleens, lymph nodes (pooled inguinal), or thymi were obtained and RBC were lysed in a hypotonic buffer for 10 min at room temperature. Cells were resuspended in staining buffer (PBS, 2% FCS, and 2 mM EDTA) at 1×10^6 cells/mL, and 100 μL of the cell suspensions was incubated for 30 min with conjugated antibodies at 4 °C. After washing, cells were quantitated by flow cytometry using a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star).

The following antibodies were used in this study for flow cytometry: CD8α (53-6.7), CD4 (L3T4), CD3ε (145-2C11), CD45.1 (A20), CD45.2 (104), CD69 (^{1}H.2F3), IFN-γ (XMG1.2), CD11b (M1/70), B220 (RA3-6B2), NK1.1 (PK 136), CD11c (N418), CD19 (1D3), PDCA-1 (eBio927), CD40 (HM40-3), CD80 (16-10A1), CD86 (GL1), MHC class II (M5/114, 15.2), Ly49H (3D10) (eBioscience), and IL15Ra (BAF551) (R&D Systems).

Statistical Analysis. The statistical significance of differences was determined by a two-tailed Student's *t* test. Differences with a P value <0.05 were considered statistically significant. All error bars represent SEM unless stated in the figure legends.

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