Survival of the fittest clone: Pro-apoptotic protein Noxa controls selection of lymphocytes under competitive conditions
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Pro-apoptotic BH3-Only protein Noxa regulates effector T cell population size upon antigen re-encounter

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Abstract

During acute T cell responses, the pro-apoptotic BH3-only molecule Noxa controls high-affinity effector CD8 T cell formation via the elimination of activated low-affinity T cells but it is unknown if Noxa deficiency also influences secondary T cell responses. In this study we elucidated the role of Noxa in memory T cell formation and reactivation during acute and chronic infections. In Noxa−/− mice, influenza infection resulted in an enlarged memory T cell compartment which contained cells of increased clonal diversity and reduced affinity. Re-infection caused strong effector T cell expansion of predominantly high affinity cells. Chronic mCMV infection also resulted in a loss of initial affinity differences between WT and Noxa−/− T cells over time and to enhanced effector T cell accumulation in peripheral organs. In a transgenic model of persistent T cell activation, effector T cell accumulation ultimately led to severe organ pathology and premature death. These results establish the pro-apoptotic molecule Noxa as an important regulatory molecule of effector population size during recall responses. Absence of this molecule leads to excessive effector cell formation and clinical consequences of deregulated immune activation.

Graphical Abstract

For a color figure, see Appendix III
Introduction

Selection and expansion of cytotoxic T cells upon antigen encounter is a highly regulated process that controls outgrowth of high-affinity cells in order to generate an effective immune response against invading pathogens. During priming in the lymph node, T cell activation, survival and proliferation are regulated by the availability of antigen, co-stimulatory molecules, nutrients and cytokines (1). Absence of any of these factors after T cell receptor (TCR) stimulation generally results in termination of cells in order to prevent improper activation and ‘abuse’ of limited nutrients in the immunological space (2,3).

Even in the presence of all these factors, outgrowth of some activated T cells is favored over others, indicating a competitive advantage of the cells that acquire dominance. The major determining factor for this competitive advantage is T cell receptor (TCR) affinity (4). Previously, several groups have shown that high TCR affinity of the favors prolonged proliferation of T cells, thus resulting in dominance of these clones by overgrowing their low-affinity competitors (5,6). Recently, we have provided a mechanistic explanation for this phenomenon by showing that competition-based apoptosis mediates clonal dominance of high-affinity clones via the termination of low-affinity T cells (7). TCR affinity was found to influence stability of the pro-survival molecule Mcl-1 via an IL-2 receptor mediated signaling loop. T cells lacking Noxa, the antagonist of Mcl-1, therefore have a competitive advantage during priming. Because of this reduced survival threshold, Noxa-deficient mice have more subdominant clones that survive the competitive environment of expanding T cells in the lymph node and contribute to the effector cell pool. This results in decreased dominance of high-affinity cells and reduced antigen-responsiveness of the effector T cell pool as a whole.

The selection of memory T cells upon antigen re-encounter is much less stringent than that of naïve cells during the primary response (8). Memory cells have reduced dependence of co-stimulation and soluble factors produced by activated professional antigen presenting cells in the lymph node (8). In addition, these cells can respond and proliferate outside of the lymph node at the site of infection (9). The competitive pressure on these cells based on antigen-affinity is therefore different than for naïve cells. However, proper control of cell death mechanisms still plays an important role in memory T cell formation and antigen re-encounter (10,11). Both death-receptor mediated apoptosis and the mitochondrial or intracellular cell death pathway have been implicated in memory T cell biology (12,13). The intracellular cell death pathway is mediated by the Bcl-2 family of pro- and anti-apoptotic proteins, which mediate their effect by controlling mitochondrial outer membrane integrity (for a review see reference (14)). The balance between these two groups of molecules determines if apoptosis is initiated and for this reason the cellular levels of these molecules need to be carefully regulated, both at the transcriptional and post-translational level, during lymphocyte activation and differentiation.

In this study we investigated if the absence of Noxa provides a selective advantages during secondary T cell responses. Our data show that upon antigen re-encounter, Noxa no longer plays a role in the control of effector T cell affinity. However, in secondary responses, Noxa controls the total effector T cell pool size by regulating death of short lived effector T cells.
Chapter V

Results

Noxa ablation leads to increased numbers of memory T cells

Previously, we observed Noxa induction after in vitro stimulation of naïve CD8 T cells. To investigate whether Noxa may also play a role in memory T cell formation, gene expression of Noxa and 39 other apoptosis genes was analyzed ex vivo in CD8 T cell subpopulations. Mice were infected with Influenza A/PR8/34 virus and ten days later, splenic short lived effector cells (SLECs), memory precursor effector cells (MPECs) and central memory (CM) cells were sorted and mRNA expression was compared with naïve T cells via multiplex ligation-dependent probe amplification RT-MLPA analysis. Of the pro-survival Bcl-2 proteins expression of both Bcl-XL and A1 was higher in all antigen-experienced T cell subsets compared to naïve cells (Figure 1a). In accordance with previous findings (15), specifically SLECs had significantly lower Bcl-2 levels than naïve CD8 T cells. Of the pro-apoptotic molecules we observed that of the BH3-only molecules only Noxa transcript amounts were higher in all antigen-experienced populations, most prominently in SLECs and MPECs.

Mcl-1, the primary antagonist of Noxa, has an important function in T cell maintenance, yet its regulation is primarily on a protein level (16-18). We found no differential mRNA expression of this gene in any of the investigated cell subsets. Using a recently described protocol for intracellular Mcl-1 staining (19) we did find differential protein expression. Mcl-1 levels were high in SLECS, whereas CM cells displayed Mcl-1 expression of similar levels as seen in naïve T cells (Figure 1b).

Next, antiviral memory formation in Noxa<sup>-/-</sup> mice was investigated. Wild-type and Noxa<sup>-/-</sup> mice were infected with influenza A/PR8/34 and memory cell formation was followed over time. As previously described (7), at the peak of infection less D<sup>b</sup>NP<sup>366</sup> CD8 T cells with decreased intensity of tetramer staining were observed in Noxa-deficient mice (data not shown). Peripheral antigen-specific memory cell numbers were quantified in the periphery two months after infection. At this time point the vast majority of influenza-specific SLECs has died and almost all cells have a memory phenotype (CD127<sup>+</sup>KLRG1<sup>-</sup>). In contrast to acute T cell responses, Noxa<sup>-/-</sup> mice displayed increased numbers of D<sup>b</sup>NP<sup>366</sup> CM and effector memory (EM) cells in the spleen and lymph nodes (Figure 1c). To assess antigen responsiveness of these cells, splenic T cells were restimulated in vitro with limiting concentrations of viral peptides and IFNγ production was used as a read-out. We observed that antigen concentrations became limiting more rapidly for Noxa deficient cells (Figure 1d), suggesting a reduced affinity of these cells for antigen. This indicates that not only the formation of acute effector cells is affected in Noxa-deficient mice, but also the generation of T cell memory.

To test the functionality of Noxa-deficient memory T cells, an in vivo cytotoxicity assay was performed. Splenocytes of naïve congenic mice (CD45.1) were pulsed with the viral peptides NP<sub>366-374</sub>, PA<sub>224-233</sub> and PB-F2<sub>62-70</sub> to mimic viral infection, or with the control peptide SIINFEKL of ovalbumin. Pulsed cells were differentially labeled with DDAO, CFSE or both and injected in WT and Noxa<sup>-/-</sup> mice (CD45.2) that had been infected with influenza virus A/PR8/34 fifty days previously. Clearance of peptide-pulsed cells was followed over time. NP<sub>366-374</sub> and PA<sub>224-233</sub> pulsed cells were cleared most efficiently, which corresponds
Figure 1. Noxa ablation leads to enhanced memory T cell formation. (a) Naïve (CD3⁺CD4⁻CD8⁺CD44⁻CD62L⁺), Central Memory (CD3⁺CD4⁺CD8⁻CD44⁺CD62L⁺), MPECs (CD3⁺CD4⁺CD8⁺CD44⁺CD62L⁻CD127⁺) and SLECs (CD3⁺CD4⁻CD8⁺CD44⁻CD62L⁺CD127⁻KLRG1⁺) were sorted to >99% purity from splenocytes of mice infected 10 days previously with influenza A/PR8/34. mRNA expression of apoptotic proteins in these cells was analyzed by RT-MLPA. Shown is the relative gene expression of pro- and anti-apoptotic molecules after log transformation, relative to averaged values of naïve cells. Shown is one of two experiments (n=3) (b) Intracellular Mcl-1 expression was determined in Naïve (CD3⁺CD8⁺CD44⁻CD62L⁺), Effector Memory (CD3⁺CD8⁺CD44⁺CD62L⁻) and Central memory (CD3⁺CD8⁺CD44⁺CD62L⁺) populations. Quantification of Mcl-1 expression relative...
to isotype control. Shown is one of two experiments (n=8) e-d Mice were infected with influenza A/PR8/34 and 84 days after infection antigen-specific cells were analyzed. (e) Absolute numbers of antigen-specific cells in (left) spleen and (right) peripheral lymph nodes. (d) IFNγ production induced by varying doses of peptide in NP366-374 re-stimulated CD8+ T cells, relative to the level of cells stimulated with saturating (500 ng/ml) peptide. e-f Mice (CD45.2) were infected with influenza A/PR8/34 and 50 days after infection injected with splenocytes (CD45.1), fluorescently labelled and pulsed with the viral peptides NP366-374, PA244-233, PB1-F262-70 or the control peptide SIINFEKL (OVA). (e) Clearance of cells pulsed with viral peptides was measured as relative decrease compared to OVA pulsed cells. (bottom right) representative FACS plots of CD45.1+ cells before and 18hrs after injection. (f) Relative number of (top) IFNγ and (bottom) IL-2 producing CD8 T cells, 42hrs after injection of pulsed cells and after 4hrs of in vitro culture in the presence of Brefeldin A. Gated is on CD45.1+ cells (n=8). Error bars represent sem.

with their previously reported immunodominance in antiviral CD8 T cell responses (20). Importantly, despite an increased number of memory T cells in Noxa-/- mice, no increased clearance of viral peptide pulsed cells was observed (Figure 1e), suggestive of the decreased functionality of the memory pool. In accordance, when cytokine production of host cells was measured after a 4hr in vitro incubation with brefeldin A, production of the memory-associated cytokine IL-2 was significantly decreased in Noxa-deficient CD8+ T cells (21). IFNγ production was maintained in these cells, illustrating that Noxa-/- memory T cells are still capable of generating effector responses.

Together these data indicate that Noxa mice have increased memory T cell formation, yet of reduced functionality and antigen-affinity.

**Noxa-/- mice have increased CD8+ memory T cell responses**

The reduced IL-2 production of Noxa deficient memory CD8 T cells suggested that Noxa-/- mice would have impaired recall responses upon viral re-infection. To investigate this, WT and Noxa deficient mice were infected intranasally with the influenza strain HKx31. At the peak of the CD8 response against the dominant NP366-374 epitope, Noxa-deficient mice generated less DNP366 specific CD8 T cells, although this difference was less pronounced than after infection with the more virulent strain A/PR8/34 (Figure 2a). Late after infection, from day 28 onwards, Noxa-/- mice contained increased numbers of influenza-specific T cells in the blood, again pointing towards a role for Noxa in memory cell formation and/or maintenance (Figure 2a). Two months after primary infection, mice were infected with the influenza strain A/PR8/34. This virus contains envelope molecules that differ sufficiently from HKx31 to avoid clearance by anti-HKx31 antibodies. However, since the two strains have identical nuclear proteins, it does induce a strong CD8 memory T cell response (22). After re-infection, a pronounced T cell response was induced in wild type mice. In contrast with our expectations, Noxa-/- mice generated a significantly larger antigen-specific CD8 T cell response, both in the spleen and lungs (Figure 2b, c). In accordance with this phenotypic observation, a significantly increased number of CD8 T cells produced IFNγ after restimulation with NP366-374 (Figure 2d).

Next the antigen-affinity of these cells was assessed. Tetramer staining is based on TCR-MHC/peptide interactions and the mean fluorescent intensity (MFI) of staining was therefore previously shown to be a reliable measure for TCR affinity (7). During acute infection, significantly reduced DNP366 staining was found for Noxa-deficient T cells after Influenza infection (Figure 2e-i). However, when the MFI of T cells was analyzed at the peak
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*Figure 2. Noxa deficient mice have enhanced recall responses. a-e* Mice were infected with influenza A/PR8/34 and reinfe cted with influenza HKx31 after 65 days. (a) relative number of D^bNP366^+ cells in the blood. (b) Absolute numbers of (left) effector and (right) D^bNP366^+ CD8 T cells in the spleen and lungs of mice, 9 days after reinfection. (c) Relative number of IFNγ producing cells after *in vitro* restimulation with NP^366-374^ peptides. (d) Representative FACS plots of CD8 T cells stained with D^bNP366^ (i) 10 days after infection with Influenza HKx31 or (ii) 9 days after re-infection with influenza A/PR8/34 (e) IFNγ production induced by varying doses of peptide in NP^366-374^ re-stimulated CD8^+^ T cells, relative to the level of cells stimulated with saturating (1000 ng/ml) peptide. Shown is one of two comparable experiments (n=8). Error bars represent sem.

of the recall response, differences between WT and Noxa-deficient mice were no longer observed (*Figure 2e-ii*). In accordance with this phenotypic observation, when a dose response assay was performed with viral peptides, differences in antigen-responsiveness were almost completely lost (*Figure 2f*).

These findings indicate that Noxa does not play a role in affinity-based T cell selection upon antigen re-encounter, but does influence the maximum amount of effector T cells that is formed.

**Noxa controls memory T cell expansion upon antigen re-encounter**

To investigate whether the increased effector cell expansion in Noxa-deficient mice upon antigen re-encounter was T cell intrinsic, an adoptive transfer model was used. Wild Type and Noxa^−/−^ mice (CD45.1) were infected with A/PR8/34 virus and 10 days after infection, splenic CD8 T cells were isolated and equal numbers of cells were injected into a congenic (CD45.2) naive host. Due to the abrupt lack of an inflammatory environment for the transferred virus-specific cells, SLECs die rapidly (23). Since, at the peak of influenza infection, the vast majority of D^bNP366^ cells has a CD127^−^ phenotype, most of the transferred D^bNP366^ cells are cleared from the recipient mouse. Antigen-specific donor T
cells could be detected up to day 28 after transfer and during this period no difference was observed in the decrease of antigen-specific cells between WT and Noxa−/− cells (Figure 3a). This excluded a role for Noxa in the contraction phase of the antiviral response, which corresponds with previous findings after HSV infection (24). No increased number of Noxa deficient memory cells was observed, but this may be due to measurement difficulties (Note the Y axis in Figure 3a).

One month after transfer, recipient mice were infected with A/PR8/34 virus to look at the ability of donor cells to form recall responses. Noxa deficient memory T cells expanded significantly more than transferred wild type cells, which was most pronounced in the lungs, the site of viral replication (Figure 3b).

Together these data indicate that enhanced expansion of Noxa deficient memory T cells upon antigen recognition is T cell intrinsic. Also the increased memory formation is most likely not the result of differences in contraction after antigenic clearance.

**Figure 3. Enhanced recall responses of Noxa CD8 T cells results from an intrinsic defect** Wild-type and Noxa−/− mice (CD45.1) were infected with influenza A/PR8/34. 10 days after infection, CD8 T cells were isolated and injected in naïve Wild-type recipients (CD45.2). 30 days after infection, recipient mice were infected with A/PR8/34. (a) Percentage of donor DbNP366+ CD8 T cells in the blood of recipient mice, relative to the total number of CD8 T cells. Total short lived effector DbNP366+ CD8 T cells are shown. b-c Donor DbNP366+ CD8 T cells (b) relative to the total number of CD8 T cells and (c) in absolute numbers.

**Noxa−/− mice generate a more diverse memory T cell compartment**

During acute influenza responses Noxa−/− mice generate a more diverse effector T cell pool, due to prolonged persistence of subdominant clones (7). Our data here indicate that Noxa mediated affinity selection also appears to play a role in memory formation, but not during recall responses. This would suggest a more diverse T cell repertoire of Noxa-deficient memory T cells, but not of effector cells after rechallenge. To elucidate this notion on a molecular level, clonal diversity of DbNP366+ CD8 T cells during memory and after recall were analyzed.

WT and Noxa-deficient mice were infected with influenza A/PR8/34 virus and DbNP366+ CD8 T cells were sorted after 84 days. Alternatively, mice were infected with influenza HKx31, re-infected with A/PR8/34 after 65 days and DbNP366+ CD8 T cells were sorted 9 days later. Clonal analysis was performed by deep sequencing and amino acid sequences of CDR3 regions were used for comparison. Chosen was to analyze only T cells that use the Vβ8.3 gene fragment, since this population has been well documented to have a
pronounced dominance upon influenza infection and generates a public response, which allows direct comparison between mice (25).

When diversity within the memory population was compared with the effector cell pool after rechallenge, more subdominant clones were observed within the memory pool (Figure 4a,b). In both populations, however, the majority of cells was derived from only a few highly expanded clones (Figure 4c). When memory cell populations were compared between Wild-type and Noxa−/− animals, the average amount of copies retrieved per clone was significantly lower in the Noxa deficient memory pool. This difference was primarily caused by an increase of low frequency (<50 copies per clone) clones and not by a decreased number of copies per clone (Figure 4d and data not shown). This difference could not be caused by

Figure 4. Increased sequence diversity in antigen-specific CD8 T cell populations DNP366+ CD8 T cells were sorted to >99% purity 84 days after influenza A/PR8/34 infection or nine days after secondary infection with influenza A/PR8/34, 65 days after primary infection with influenza HKx31. a-b frequency of individual clones (a) during memory and (b) after viral rechallenge. Shown is pooled data of three mice per genotype with population means. Equal numbers of clones were sequenced (6074 ±398 clones per mouse). c Clones were divided in four categories (1-2 times, 3-20 times, 20-50 times or >50 times retrieved) based on the frequency with which they were retrieved. Relative contribution of each of these categories is given. (RI: Re-Infection). d Average number of clones per category shows specific increase of low frequency clones (3-20 times retrieved) in Noxa deficient mice.
‘pollution’ of naïve clones, as similar numbers of clones that were received only 1-2 times were observed in Wild-type and Noxa deficient populations. These data correspond surprisingly well with our previous findings that Noxa mice allow survival of more subdominant clones in the effector cell pool during acute infection (7).

When rechallenged cells were compared, differences in diversity between Wild Type and Noxa deficient cells were no longer observed. This indicates that the previously described narrowing of the antigen-responsive repertoire (26) is not dependent on a Noxa mediated selection mechanism. This also corresponds with our findings after reinfection, which shows only marginal differences in antigen-affinity between Noxa−/− and Wild-type mice (Figure 2f).

**Noxa ablation leads to increased memory inflation during chronic mCMV infection**

Primary infection of Noxa deficient animals does not lead to increased effector cell numbers, but to an effector pool of increased diversity and reduced affinity. Recall responses, on the other hand, result in loss of diversity differences but cause enhanced effector T cell numbers. Also, aged Noxa-deficient mice accumulate large numbers of memory cells (7). These data indicate that during chronic infection, apoptosis-mediated selection based on antigen-affinity no longer play a role but may lead to accumulation of effector cells in the absence of Noxa. To study this notion, WT and Noxa-deficient mice were infected with mouse cytomegalovirus (mCMV).

CMV causes a chronic infection that is never cleared. After the acute phase upon primary infection, CMV remains latently present in its host, but is regularly reactivated which causes flares of the antiviral response (27). This continuous boost of the immune system causes a gradual increase of CD127−KLRG1+ CMV-specific CD8 T cells, a so called ‘memory inflation’, which will ultimately comprise a very significant percentage of the total CD8 T cell pool both in humans and in mice (27). We studied CD8 T cells directed against three different viral Kb epitopes; one epitope which is only dominant during the acute phase of mCMV infection (m57816-824), one epitope that is only immunodominant during memory inflation (IE3/m122416-475) and an epitope that is dominant during both responses (m139416-423) (28).

During acute infection we observed reduced numbers of CD8+ T cells directed against m139, whereas total effector T cell numbers were not different (Figure 5a and data not shown). These results are comparable with our findings after influenza infection (7). Responses against m57 were only reduced early during mCMV infection, suggesting differences in selective pressure between cells for these two epitopes. When intensity of tetramer staining was assessed, clearly a reduction in tetramer binding was observed in Noxa−/− mice for both epitopes (Figure 5b,c). When splenic T cells were restimulated in vitro on day 8 after infection, a clear reduction in peptide responsiveness was observed (Figure 5d). Together these data indicate that also during primary mCMV infection Noxa-deficient mice generate an effector T cell response of reduced affinity.

Subsequently, the antiviral CD8 T cell formation was analyzed during the chronic phase of mCMV infection. Previously we observed that aged Noxa−/− mice accumulate effector T cells, of which we hypothesized that this was antigen driven (7). During mCMV infection both genotypes showed memory inflation for the epitopes m139 and IE3, even though the ratio between antigen-specific cells appeared to be skewed towards IE3 in the
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Figure 5. Acute mCMV infection of Noxa<sup>−/−</sup> mice leads to the formation of effector cells of reduced antigen-affinity. a-d Mice were infected i.p. with mCMV and antigen specific responses were followed for three viral epitopes. Shown is one of three experiments (n=8). (a) Number of (left) K<sup>b</sup>m139<sup>+</sup>, (middle) K<sup>b</sup>m57<sup>+</sup> and (right) K<sup>IE3</sup>CD8<sup>+</sup> T cells, relative to the total number of CD8 T cells. (b) Representative FACS plots of tetramer stained CD8 T cells eight days after infection. (c) Quantification of the mean tetramer staining intensity (MFI) eight days after infection. (d) IFNγ production induced by varying doses of peptide in m57<sub>816-824</sub> re-stimulated CD8<sup>+</sup> T cells, relative to the level of cells stimulated with saturating (500 ng/ml) peptide.

Noxa mice (Figure 6a). At various timepoints after infection, peptide restimulation assays were performed to investigate whether antigen-responsiveness differed between Wild-type and Noxa-deficient cells. This revealed that during memory inflation the affinity differences that occurred after acute infection were lost (Figure 6b). Accordingly, no differences in tetramer staining intensity were observed (data not shown).

The loss of affinity differences may be the result of specific outgrowth of antigen-specific repertoire under influence of constant restimulation by antigen, in a process that is not controlled by Noxa. To investigate whether inflating CD8 T cell pools increase their affinity in Wild Type mice, peptide restimulation dose-response curves were compared after various time points after infection. These data revealed that indeed, for the inflationary epitopes, relative peptide responsiveness increased over time, whereas it remained unaltered for the non-inflationary epitope m57 (Figure 6c).

Even though qualitatively no differences were observed between Noxa-deficient and Wild-type effector cells, quantification of absolute numbers showed enhanced accumulation of CD8 T cells in multiple compartments of Noxa-deficient mice (Figure 7a). Phenotypic analysis of these cells showed that this difference was primarily the result of an accumulation of KLRG1<sup>+</sup>CD127<sup>+</sup> short lived effector cells. No differences were observed in the lymph node, which corresponds with the notion that when no acute infection is taking place, low numbers of effector cells are present in these organs.
Antigen-specific analysis of CD8 T cells revealed that it was primarily the IE3-directed CD8 T cell population that accumulated in the organs of Noxa<sup>−/−</sup> mice (Figure 7b), which is also the most prominently inflationary pool (Figure 6a) which may explain the relative difference in response against this epitope and the m139 epitope. Phenotypic analysis of these cells showed that in Noxa<sup>−/−</sup> mice IE3-specific cells contained more short lived KLRG1<sup>+</sup>CD127<sup>−</sup> cells (Figure 7b), which were predominantly CD27<sup>−</sup> (data not shown). Interestingly, also the non-inflationary m57-directed cells were increased in the Noxa<sup>−/−</sup> mice. This is corresponds with our observation that Noxa-deficient mice have increased numbers of memory cells after acute influenza infection. Together these data indicate that Noxa-deficient mice accumulate memory CD8 T cells and that chronic infection leads to continuous reactivation and accumulation of short lived effector cells in these animals.

Figure 6. Chronic mCMV infection leads to increased affinity of the antigen-specific effector cell pool. a-c Mice were infected i.p. with mCMV and antigen specific responses were followed for three viral epitopes. Shown is one of two experiments (n=8). (a) Number of (left) K<sup>b</sup>m139<sup>+</sup>, (middle) K<sup>b</sup>m57<sup>+</sup>, and (right) K<sup>b</sup>IE3<sup>+</sup>CD8<sup>+</sup> T cells, relative to the total number of CD8<sup>+</sup> T cells. (b) IFNγ production induced by varying doses of peptide in m57<sub>816-824</sub>, m139<sub>419-426</sub> or IE3<sub>466-475</sub> re-stimulated CD8<sup>+</sup> T cells, relative to the level of cells stimulated with saturating (500 ng/ml) peptides, 171 days after infection. (c) IFNγ production induced by varying doses of peptide in m57<sub>816-824</sub>, m139<sub>419-426</sub> or IE3<sub>466-475</sub> re-stimulated Wild-type CD8<sup>+</sup> T cells, relative to the level of cells stimulated with saturating (1000 ng/ml) peptides, 114 and 171 days after infection.
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Persistent activation of the T cell compartment leads to severe pathology in Noxa\(^/-\) mice

mCMV infection represents a model in which the CD8 T cell compartment is continuously triggered by low doses of antigen. Other infections, such as HIV infection in humans, lead to a constant hyperactivation of the immune system due to the continuous presence of high viral titers. To investigate how Noxa deficient animals perform during persistent high-grade immune activation, a transgenic mouse model was used to increase immunological pressure on the T cell compartment. Co-stimulation via CD27 on naïve T cells directs differentiation into effector cells (29). Mice that constitutively express the CD27 ligand CD70 on B cells (CD70\(^{TG}\) mice) possess an enlarged fraction of effector T cells, which is driven by hyper-responsiveness to environmental antigen (30,31). Therefore, this model for chronic T cell activation was chosen to investigate the role of Noxa during high-grade immune activation and Noxa\(^/-\) mice were crossed with CD70\(^{TG}\) mice.

Figure 7. Noxa\(^/-\) mice accumulate effector CD8 T cells during chronic mCMV infection a-d Mice were infected i.p. with mCMV and antigen specific responses were followed for three viral epitopes. Shown is one of two experiments (n=8). (a) Absolute number of (left) effector CD8 T cells and (right) Short lived effector cells, 171 days after infection. (b) Absolute numbers of antigen-specific cells in the spleen and liver of mice, 171 days after infection.

**Figure 7. Noxa\(^/-\) mice accumulate effector CD8 T cells during chronic mCMV infection a-d Mice were infected i.p. with mCMV and antigen specific responses were followed for three viral epitopes. Shown is one of two experiments (n=8). (a) Absolute number of (left) effector CD8 T cells and (right) Short lived effector cells, 171 days after infection. (b) Absolute numbers of antigen-specific cells in the spleen and liver of mice, 171 days after infection.**
Under homeostatic conditions, CD70\textsuperscript{TG} mice remain relatively healthy for at least 6-8 months but eventually die from opportunistic infections as a result of T cell depletion (31). In stark contrast with this gradual phenotype, Noxa\textsuperscript{-/-} x CD70\textsuperscript{TG} (Noxa70) mice displayed severe organ pathology, including splenomegaly, lung damage and liver cirrhosis already at early age (Figure 8a and data not shown). Pathology quickly exacerbated, resulting in premature death with a median survival of only 16 weeks (Figure 8b). Noxa70 mice accumulated high

![Figure 8](image)

**Figure 8. Noxa ablation causes increased SLEC survival culminating in lethal pathology upon chronic T cell activation.** (a) Representative spleens of WT, Noxa\textsuperscript{-/-}, CD70\textsuperscript{TG} and Noxa70 mice at 12 weeks of age. (b) Kaplan-Meier survival plot for mice of indicated genotypes housed under SPF conditions. Wild type (n = 8). Noxa\textsuperscript{-/-} (n = 12), CD70\textsuperscript{TG} (n = 7), Noxa70 (n = 21). (c) Haematoxylin and eosin (HE) analysis of liver sections from CD70\textsuperscript{TG} and Noxa70 mice at 15 weeks of age, showing increased cellular infiltrates in Noxa70 compared to CD70\textsuperscript{TG} mice. (d) Activated caspase-3 and haematoxylin staining in spleen sections of mice at 15 weeks of age, showing reduced numbers of apoptotic cells (marked by arrowheads) in Noxa70 compared to CD70\textsuperscript{TG} mice. a-d for color figures, see Appendix III (e) Effector memory cell (CD4\textsuperscript{4}CD62L\textsuperscript{−}) and (f) short lived effector cell (CD4\textsuperscript{4}CD62L\textsuperscript{−} KLRG1\textsuperscript{−}) numbers in spleen and liver of WT, Noxa\textsuperscript{-/-}, CD70\textsuperscript{TG} and Noxa70 mice at twelve weeks of age (n = 4-7). Significant differences are compared to Noxa70 mice. (g) Representative FACS plots for short lived effector cell accumulation in Noxa70 mice. Shown are splenic CD4\textsuperscript{4}CD44\textsuperscript{−}CD62L\textsuperscript{−} T cell subsets. (h) Cytokine production of CD4 and CD8 T cells from the spleens of WT, Noxa\textsuperscript{-/-}, CD70\textsuperscript{TG} and Noxa70 mice stimulated with PMA/Ionomycin in the presence of Brefeldin A. Number of (left) IL-10 and (right) IFN\textgamma{} producing cells as a percentage of the total CD4 or CD8 T cell pool is given. Significant differences are compared to Noxa70 mice. (* p<0.05, ** p<0.005).
numbers of lymphocytes in spleen, liver and lung (Figure 8c and data not shown). Activated caspase-3 staining in the spleen showed less apoptotic cells in Noxa70 mice, indicating that the accumulation of lymphocytes was the result of decreased apoptosis compared to CD70Tg mice (Figure 8d).

Phenotypic analysis of these cells revealed that it were primarily effector CD4 and CD8 T cells of a KLRG1+ phenotype that accumulated (Figure 8e-g), which corresponds with our findings in mice chronically infected with mCMV. In accordance with their effector phenotype, Noxa70 T cells expressed increased levels of pro-inflammatory cytokines, whereas inhibitory mediators such as IL-10 were unchanged compared to the CD70Tg (Figure 8h and data not shown). Even though extensive organ pathology was observed, there was no direct evidence that these mice suffered from auto-immunity, since no T cell infiltrates were observed in the kidney or brain.

Together these data indicate that Noxa plays a role in the control of effector T cell population size under conditions of persistent high antigenic pressure. Ablation of this molecule leads to severe organ pathology under conditions of chronic immune activation.

Discussion

T cell expansion and differentiation into effector cells upon antigen encounter is markedly different between primary and secondary responses. When a naïve cell is activated, multiple layers of regulation ensure the outgrowth of only a limited number of clones from the vast pool of different cells within the naïve T cell compartment (REF). The primary checkpoint appears to be the selection based on antigen-affinity, which is mediated by differences in survival and proliferation of clones competing for antigen (REF, REF). This selection and expansion therefore takes place within the lymph node. Here, naïve clones are clustered in specific zones and the presence of specialized hematopoietic cell subsets such as dendritic cells and CD4 T cells generates the required immunological environment (REF, REF).

Activation of memory clones, on the other hand, is subjected to other restrictions. Expansion of these cells requires less help from specialized immune cells and appears to be limited primarily by the availability of antigen (REF, REF). These cells have already been selected based on their antigen-affinity and their proven selectivity therefore requires less regulation based on that feature. Expansion of these cells therefore does not necessarily take place in the lymph node, but may also occur in the periphery (REF) where much less interclonal competition occurs.

Our data here give Noxa a central role in mediating T cell responses both during primary and secondary antigen encounter. During T cell priming, Noxa mediates limits clonal diversity both during the formation of effector (7) and memory (shown here) CD8 T cell populations. During secondary expansion, affinity-based interclonal competition is less stringent than during priming and Noxa does not play a major role during outgrowth of dominant clones in these responses. However, total effector population size during secondary expansion is controlled by Noxa. Ablation of this molecule leads to accumulation of short lived effector cells in peripheral tissues, leading to severe organ pathology and premature death in a transgenic model of high antigenic pressure.

An open question is whether the role of Noxa during primary and secondary effector T cell formation comprises two different mechanisms, or represents two aspects of the same
apoptotic checkpoint. Previously we showed that the Noxa/Mcl-1 axis mediates affinity based T cell selection based on IL-2R mediated control of Mcl-1 stability. Even though re-activated T cells also express high levels of the IL-2 receptor, IL-15 appears to play a more prominent role in the maintenance of memory cells and the early phases of their expansion upon antigen re-encounter (32). However, since Noxa was also shown to play a role in mediating NK cell apoptosis upon IL-15 deprivation (REF), Noxa may also mediate IL-15 responses of re-activated memory T cells. IL-15 is a cytokine which is highly produced by infected tissues (REF). We therefore hypothesize that Noxa controls maximum population size by mediating apoptosis under conditions of limiting tissue-IL-15.

Alternatively, the role of Noxa during recall responses may be mediated by a completely different mechanism than during priming. Previously it was shown that Th2 CD4 memory cell formation is dependent on repression of Noxa by the molecule Bmi1 (33). Even though we did not find repressed expression of Noxa in resting memory CD8 T cells, it is possible that a similar mechanism may play a role during reactivation of these cells. However, since Noxa and its antagonist Mcl-1 are generally considered to be direct stress-response proteins that can mediate apoptosis very rapidly (18), a post-transcriptional regulation of these molecules seems most likely to us.

It remains to be investigated why Noxa does control the maximum population size during secondary, but not during primary T cell expansion. This may be the result of redundancy within the BH3-only family of proteins. During primary effector cell formation the pro-apoptotic molecules Puma and Bim, which have partly overlapping functions with Noxa, play a prominent role (24,34). Ablation of Bim or Puma leads to a pronounced increase of antigen-specific cells during acute infection. The role of these molecules in recall responses is less clear, but it appears likely that Noxa plays a less redundant role in this phase, allowing for enhanced T cell expansion in Noxa-deficient mice upon antigen re-encounter.

Importantly, ablation of Noxa under conditions of high amounts of T cell activation did not simply lead to a passive accumulation of effector memory cells, but resulted in liver and lung destruction (Fig. 7). This phenotype is distinct from other knock-out mice that accumulate lethal numbers of lymphocytes, such as the FAS/Bim double knock-out, which acquire a severe auto-immune phenotype (35-37). In Noxa70 mice, organs commonly targeted by self-reactive cells such as the kidneys, remained free of SLECs even at the final stages of disease. Noxa therefore seems to have a non-redundant function in controlling SLEC numbers. We present persistent CD27 stimulation as only one possible way in which a reduced threshold for activated T cell survival can lead to pathology. Increased CD70 expression has been well documented, however, in a variety of human diseases such as HIV infection and SLE (38). The fact that Noxa ablation induced effector T cell accumulation both under conditions of chronic CD27 stimulation and in a model for chronic infection with mCMV virus indicates that proper control of activated T cell expansion by the Noxa may be relevant for human disease.

In conclusion, we present Noxa as a molecule that controls the quality and quantity of T cells during expansion upon antigen encounter. Ablation of this molecule leads to a more diverse memory repertoire of reduced affinity. Re-encounter of antigen can overcome this deficiency, yet comes to a price; enhanced effector cell formation and lethal pathology after chronic immune activation. A proper control of apoptotic molecules therefore appears to be
essential for effector and memory T cells to maintain the fine balance between too little and too much cells upon pathogen encounter.

**Materials & Methods**

**Mice**

C57BL/6 (B6), OT-1 and B6 Ly5.1 JAX® mice were purchased from Charles River and kept as breeding colonies in our local animal facility. Only these mice, which were kept under identical conditions as our transgenic and gene targeted mice, were used as WT controls in our experiments. Noxa−/− mice were a kind gift from Dr. A. Strasser (WEHI, Melbourne) and provided by Dr. M. Serrano (CNIO, Madrid). Noxa5.1 mice were generated by crossing Noxa−/− mice with B6 Ly5.1+ mice from our in house colony. B cell specific CD70TG mice were generated as previously described (Hendriks et al. Nat. Immunol. 2000, Arens et al. Immunity 2001). Noxa70 mice were generated by crossing Noxa−/− mice with CD70TG mice. Mice were used at 6-12 weeks of age, unless stated otherwise, age- and sex-matched within experiments and were handled in accordance with institutional and national guidelines. All mice were either generated in B6 mice or backcrossed at least ten times on this background.

**Flow Cytometry**

Single-cell suspensions were obtained by mincing the specified organs through 40 μm cell strainers (Becton Dickinson). Erythrocytes were lysed with an ammonium chloride solution (155 mM NH4Cl, 10 mM KHCO3, and 1 mM EDTA) and cells were subsequently counted using an automated cell counter (SCHÄRFE SYSTEM). Cells (5 x 10^5- 5 x 10^6) were collected in staining buffer (PBS with 0.5% bovine serum albumin (Sigma)) and stained for 30 min at 4°C with antibodies in the presence of anti-CD16&CD32 (clone 2.4G2, anti-mouse FcγRII&RIII (a kind gift of Louis Boon, Bioceros, Utrecht, the Netherlands). For a list of the monoclonal antibodies used see Supplementary Table S1. FACS™ experiments were performed on a FACSCalibur™ or FACSCanto™ (Becton Dickinson) and analysed with FlowJo™ software (TriStar). APC conjugated Db and Kb tetramers were home made. T cell subsets were sorted to >99% purity with a FACSArria™ (Becton Dickinson).

**RT-MLPA analysis**

Total RNA was extracted using the trizol isolation method (Invitrogen). mRNA levels were analyzed with the Apoptosis Mouse mRNA RT-MLPA kit (MRC-Holland, http://www.mlpa.com) according to the manufacturer’s instructions. Samples were run through a Genescan and analyzed with GeneMapper (Applied Biosystems GmbH; http://www.appliedbiosystems.com) and subsequently Excell software (Microsoft).

**Flow cytometric measurement of intracellular proteins**

To determine direct ex vivo cytokine production, splenocytes were plated at 1 x 10^6 cells/well in a 96-well round-bottom plate and stimulated with 10 ng/ml PMA and 1 μM ionomycin (Sigma) or with peptides (ASNENMDAM, SCLEFWQRV, RALEYKNL and TVYGFCCLL; Genscript, http://www.genscript.com) for 6hrs at 37°C. During the final 4 hours, 1 μg/ml of the protein-secretion inhibitor Brefeldin A (Sigma) was added. Thereafter, cells were washed
and stained with anti-CD4 or anti-CD8, followed by fixation and permeabilization (Becton Dickinson). Cells were then incubated for 30 min with fluorescently labelled antibodies against IFNγ, TNFα, IL-2, IL-10 or IL-17 thoroughly washed and analyzed by flow Cytometry. For intracellular Mcl-1 staining, after permeabilization, cells were incubated for 30 min with anti-Mcl-1 (Rockland), followed by staining with PE labelled anti-rabbit antibodies (BD bioscience).

**Viral infection**

Influenza A virus of the strains A/PR8/34 (H1N1) and HKx31 (H2N3) were generated in LLC-MK2 cells and TCID₅₀ was determined in wild type B6 mice. Mice were infected intranasally with 10 x TCID₅₀ under general anaesthesia. BAC derived mCMV strain MW97.01 (a kind gift from Dr. S. Jonjić, Univ. of Rijeka, Croatia), which was experimentally shown to be identical to the wild-type Smith Strain, was generated in primary mouse embryonic fibroblasts (MEFs). PFU’s were determined by standard plaque assay on MEFs and 10⁶ PFU’s per mouse were injected intraperitoneal.

**Adoptive transfer and in vivo cytotoxicity**

For adoptive transfer, Wild-type and Noxa mice (both CD45.1) were infected intranasally with influenza A/PR8/34. Ten days after infection, CD8⁺ T cells were isolated from spleens by positive selection for CD4⁺ and CD8⁺ T cells using the MACS cell separation system (Myltenyi). Cells were injected intravenous in Wild-type (CD45.2) recipient mice and contraction was followed in the blood. For in vivo cytotoxicity, splenocytes from naïve Wild-type (CD45.1) mice were isolated and labeled with CFSE, DDAO (Both Molecular Probes), both or nothing. Fractions were subsequently incubated for 2hrs with the viral peptides NP₃₆₆-₃₇₄, PA₂₂₄-₂₃₃ and PB-F1₂₆₂-₇₀ or the control peptide SIINFEKL in RPMI, washed and 9*10e⁶ cells were injected in wild-type or Noxa⁻/⁻ recipients (CD45.2) that had been infected with influenza 50 days previously. Ratio of labeled cells within the CD45.1 donor fraction was used as a read-out for cellular clearance.

**PCR analysis of TCR clones.**

Total RNA was extracted from CD3⁺CD4⁻CD8⁺DβNP₃₆₆⁺ sorted T-cells using the Trizol™ (Invitrogen) isolation method. cDNA was generated using SuperscriptII-RT and oligo-dT primers according to the manufacturer’s protocol (Invitrogen, #18064). The Complementarity Determining Region 3 (CDR3) of the β-chain was used as a unique tag for clonal expansions. The TCR of the … tetramer responding cells were amplified using a primer specific for the Vβ8.3 gene segment (GCCTCCCCTCGCAGCATCA GTGCTGGCAACCTCAGATAGGA) and a primer specific for the Constant segment (5'-AAGGAGACCTTTGGTGGAGTG-3'), using the following PCR conditions: 4 µL cDNA (out of a total volume of 50 µL) was amplified in the presence of 10 pmol of both primers, 1 mM MgCl₂, 0.1 mM dNTP’s, 1x Buffer B (Solis BioDyne, Tartu, Estonia), and 2 U of Hotfire-Polymerase (Solis BioDyne) in a volume of 40 µL. Amplification was performed on a T3000 thermocycler (Biometra, Goettingen, G) in 35 cycles (hot start (96°С 15 min, then 40x (96°С for 30 sec., 60°С for 1 30 sec, 72°С for 60 sec), and 10 min at 72°С). Amplification-products were purified using AMPure SPRI beads (Agencourt Bioscience, Beverly, MA). The primers were tailed using the
primerA and primerB sequences that allow high throughput sequencing using a Genome Sequencer FLX (Roche Diagnostics, Mannheim G) and identification of the CDR3 regions as described earlier (39). Preparation of the samples and sequencing was performed according to the manufacturer’s protocol for amplicon sequencing on the FLX platform.

**Immunohistochemistry**

For histochemical analysis, sections were stained with haematoxylin and eosin. For immunohistochemical analysis, sections were stained with primary antibodies against cleaved caspase-3 (Cell Signaling) and subsequently with PowerVision Anti-Rabbit Poly-HRP (Immunologic). Cells were visualized with DAB (Sigma-Aldrich) and counter-stained with haematoxylin.

**Statistical analysis**

Statistical analysis of the data was performed using the unpaired Student’s t-test, Wilcoxon rank-sum test or one-way ANOVA test where applicable. Asterisks denote significant differences (* p<0.05, ** p<0.005, *** p<0.0005).

**Reference List**


