

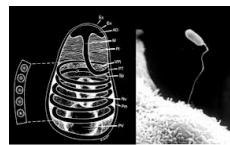
# Upregulation of innate immune response after oral microsporidia infection

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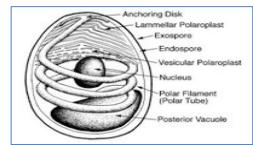
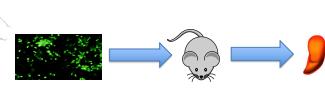
## Abstract

Microsporidia are obligate intracellular parasites from the Fungal kingdom that infect a range of animals, including humans. Because infection is usually asymptomatic in immunocompetent individuals, importance of the unicellular pathogen was thought to be restricted to agricultural animals. After the AIDS pandemic, the importance of this pathogen was recognized and more widely publicized. Recently, an increasing number of cases have been recorded in organ transplant and cancer patients. Immunocompromised murine models show that protective immunity to *Encephalitozoon cuniculi* (*E. cuniculi*) one of the Microsporidia species, which also affects HIV infected individuals. In previous studies with murine models and *E. cuniculi* infection, it has been demonstrated that protective immunity is pre-dominantly dependent on cytotoxic CD8 T cells. However, these mice are able to survive for 40 days in the absence of CD4 and CD8 T cells suggesting an important role for innate immune response. Natural Killer (NK) cells are one of the main components of the innate immunity and have been shown to play an important role against cancer and various infections. Their role in the immune response is similar to that of cytotoxic T cells in that they both trigger cytokine release, causing apoptosis. However NK cells do not require other cells to activate them; they can recognize stressed cells in the absence of antibodies and Major Histocompatibility Complex (MHC) markers, prompting a faster response. Currently there are no known studies of the NK cells in relation to microsporidia infection. Because of their ability to circulate and act on infected cells faster, it is important to investigate the role of NK cell response to infection with *E. cuniculi*.



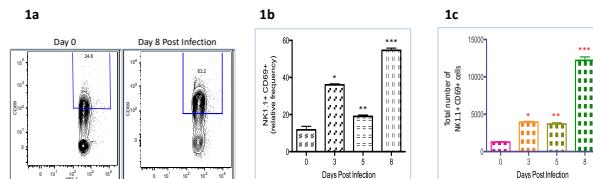
## Methods

IFN  $\gamma$  reporter YFP (Great) mice were infected orally with  $2 \times 10^7$  *E. cuniculi* spores. The spleens were removed and processed for staining on days 0, 3, 5, and 8 post infection. One million cells were plated and stained with live/dead zombie, CD69, KLRG1, and NK1.1. A student t test was performed between day 0 and days 3, 5, and 8 post infection for each set of data. Those determined statistically significant are with a 95% confidence interval. The p value can be seen at the figure legend below each set of data.



## Results

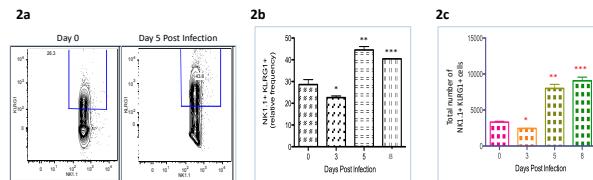
Because NK cell activity is usually seen with a rapid response, the kinetic was limited to day 8 post infection. We analyzed NK cells using an NK1.1 marker at different time points and determined there was no significant increase in total number of NK cells (data not shown). In previous research, it has been widely supported that CD69 is an activation marker on NK cells, triggering NK-cell-mediated cytolytic activity. We investigated the NK cell response by analyzing the expression of CD69. As shown in Figure 1a, there were 40% more NK cells activated at day 8 post infection. The peak of activated NK cells is also determined at day 8 by frequency and total number of NK1.1+ CD69+ cells (Figure 1b, 1c).



Three mice were analyzed per time point, with analysis gated on live, single celled lymphocytes positive for NK1.1 and CD69. Relative frequency of NK1.1+ CD69+ at days 0 and 8 post infection (Figure 1a). Relative frequency of NK1.1+ CD69+ cells at day 0 and different time points post infection (Figure 1b). Total number of NK1.1+ CD69+ cells at day 0 and different time points post infection (Figure 1c).

\*day 0 to day 3: 0.0004  
\*\*day 0 to day 5: 0.0024  
\*\*\*day 0 to day 8: 0.0018  
\*\*\*\*day 0 to day 8: 0.0002  
\*\*\*\*\*day 0 to day 8: 0.0004

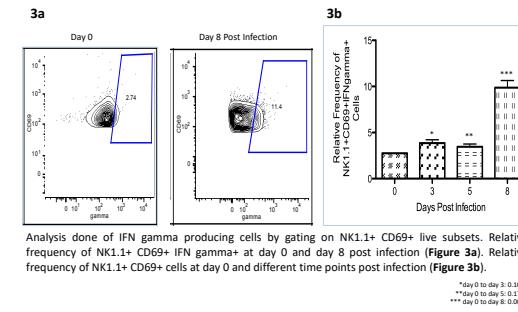
It has been previously determined that splenic NK cells expressing KLRG1 represent a mature phenotype. We analyzed NK cells for maturation with the KLRG1 marker and compared expression at different time points post infection. There is a significant increase in frequency of NK1.1+ KLRG1+ cells from day 0 to day 5 post infection (Figure 2a). However, over the days post infection both an increase and decrease in frequency of both NK1.1+ CD69+ and NK1.1+ KLRG1+ (Figure 2b) subsets is seen. Both markers were mutually exclusive, so NK cells did not express both CD69 and KLRG1, distinguishing two separate populations. This decrease in frequency can be explained by migration of the cells post infection. Because mice were infected orally, NK cells are most likely being recruited at the site of infection at gut mucosae early for an innate response, and are circulating later on in the spleen.



Three mice were analyzed per time point, with analysis on live, single celled lymphocytes positive for NK1.1 and KLRG1. Relative frequency of NK1.1+ KLRG1+ at day 0 and day 5 post infection (Figure 2a). Relative frequency of NK1.1+ KLRG1+ cells at day 0 and different time points post infection (Figure 2b). Total number of NK1.1+ KLRG1+ cells at day 0 and different time points post infection (Figure 2c).

\*day 0 to day 3: 0.0081  
\*\*day 0 to day 5: 0.0042  
\*\*\*day 0 to day 5: 0.0092  
\*\*\*\*day 0 to day 5: 0.0077  
\*\*\*\*\*day 0 to day 8: 0.0063  
\*\*\*\*\*day 0 to day 8: 0.0035

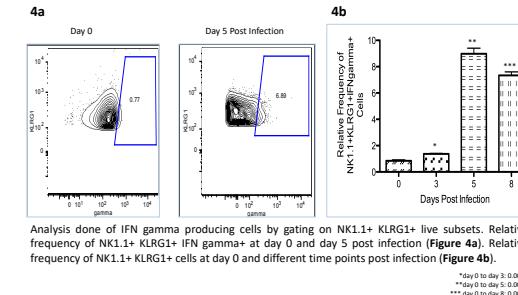
It has been shown that NK cells are vital components of the innate immune system by producing a variety of cytokines. Specifically, murine models demonstrate a dependence on NK cell-derived cytokines in early responses to obligate intracellular parasites; one of the most prominent cytokines is IFN gamma. Using IFN gamma reporter YFP mice we analyzed the IFN gamma producing cells within each NK1.1+CD69+ and NK1.1+KLRG1+ subset. A five fold increase in gamma producing NK1.1+CD69+ cells can be seen from day 0 to day 8 post infection (Figure 3a). The percentage of gamma producing NK1.1+CD69+ cells is highest at day 8 post infection, with a relatively steady percentage from days 0 to day 5 post infection (Figure 3b).



Analysis done of IFN gamma producing cells by gating on NK1.1+ CD69+ live subsets. Relative frequency of NK1.1+ CD69+ IFN gamma+ at day 0 and day 8 post infection (Figure 3a). Relative frequency of NK1.1+ CD69+ cells at day 0 and different time points post infection (Figure 3b).

\*day 0 to day 3: 0.0041  
\*\*day 0 to day 5: 0.0172  
\*\*\*day 0 to day 8: 0.0002  
\*\*\*\*day 0 to day 8: 0.0005

A significant five fold increase in gamma producing cells can also be seen in the NK1.1+KLRG1+ subset from day 0 to day 5 post infection (Figure 4a). There is a peak in the relative frequency of NK1.1+ KLRG1+ IFN gamma producing cells at day 5 post infection, and though there is a decrease at day 8 post infection the relative frequency is still close to that at peak of infection (Figure 4b). This increase suggests that the functionality of NK1.1+KLRG1+ IFN gamma+ cells remains for a few days after the peak of the immune response to the infection.



Analysis done of IFN gamma producing cells by gating on NK1.1+ KLRG1+ live subsets. Relative frequency of NK1.1+ KLRG1+ IFN gamma+ at day 0 and day 5 post infection (Figure 4a). Relative frequency of NK1.1+ KLRG1+ cells at day 0 and different time points post infection (Figure 4b).

\*day 0 to day 3: 0.0059  
\*\*day 0 to day 5: 0.0007  
\*\*\*day 0 to day 8: 0.0003

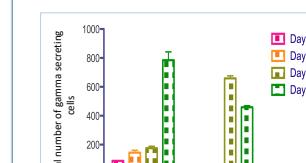


Figure 5: Total number of IFN gamma secreting NK1.1+ CD69+ and NK1.1+ KLRG1+ subsets at different time points post infection. Gates were drawn on the live NK1.1+ cells expressing CD69 or KLRG1 followed by IFN gamma+ gating.

In terms of peak of functionality, the same pattern can be seen from the frequency in with the total number of both NK1.1+CD69+IFN gamma+ and NK1.1+KLRG1+IFN gamma+ cells. There is a peak of gamma secreting NK1.1+CD69+ cells at day 8 post infection, and a peak of gamma secreting NK1.1+ KLRG1+ cells at day 5 post infection (Figure 5).

Our data suggest that mature (KLRG1+) NK cells secrete IFN gamma earlier than activated (CD69+) NK cells. Because there was such a high increase in total number of IFN gamma+ CD69+ and KLRG1+ NK cells, we can infer that both are relevant to the immune response against infection with *E. cuniculi*.

## Conclusion

Our data show that although there was no increase in total number of circulating NK cells after infection, the number of activated NK cells was increased. Effectively, an increase in both the number of activated (NK1.1+ CD69+) and mature (NK1.1+ KLRG1+) NK cells was detected at day 8 post infection. This increase in mature and activated NK cells was correlated with their ability to produce IFN gamma in response to infection. In previous studies, IFN gamma was shown to be crucial in the immune response against *E. cuniculi* infection.

Because the peak of the NK cell was detected at the last time point of our kinetic, it would be interesting to extend the kinetic. Moreover, because one of the main NK cell functions is their capability to lyse infected cells via secretion of perforins and granzymes, the expression of these molecules should be investigated. Furthermore, studies have determined that Ly49H is a memory NK cell marker for memory in the murine cytomegalovirus (MCMV), and it would be interesting to explore NK memory in response to *E. cuniculi* infection.

## References

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