NK cells accumulate in infected tissues and contribute to pathogenicity of Ebola virus in mice.

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Abstract

Understanding the immune parameters responsible for survival following Ebola virus (EBOV) infection is paramount for developing countermeasures. In lethal EBOV infections, levels of both NK and T cells decline drastically in the circulation and lymphoid tissues before death. However, the fate of these lymphocyte in viral replication sites remains unknown. In this study, RT-PCR and FACS analysis were used to investigate lymphocyte frequencies in various mouse infected tissues after challenge with mouse-adapted EBOV (MA-EBOV). A decrease in NK cell numbers from the systemic circulation was observed concomitant to an increase of these cells in tissues that are supporting active replication of EBOV. Unexpectedly, NK accumulation in virus replication sites correlated with enhanced EBOV disease progression in specific conditions; at high challenge dose, NK depleted mice displayed lower viremia, liver damage and higher hepatic T cell level. Up regulation of UL16 binding protein 1 (ULBP-1) was detected on hepatic T cells.
suggesting that NK cells participate in their elimination. Overall, this study supports the concept that NK cells accumulate in EBOV infected tissues and that they can contribute to viral pathogenicity.

**Importance:**

Ebola virus (EBOV) outbreaks can claim numerous lives and also devastate the local health infrastructure, as well as the economy of affected countries. Lethal EBOV infection has been documented to decrease the level of several immune cells in the blood that are necessary to defend the host. This decrease in immune cells is however not observed in individuals surviving EBOV infection. Having a better grasp of how these immune cells are lost is therefore of high importance to develop and improve new and existing therapeutics. The significance of our research is in identifying the mechanism responsible for the apparent loss of immune cells in lethal EBOV infection. This will allow therapeutic options aiming at preventing the loss of these immune cells and therefore allowing infected individuals to better fight the infection.

**Keywords:** Ebola virus; Lymphopenia; NK cells; autologous killing; ULBP-1

**Introduction**

Untreated Ebola virus (EBOV) infection can lead to a haemorrhagic fever, multiple organ failure followed by septic shock and death in up to 90% of cases (1). During the 2014-2016 EBOV, the safety and efficacy of several therapeutic and prophylactic countermeasures were clinically evaluated (2). Although the protective efficacy of the (VSV) vaccine was clinically demonstrated(3), statistically significant survival benefits is yet to be demonstrated by any therapeutics against in efficacy trial (4, 5). Understanding the immune properties specific to
surviving or succumbing to an EBOV infection is important for the optimisation of therapeutic products.

Lethal EBOV infections are characterized by an increase in granulocytes and a concomitant decrease in total lymphocyte counts in the systemic circulation. Among the lymphocyte populations, the relative frequency of both T and NK cells drastically decline in the blood of infected individuals before death. A decrease in T cell levels was documented in human infections (6), while depletion of both T and NK cells was observed in non-human primates (NHP) (7, 8) and rodents (9) infected with EBOV. Interestingly, this decrease was only observed in lethal human infections but not in human survivors (6). This is of particular interest as both NK and T cells are important players in EBOV clearance (10–12).

The mechanism responsible for the decline in circulating T and NK cells is suspected to be indirect since neither T nor NK cells are infected by EBOV (7, 13). Apoptosis is currently believed to be one of the possible mechanisms responsible for the loss of T and NK cells. Numerous mechanisms such as FAS (6, 7, 14), TRAIL (7) and superantigen (15) mediated cell death have been proposed to explain T and NK cell apoptosis.

During EBOV infection, lymphocyte apoptosis has been mainly studied in peripheral blood and lymphoid tissues including the spleen and lymph nodes of infected animals. (6–9, 16). However, the lymphopenia observed in peripheral blood and lymphoid tissues might not reflect the fate of lymphocyte populations in non-lymphoid tissues such as the liver, lung and kidney where EBOV also replicates. T and NK cells migrate to infection sites in response to chemokines secretion. In addition, tissue resident T and NK cell populations have been described in various non-lymphoid tissues including the skin, liver, gut, uterus and lung (17, 18). Tissue resident lymphocytes and their circulating counterparts often have distinct phenotypic and functional properties (19, 20),
which could result in these pools of lymphocytes having different fates during an EBOV infection.

This study documents the fate of lymphocytes populations in various viral replication sites. This study also investigates the contribution of NK cells mediated killing of immune cells to the EBOV-induced lymphopenia. Here, both flow cytometry (FACS) and reverse transcription (RT)-PCR were used to monitor the presence of NK cells in the blood and tissues of mice infected with mouse-adapted (MA)-EBOV when lymphopenia was detectable. Depletion studies of NK cells in mice were performed to investigate NK cell function during MA-EBOV infection. The level of activating NK ligands was also monitored in MA-EBOV-infected tissues to inform on putative bystander NK cell targets.

Results

**NK cells accumulate in viral replication sites during MA-EBOV infection**

We sought to examine the fate of lymphocytes outside of the peripheral blood and lymphoid tissues during MA-EBOV infection. First, the kinetic of lymphopenia in peripheral blood was monitored in mice infected with a lethal dose of MA-EBOV (100LD50). As previously reported, the total lymphocyte percentage declines starting 3 days post infection and remain low around 30%. Similarly, total lymphocyte count decrease from 3 days post challenge but started rising 5 days post infection (data not shown). Based on the above kinetics, the presence of T and NK cells was then monitored by RT-PCR and FACS in various tissues four days post MA-EBOV (100LD50) challenge in subsequent experiments. The entire B cell population was used as a control as its frequency within the lymphocyte population remains relatively stable during EBOV infection. Mice were either mock infected or infected with MA-EBOV. Four days post infection,
the spleen, kidney, lung and liver were collected. The frequency of T, NK and B cells were first
analysed by RT-PCR. mRNA levels were normalized with actin to ensure that the loss of specific
immune subset would not artificially increase the frequency of other subsets. CD3epsilon and
CD19 mRNA levels decreased in all tissues assessed except for the liver and kidney where no
statistically significant change in CD3 and CD19 mRNA level was observed (Fig 1), thus
confirming the overall lymphopenia. In contrast, NKp46 mRNA level decreased in the spleen of
MA-EBOV infected mice, while its level rose 1.7, 2.4 and 3.5-fold respectively in the kidney,
lung and liver of infected mice (Fig 1), indicating an accumulation of NK cells to these organs at
that time point. To further document the frequency of T, NK and B cells in vivo, each respective
population was measured by FACS in the above mentioned tissues of MA-EBOV and mock
infected mice. Within all tested tissues, there was no significant difference in the frequency of
live haematopoietic cells (live CD45+), which include both lymphocytes and granulocytes. As a
result, frequencies of other immune subsets were expressed as percentage of live CD45+ cells.
The frequency of T and NK cells decreased while the frequency of B cells slightly increased in
the spleen of MA-EBOV infected mice. In the remaining MA-EBOV infected tissues (liver, lung
and kidney), there was a trend toward lower B cell frequency and a sharp decline in T cell
frequency. Conversely, in all these sites, the frequency of NK cells significantly increased,
suggesting an NK cell accumulation within these tissues (Fig 2). Taken together, both FACS and
RT-PCR results suggest that NK but not T cells accumulates in MA-EBOV infected tissues, also
indirectly reinforcing previous studies indicating that T cell depletion is due to cell death.

Levels of IFNα and KC, which modulate NK cell migration, are elevated after MA-EBOV
infection.
During hepatitis B virus (HBV) and murine cytomegalovirus (MCMV) infection, NK cell accumulation in the liver is dependent on interferon alpha (IFNα) and IL-8 production (21, 22). To investigate a possible role of these cytokines in promoting NK cells accumulation in the liver during MA-EBOV replication, circulating levels of IFNα, IL-8 and its murine homologue KC/CXCL1 and MIP2/CXCL2 (23) were measured by ELISA four days post challenge in mice. Elevated levels of IFNα, KC but not IL-8 or MIP2 were observed in sera from MA-EBOV challenged mice (Fig 3). These results suggest that elevated IFNα and KC production could both contribute to NK cell accumulation in the liver during MA-EBOV infection.

**The role of NK cells during EBOV infection is dependent on viral load.**

To better understand the role of NK cells during EBOV infection, NK depletion studies in BALB/c were performed using anti-asialo GM1 antibodies. NK depleted mice were infected with either a low dose (1 LD50) or a high dose (100 LD50) of MA-EBOV. At low viral load, fewer NK depleted mice survived the challenge compared with their mock depleted counterparts (Fig 4a). This suggests that NK cells can interfere with the progression of MA-EBOV-induced disease in these conditions. Interestingly, in mice infected with a 100-fold higher dose of MA-EBOV, no difference in survival rate was observed with or without NK cell depletion. However, NK depleted mice had a delayed time to symptom (weight loss) onset and to death than the control mice; 7.6+/− 0.2 days vs 5.8 +/-0.4 days, (p<0.001) (Fig 4b). Anti-asialo GM1 have been reported to deplete both NK and basophils(24). To ensure that the detrimental effect observed was due to NK cells, the latter challenge experiment was repeated in C57BL/6 using two distinct NK depleting antibodies. Both anti-asialo GM1 and anti-NK1.1 delay the mean time to death of MA-EBOV (100LD50) infected mice compare with mock treated ones from 7.2 to 8.1 and 7.9...
days post challenge respectively (**Fig 4c**). This delayed time to death suggests that with higher initial viral load, the NK cell response can be detrimental to the host. Interestingly, in the mouse model of LCMV infection, NK cells differentially affect the host immune response depending on the challenge dose (25).

**NK depletion delays liver damage during MA-EBOV infection.**

To investigate the mechanism behind NK cell mediated disease aggravation, viral load and liver damage were monitored in mock and NK depleted mice infected with MA-EBOV (100 LD50). Based on elevated alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels, no significant liver damage is detectable four days post MA-EBOV challenge. As a result, the above parameters were assessed five days post challenge. Viremia, ALP and ALT levels were all greatly reduced (p values of 0.04, 0.02 and 0.05 respectively) in NK depleted mice (**Fig 5a-c**), further supporting the idea that NK cells can play a detrimental role in specific conditions related to Ebola virus replication.

Both T and B cells are involved in controlling viremia during EBOV infection (12, 26, 27). To probe the decrease viremia and liver damage in NK depleted mice, hepatic level of both T and B cells were compare by RT-PCR between mock and NK depleted mice infected with MA-EBOV. Although no difference in hepatic B cell level was detectable, there was on average a 1.56-fold increase in hepatic T cell level in NK depleted mice compare with their mock depleted MA-EBOV infected counterpart (**Fig 5d**). This result may indicate a direct or indirect pathogenic effect of NK cells toward hepatic T cells.

**ULBP-1 is overexpressed on haematopoetic cells in the liver of MA-EBOV infected mice.**

The phenomenon of NK cell mediated pathogenicity was further investigated. We hypothesized that NK cell killing of hepatic T cells in MA-EBOV infected mice was responsible for their
detrimental effects at higher loads of MA-EBOV. Unfortunately, increase NK killing of hepatic T
159 cells from MA-EBOV infected mice could not be directly demonstrated using in vitro killing
160 assays due to the limited number of lymphocytes which could be isolated from livers. Instead,
161 expression of activating NK receptors and ligands was monitored on hepatic NK and T cells
162 respectively. Surface expression of activating TRAIL receptors or activating NKG2D ligands is
163 sufficient for target cells to become sensitive to lysis by autologous NK cells expressing TRAIL
164 or NKG2D respectively. Levels of NKG2D and TRAIL were first monitored on hepatic NK cell
165 by FACS in mock or MA-EBOV infected mice (100LD50). In MA-EBOV infected mice, only
166 5% of NK cells in the liver expressed TRAIL on their surface (Fig 6). In contrast, an average of
167 90% of NK cells expressed NKG2D in the liver of MA-EBOV challenged mice (Fig 6). Due to
168 high level of NKG2D expression on NK cells, the amount of NKG2D ligands on T and B
169 lymphocytes from the liver was measured in mock or MA-EBOV infected mice (100LD50). Of
170 note, activating TRAIL receptors level were not measured on immune cells due to the low
171 expression level of TRAIL on NK cells. There were no to little increase in the surface expression
172 of H60 and Rae-1, two NKG2D activating ligands, on T and B cells in the liver of infected mice
173 compared to their naïve counterparts. In contrast, surface expression of UL16 binding protein
174 (ULBP-1), a third NKG2D activating ligands was significantly increased on the surface of T and
175 B cells in the liver but not in other tested tissues from infected mice (Fig 7a-d). To confirm the
176 above FACS results, ULBP-1 mRNA levels were compared between naïve and MA-EBOV
177 infected mice (100LD50). Similarly to the FACS results, there was a wide range of ULBP-1
178 induction level in infected mice, overall there was a 2.66 fold increase in ULBP-1 expression in
179 the liver of MA-EBOV infected compared with naïve mice (Fig 7e). It is worth pointing out that
180 there was no difference in the ULBP-1 mRNA level between mock and NK depleted mice post
MA-EBOV challenge (Fig 7e). ULBP-1 is probably induced by direct contact with MA-EBOV viral particles or by MA-EBOV induced inflammation. Despite reduced NK killing of ULBP-1 cells, lower ULBP-1 induction in NK depleted mice is probably responsible for the similar ULBP-1 expression level between mock and NK depleted mice after MA-EBOV challenge. ULBP-1 induction on T cells has previously been shown to render them sensitive to autologous NK cell mediated killing (28). Therefore, the above results suggest that NK killing of ULBP-1 positive T cells in the liver may contribute to MA-EBOV immune evasion and viral pathogenicity.

Discussion

NK cells possess multiple antiviral properties. They can kill infected cells, secrete inflammatory cytokines and shape the adaptive response by interacting with dendritic cells (29–31). Accordingly, the beneficial impact of NK cells during MA-EBOV infection has previously been demonstrated in mice treated with virus like particle (VLP) or recombinant vesicular stomatitis virus encoding EBOV GP (VSV-GP) days before or after challenge respectively (11, 13). The present study indicates that the NK cell depletion in peripheral blood and the spleen of MA-EBOV infected mice was concomitant with an accumulation of NK cells in non-lymphoid tissues. The sharpest increase in NK cell frequency was observed in the liver, which is a major replication site early after EBOV infection (7). This observation suggests that NK cell accumulation may directly correlate with viral load and EBOV mediated inflammation. NK cells migrate in an IFNα and IL-8-dependent manner to the liver of HBV and MCMV infected individuals and mice, respectively, (21, 22). MA-EBOV infection induces secretion of IFNα and KC, the murine IL-8 homolog, suggesting that NK accumulation might results from circulating
NK migration to replication sites. It is worth pointing out that proliferation of tissue resident NK cells may also contribute to NK accumulation. Due to the lack of specific markers to differentiate tissue resident from circulating NK cells in our study, the contribution of each mechanism cannot be determined. Interestingly, a decrease in the number of circulating NK cells has also been described in NHPs infected with Lassa virus (32), suggesting that vast NK cell accumulation in sites of viral replication might be a common feature of viral haemorrhagic fevers.

Although the antiviral properties of NK cells are well documented, data obtained in this study indicates that the activity of NK cells can also be detrimental to the host. Infection of perforin deficient mice previously suggested that killing of non-infected (bystander) cells may be involved in MA-EBOV disease aggravation (13). Here, NK depletion studies in conjunction with the significant increase in ULBP-1 surface expression on T cells in the liver of MA-EBOV infected mice suggests NK cells killing of T cells. Although sufficient T and NK cells could not be obtained from liver to demonstrate in vitro NK killing of hepatic T cells from MA-EBOV infected mice, Cerboni and colleagues previously demonstrated NK killing of ULBP-1 positive T cells. Indeed, in vitro T cell activation leads to a similar increase in ULBP-1 surface expression, as well as an increase in susceptibility to autologous NK cells lysis via NKG2D (28). It is worth noting that NK killing of T lymphocytes within the liver of infected mice may contribute to MA-EBOV immune evasion. T cells are recruited to infection sites independently of antigen specificity (33, 34). Even though the role of these non-antigen specific infiltrating cells is not fully understood, they are thought to participate in controlling invading pathogens by producing anti-viral cytokines in response to the inflammatory cytokines milieu (35). Therefore, NK killing of T cells within the liver of infected mice, independently of their antigen specificity, may
facilitate viral replication and therefore exacerbate liver damage as MA-EBOV replication in hepatocytes leads to necrosis.

Additional mechanisms may also be involved in the observed NK cell mediated MA-EBOV disease exacerbation. Indeed, no significant ULBP-1 induction on T cells was detected in the lung of MA-EBOV infected mice despite a reduction in pulmonary T cells frequency. In addition, MA-EBOV replicates heavily in the liver but not in the lungs of intraperitoneal challenged mice, resulting in substantially more immune activation including cytokines in the liver. Taken together the above observations suggest that distinct mechanisms in the lung versus the liver of MA-EBOV infected mice could be at play.

NK killing of infected hepatocytes and epithelial cells could aggravate liver damage, while NK killing of infected antigen presenting cells such as dendritic cells and macrophages might dampen the cellular response against MA-EBOV. In addition, T cells apoptosis due to improper activation rather than direct killing by NK cells may also be responsible for the decrease in T cells frequency in MA-EBOV infected mice. Of note, five days post MA-EBOV infection, level of virus specific IgG are very low. As a result, NK mediated antibody-dependent cellular cytotoxicity (ADCC) is not expected to significantly contribute to the disease aggravation observed in our experiments.

Together, our work suggests the following model in which after low MA-EBOV challenge dose, NK cells participate in viral clearance and therefore improve survival. In contrast, in untreated mice challenged with high dose of MA-EBOV, NK cells contribute to viral pathogenicity. Treatments, such as VLP or VSV-GP administration close to the time of challenge, also promote a beneficial role of NK cells in mice challenge with high infectious dose by reducing the initial viral load (11, 13).
Data presented in this manuscript are restricted to the mouse model of EBOV infection and would therefore need confirmation in EBOV infected individuals. However, data from EBOV infected NHPs and individuals indicates that NK cell mediated disease aggravation is probably not limited to the rodent model of EBOV infection. An increase in circulating IFNα levels in NHPs challenged with a lethal dose of EBOV was previously reported (7). Wauquier and colleagues also demonstrated that KIR2DS1 and KIR2DS3, two activating killer immunoglobulin like receptors (KIR), which are expressed on NK cells and a subset of T cells, were more frequent in lethal EBOV infections than in EBOV survivors and the non-infected population (36). Whether additional NK activating ligands such as NKp44L and NKp30L, which do not have known murine equivalents (37), are also involved in disease aggravation in EBOV infected individuals will require further investigation. It is worth noting that NK cell disease aggravation has been previously described in the context of other infections. In the mouse model of LCMV infection, viral challenge also induces expression of NKG2D ligands on T cells. Furthermore, NK depletion resulted in lower viremia, liver damage and virus specific T cell response (38). NK cells also exacerbate disease progression in Semliki Forest virus (39) and Streptococcus pneumonia (40) infected mice. In addition, NK killing of CD4 T cells has also been postulated to participate in the decline of CD4 T cells in HIV-1 infection (41, 42).

Overall, the evidence presented herein supports the idea that the decrease in NK cell numbers from the systemic circulation is associated with an NK cell accumulation in non-lymphoid tissues supporting EBOV replication. This study also indicates that relocated NK cells can participate in MA-EBOV pathogenicity under specific conditions. Early reduction of initial viral load would influence the balance of NK cell-mediated functions and could led to better clinical management of infected individuals.
Methods

Mice and virus strains.

Five to six weeks old female BALB/c and C57BL/6 mice were purchased from Charles River (Quebec, Canada). Mice were infected with either 100 LD50 (high dose) or 1 LD50 (low dose) of mouse adapted (MA) – EBOV. The MA-EBOV strain used has been previously described (43) and is derived from the Mayinga strain of EBOV.

Antibodies

CD3 PE (500A2), NKp46 BV605 (29A1.4), CD19 PercPCy55 (1D3), CD45 alexa700 and CD45 APC-Cy7 (30-F11), CD8 PE-Cy7 (53-6.7), CD4 BV605 (RM4-5) antibodies were purchased from BD Biosciences (San Jose, CA), while CD3-PeCy7 (17A2), NKP46-FITC (29A1.4) and TRAIL-PE (N2B2) were from Biolegend (San Diego, CA). Fixable viability Dye eFluor 506 (ebioscience, San Diego, CA) or LIVE/DEAD fixable far red Dead cell stain kit (Life technologies, Burlington, Canada) were used to exclude dead cells.

Flow cytometry

Spleen, lung, liver and kidney from infected mice were harvested four days post MA-EBOV infection (100 LD50). Corresponding tissues from healthy mice were used as control. Tissues were homogenized using a plunger and a single cell suspension was obtained after filtering the homogenate through a 22 µM cell strainer. After extensive washes with PBS, samples were stained with the designated antibody cocktail and viability dye to exclude dead cells. After staining, all samples were analyzed using a LSR II (BD Biosciences, San Jose, CA). Analysis was performed using the Flow Jo software (TreeStar, Ashland, OR).
Spleen, lung, liver and kidney from naive or infected mice were harvested four days post mock or MA-EBOV infection (100 LD50), respectively, and preserved in RNAlater (Qiagen, Toronto, Canada) until use. RNA was purified from 30 mg of each tissue using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. One µg of purified RNA was transcribed into cDNA using the high capacity RNA to cDNA kit (Life Technologies, Burlington, Canada) according to the manufacturer’s instructions. Following reverse transcription (RT), 1 µl of cDNA was analysed by PCR using the Taqman gene expression master mix (Life Technologies) and with the following forward (F-) and reverse (R-) primers and probes (P-) in the 5’ to 3’ orientation; CD3 epsilon (F- GCCCAGAGGGCAAACAAG; R- TGCGGATGGGCTCATAGTCT; P- AGCGGGCACACCTGTTCCCA), CD19 (F- CGCCAGGAGATTCTTCAAAGTG; R- AGAGCACATTCCCGTACTGGTT; P- CCTCCCTCGGGAAACGGGACC), NKP46 (F- GACTCTCCGAAACCATCA; R- GTTCACCGAGTTCCATTG; P- TGGGCAAAACCAGCATCATGG). The beta actin (Mm01205647_g1) primer/probes mix was from Life Technologies. PCR plates were run onto a thermocycler StepOnePlus (Applied Biosystems, Burlington, Canada). CD3 epsilon, CD19 and NKP46 mRNA level were used to monitor T, B and NK cell frequencies, respectively, by RT-PCR, while beta actin mRNA was used as a loading control. Fold change between tested mice were calculated based on ΔΔCt method.

For ULBP-1 quantification, RT-PCR analysis was performed as described above except that ULBP-1 (Mm_ULBP1_1_SG) and actin beta (Mn_Actb_1_SG) Quantitect primers were obtained from Qiagen, and the PCR reactions were performed using the iQ SYBR Green supermix (Biorad, Mississauga, Canada) according to the manufacturer’s instructions.

**NK Depletion studies**
For NK cell depletions, mice received either 40 µl of polyclonal rabbit anti asialo GM1 antibodies (Wako BioProducts, Richmond, VA) 1 day before MA-EBOV challenge and on day 5, 11 and 18 post infection or 100 µg anti mouse NK1.1 (PK136) antibodies (Cedarlane, Burlington, Canada) 2 days before and on the day of challenge as previously reported (44). Both NK depleted mice and control mice were monitored daily for survival, weight loss and signs of disease for 28 days after MA-EBOV infection. NK cell depletion efficiency was confirmed by FACS using unchallenged mice.

Viremia

Mice were bled via the retro-orbital route into EDTA tubes (BD Biosciences, San Jose, CA). Viremia was determined by RT-qPCR as previously reported (45). Briefly, RNA was extracted using QIAmp Viral RNA Mini Kit (Qiagen). Level of EBOV RNA polymerase was detected by RT-qPCR.

Blood count and Liver damage

Mice were bled via the retro-orbital route and sera was collected using serum separation tubes (BD Biosciences). Blood counts were measure using the VetSan HM5 (Abaxis Veterinary Diagnostics, Union City, CA), while alanine aminotransferase (ALT) and alkaline phosphatase (ALP) measurement was measured using a VetScan VS2 (Abaxis Veterinary Diagnostics).

Cytokines serum level

IFNα and IL-8 ELISA kits were purchased from ebiosciences and Mybiosource, while KC and MIP2 ELISA kits were from Cedarlane. Serum levels were measured according to the manufacturers’ instructions.

Ethic statement
All infectious animal work was performed in the biosafety level 4 biocontainment laboratory at the Public Health Agency of Canada. The procedures described in this manuscript were approved under the animal use document H13-009 by the Canadian Science Centre for Human and Animal Health Care Committee following the guidelines of the Canadian Council on Animal Care. Animals were all acclimatized for at least seven days prior to the start of any experiments. Animals were fed and monitored daily before and during the course of the experiments.

**Statistical Analysis**

Differences in immune cells frequency were analyzed using a two-way ANOVA test followed by a Bonferroni test, while differences in mRNA levels, NKG2D ligands, weight loss, cytokines levels, ALT, ALP and viremia were analysed using unpaired t-tests. For RT-PCR results, mRNA level of each sample was first normalized using actin (using the $2^{-\Delta \Delta Ct}$ formula) as previously described (46). All statistical analyses were performed using GraphPad Prism version 5.03 software. Throughout the manuscript *, ** and *** indicates statistically significant differences with $p <0.05$, 0.01 and 0.001 respectively, while ns indicates non-statistically significant difference ($p >0.05$).

**Authors contributions:** H.F.-B. and G.K. designed the study. H.F.-B., X.Q, S.H., A.B, J.A., G.W., B.C., E.M., C.EH and B.P. performed experiments. H.F.-B., G.I. and G.K. analyzed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

**Additional Information**

**Competing financial interests**

The authors declare no competing financial interests.

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Figures Legend

Fig 1: NK cell accumulates in MA-EBOV infected tissues.
Level of CD3Ɛ, CD19 and NKp46 mRNA were compared in spleen, kidney, lung and liver from naïve and MA-EBOV infected (100LD50) mice 4 days after infection using RT-PCR. 6 mice were used per group and actin was used to normalize mRNA for each sample. Relative mRNA expression levels (mean +/- SEM) are indicated.

Fig 2: NK cell frequencies increased in MA-EBOV infected tissues.
Frequency of live (live/dead low) B (CD45+ CD19+), T (CD45+ CD3+) and NK (CD45+ NKp46+) cells in spleen, kidney, lung and liver was measured by FACS in naïve and MA-EBOV infected (100LD50) mice 4 days after infection. (a.) Gating strategies for B, T and NK cells
frequency. Representative plots of T, B (b.) and NK cell (c.) frequency from the lymphocyte gate are illustrated. (d.) Summary graphs (n=6 per group) of B, T and NK cells frequency (mean +/- SEM) are shown.

**Figure 3: IFNa and KC are induced after MA-EBOV challenge.**

Circulating level (mean +/- SEM) of various cytokines were measured by ELISA in sera of naive (open bars) and MA-EBOV (100LD50) infected mice (black bars) 4 days post challenge. Results are representative of a single experiment with 8 mice per group.

**Figure 4: NK cells can have beneficial or detrimental roles depending on MA-EBOV infectious dose.**

BALB/c (a.-b) and C57BL/6 mice (c.) were treated with PBS (black lines) or one of two NK depleting antibodies including asialo GM1 (grey lines) or anti NK1.1 (PK136) (dotted lines). Survival curves (left) and weight loss (right) are illustrated. (a.-b) BALB/c Mice (n=12 per group) were infected with a low dose (1LD50) (a.) or high dose (100LD50) of MA-EBOV (b.) and monitored for 28 days post infection. (c.) C57BL/6 mice were infected with MA-EBOV (100LD50) and monitored post infection. ns indicates non-statistically significant difference (p >0.05).

**Figure 5: NK cells contribute to MA-EBOV pathogenicity.**

(A-C.) Mock (black) and NK depleted mice (grey) were infected with high dose (100LD50) of MA-EBOV. Five days post challenge, viremia (a.), ALP (b.), ALT (c.) were measured by RT-
PCR and using a Vetscan respectively (n=8 per group). Means +/- SEM are shown, dotted lines represent the average values from naïve mice (n=6). (d.) Level of CD3Ɛ and CD19 mRNA were compare by RT-PCR between mock and NK depleted mice challenge with MA-EBOV (n=6 per group). Relative mRNA levels (mean +/- SEM) are illustrated. P values are indicated where the differences fell short of statistical significance. NK depletion was achieved by injecting anti-asialo GM1 antibodies.

Figure 6: Abundant NKG2D but limited TRAIL expression is detected on liver NK cells from MA-EBOV infected mice.

On day 4 after MA-EBOV infection (100LD50), TRAIL (a.-b.) and NKG2D (c.-d.) surface expression on NK cells were measured by FACS, with naive mice used as negative controls. Representative (a., c.) and summary graphs (n=6/group) (mean +/- SEM) (b., d.) are illustrated.

Figure 7: ULBP-1/MULT-1 is upregulated in the liver on MA-EBOV infected mice.

Level of NKG2D ligands (ULBP-1, H60 and Rae-1) were measured by FACS on live T (Live CD45+ CD3+ NKp46-) and B cells (Live CD45+ CD3- NKp46-) from spleen, lung and liver of naïve or MA-EBOV infected mice (100LD50) 4 days post challenge. (n=6/group). (a.) Gating strategies for H60, Rae-1 and ULBP-1 expression level. (b.) ULBP-1 expression (mean +/- SEM) on T and B cells from the spleen, lung and liver of mock and MA-EBOV infected mice are depicted. Summary graphs (b.) as well as representative plots (c.) are displayed for ULBP-1 (n=9/group). (d.) H60 and Rae-1 levels (mean +/- SEM) on hepatic lymphocytes are illustrated. (e.) Change in ULBP-1 mRNA were also measured by RT-PCR between naïve and MA-EBOV.
infected mice (mock or NK depleted). Relative mRNA levels (mean +/- SEM) are illustrated. NK depletion was achieved by injecting anti-asialo GM1 antibodies.
a. Singlet

FSC-A
FSC-A
CD45
Live/Dead
SSC-A
FSC-H
Singlet
Lymphocytes
Live CD45+

T, NK and B cells

b. Spleen

Kidney

Lung

Liver

CD3

CD19

NAIVE

MA-EBOV

Spleen

Kidney

Lung

Liver

CD19

NKp46

NAIVE

MA-EBOV
c.

d. Susbet freq (% of live CD45)

B cells

T cells

NK cells

Spleen

Kidney

Lung

Liver

Naive

MA-EBOV
a. 1LD50 BALB/c

% survival

Time post infection

b. 100LD50 BALB/c

% initial weight

p < 0.01

c. 100LD50 C57BL/6

% initial weight

Time post infection

- PBS
- Anti Asialo GM
- Anti NK1.1
a. Naive MA-EBOV

TRAIL

|    | Naive | MA-EBOV |
|----|-------|---------|
| 1.48 | 1.46  | 0.85    |
| 7.02 |       | 0.92    |

b. TRAIL freq (% NK)

***

c. NKG2D

|    | Naive | MA-EBOV |
|----|-------|---------|
| 6.67 | 8.02  | 14.3    |
| 2.09 |       | 31.5    |
| 2.09 |       | 3.64    |

d. NKG2D freq (% NK cells)

**MA-EBOV**
