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Short note

Evaluation of the hepatic NK cell response during the early phase of *Fasciola hepatica* infection in rats

Omar TLIBA[a], Alain CHAUVIN[b], Yves LE VERN[c], Chantal BOULARD[a], Pierre SIBILLE[a]*

[a]Unité de Pathologie Aviaire et Parasitologie, INRA, 37380 Nouzilly, France
[b]Unité associée INRA/ENVN Interactions hôte-parasite-milieu, École Nationale Vétérinaire de Nantes, BP 40706, 44307 Nantes Cedex 03, France
[c]Service commun de Cytométrie de Flux, INRA, 37380 Nouzilly, France

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Abstract – The in situ distribution of NK cells in rat liver during the first 28 days of an experimental infection with *F. hepatica* was investigated. NK cells were distributed homogeneously throughout the hepatic parenchyma in uninfected animals. The total number of hepatic mononuclear cells increased significantly following infection, but the proportion of NK cells did not change. After infection, these cells were found around the portal space, around the centrolobular vein, in the periportal fibrosis and in the band of collagen. However, no NK cells could be detected in or around the granuloma during infection. The frequency of both IL-2- and IFNγ-producing NK cells was higher on day 7 postinfection (pi) but only the percentage of IFNγ$^+$CD161$^+$ subsets remained elevated thereafter, whereas the percentage of both IL-2$^+$CD161$^+$ and IL-4$^+$CD161$^+$ subsets returned to the baseline. The number of CD161$^+$IL10$^+$ cells did not change significantly. These results suggest that NK cells could be another source for the early production of IFNγ but provide no evidence that these cells are involved in early events associated with granuloma formation.

***Fasciola hepatica* / NK cells / cytokine / flow cytometry / immunohistochemistry**

Résumé – Évaluation de la réponse des cellules NK hépatiques pendant la phase précoce d'infestation par *Fasciola hepatica* chez le rat. Nous avons suivi la localisation et la distribution hépatique des cellules NK durant les 28 premiers jours d'une fasciolose expérimentale chez le rat. Chez les animaux non infestés, les cellules NK sont distribuées de façon homogène dans tout le foie. Chez les animaux infestés, ces cellules sont localisées préférentiellement au niveau des espaces portes, autour des veines centrolobulaires, dans les bandes de collagène et dans la zone de fibrose périportale. En revanche, aucune de ces cellules n'a été détectée ni autour ni au sein du granulome. Le

*Correspondence and reprints
Tel.: (33) 2 47 42 77 47; fax: (33) 2 47 42 77 74; e-mail: sibille@tours.inra.fr

pourcentage des cellules NK n'a pas changé durant l'infestation, en dépit de leur augmentation significative en nombre absolu. Tandis que la fréquence des cellules NK productrices d'IL-2 et d'IFN-γ a augmenté au 7e jour après infestation, seule l'augmentation de celles qui produisent de l'IFN-γ a persisté, la fréquence des cellules produisant l'IL-2 diminuant par la suite. Les cellules NK productrices d'IL-4 présentaient la même cinétique que l'IL-2, alors que celles qui produisent de l'IL-10 n'ont montré aucun changement. L'ensemble de ces résultats montre que les cellules NK constituent une autre source précoce d'IFN-γ, mais que leur participation à la formation précoce du granulome apparaît peu probable.

***Fasciola hepatica* / cellule NK / cytokine / cytométrie de flux / immunohistochimie**

1. INTRODUCTION

The common liver fluke, *Fasciola hepatica,* is a parasitic helminth which induces both acute and chronic infections in livestock and causes severe financial losses to farmers.

In contrast to the relatively low frequency of NK cells in the peripheral lymphatic system, they are quite abundant in the liver [1, 7]. This has led to the hypothesis that optimal immune responses in the liver require the presence of NK cells. The role of these cells in the immune response has long been an area of intense interest since these cells may provide a first line of defence against invading infectious pathogens such as viruses, bacteria and parasites [3, 23] by exerting effector functions without having to be primed [9]. The activation of NK cells in response to certain pathogens involves an increased cytolytic capacity as well as the induction of cytokine production. Special attention was given to the cytokines, particularly IFNγ, which are produced by NK cells before T cell-derived cytokines are available. It has become increasingly clear that the early cellular and molecular events after infection are not only important for the regulation of the early defence against infection but also strongly influence the nature of the immune response and the ultimate outcome of disease [14, 19]. Initially, using competitive RT-PCR, in *F. hepatica* experimentally infected rats, we observed that IFNγ is up-regulated as early as 4 days postinfection (pi) and that the high levels of this cytokine were reached by 7 days pi [21]. The NK cells appear as a logical candidate for the production of IFNγ at this stage of infection [16, 17]. Thus, we aimed at following the early cytokine production by NK cells during the two first weeks after a primary *F. hepatica* infection in rats and to characterise their hepatic distribution.

2. MATERIALS AND METHODS

We used approximately 10 week old inbred Wistar rats of both sexes (reared at INRA, Nouzilly, France). Two groups of four rats were orally infected with 60 metacercariae [15] and killed by chloroform inhalation after 7 and 14 days of infection. One group of four healthy rats was not infected.

In order to locate the NK cells in hepatic parenchyma, we used Alcaline Phosphatase Anti Alcaline Phosphatase complex technique (APAAP) as previously described [20]. The optimal dilution of the mouse monoclonal antibody (mAb) 10/78, that recognises the CD161 molecule expressed on all rat NK cells (Serotec, Argène Varihles, France) [26], used for the immunohistochemistry assay was 1/50.

Liver perfusion and cell preparation were performed as previously described [24]. The viability of the recovered cells was > 90%. The purified HMNC were cultured with either 10 ng/mL PMA plus 0.5 μg/mL ionomycin and 2 μg/mL Brefeldin A (Sigma,

Saint Louis, USA) for 18 h before being stained.

After culture, cells (10^5/tube) were washed with PBS containing 5% of Fetal Calf Serum at 250 *g* for 7 min, then, they were stained with 1/100 dilution of mAb 10/78. After a 30 min incubation on ice, the cells were washed again and stained with 100 µL of Donkey anti mouse Ig conjugated to Phycoerythrin (Interchim, Montluçon, France, 1/200 dilution). Before intracellular cytokine staining, the cells were fixed with 2% paraformaldehyde, permeabilised with 0.5% saponin (Sigma). IL-2, IL-4 and IL-10 staining was performed as previously described [22] by incubating cells with A38-3, B11-3B and A5-6 (Becton Dickinson, Le Pont de Claix, France; 1/50, 1/300 and 1/50 dilutions, respectively) 45 min on ice followed by Streptavidin-FITC (Dako, Denmark) incubation for 15 min. IFNγ positive cells were directly stained with DB-1F (Serotec, 1/20 dilution) for 30 min on ice. Finally, cytokine producing NK cells were analysed by a double colour flow cytometry of gated $CD161^+$ using FACStar Plus (Becton Dickinson). For each sample, 15 000 events were recorded.

3. RESULTS

In control animals, NK cells were distributed homogeneously throughout the hepatic parenchyma (Fig. 1a). After one week of infestation, NK cells were located around the portal space and the centrolobular vein, suggesting that they had been

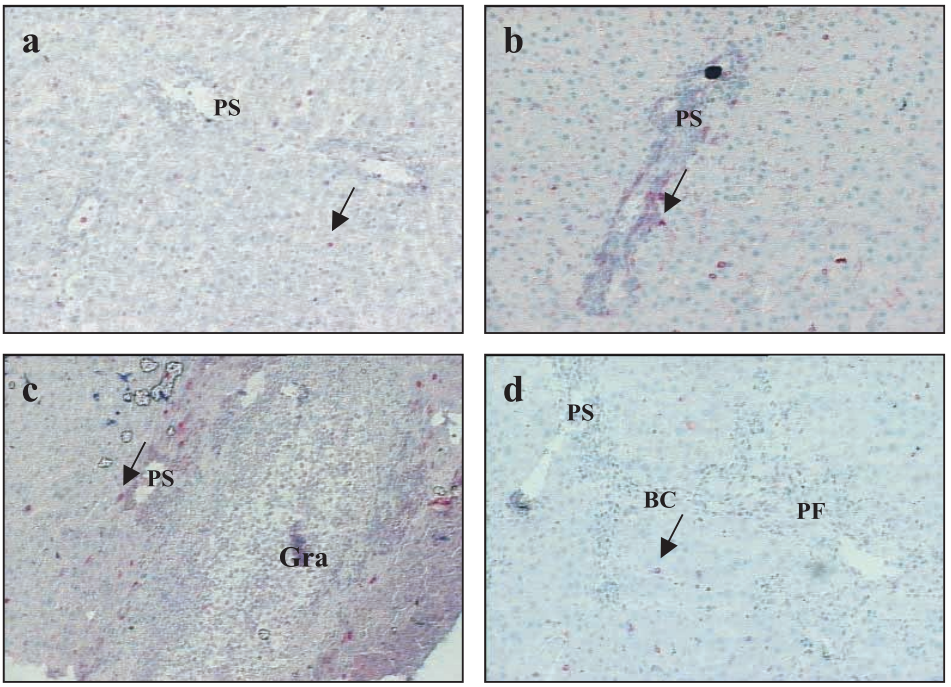

Figure 1. Immunohistological staining of frozen sections in hepatic parenchyma showing CD161 positive cells: (a) in an uninfected control animal (× 200), (b) at 7 days post infection (DPI) (× 200), (c) at 14DPI (× 200) and (d) at 28DPI (× 200). PS: portal space, Gra: granuloma, PF: periportal fibrosis, BC: bands of collagen, arrow: NK cells.

recruited recently (Fig. 1b). On days 14 and 28 pi, a few NK cells were observed in the periportal fibrosis and in collagen band (Figs. 1c and 1d) whereas no NK cells could be detected in or around the infection induced granuloma.

No significant change in the percentage of $CD161^+$ cells could be observed, despite an increase in the total number of recovered NK cells (Tab. I). The frequency of IFNγ-producing NK cells was significantly higher on day 7 pi and remained elevated thereafter. In contrast, the proportion of IL-4-producing NK cells increased on day 7 pi but returned to the baseline one week later. The $CD161^+IL10^+$ subset showed no significant change.

4. DISCUSSION

The present work showed no increase in the percentage of NK cells, whereas their absolute number was enhanced. This suggests that the recruitment of NK cells is non-selective and is related to the release of parasite products in the liver and/or to the local secretion of cytokines by the immune cells in this organ. We also demonstrated that the early increase of IFNγ mRNA previously observed by RT-PCR [21] could be related, at least in part, to NK cells. Moreover, the increase in the production of different cytokines by NK cells (IL-2, IL-4 and IFNγ) on day 7 pi indicates that in vivo NK cell activity is higher than the baseline level during the early hepatic phase of *F. hepatica* infection in rats. This is in agreement with the results obtained by an other study which showed that NK activity was enhanced in mice infected with *Trichinella spiralis* [4]. Several molecules may act as positive regulators of NK cell function. IL-1, IL-2, IL-7, IL-12, IL-15, TNFα, IFNα and IL-18 were reported to augment the production of IFNγ by NK cells [5, 6, 8, 10–13]. In vivo depletion of IL-12 substantially diminishes the NK cell activity response and/or early IFNγ response in mice infected with *Leishmania major* and *Trypanosoma cruzi* [16, 18]. In addition, IL-12 can induce the co-production of Th1 and Th2 cytokines by human lymphoid cells [25]. This could account for the production of both IL-4 and IFNγ by NK cells on day 7 pi. In *F. hepatica* infected rats, IL-12 mRNA increases as early as 4 days pi [21], however, the definitive role of these mediators in early trematode infection has yet to be demonstrated. Although the frequency of $CD161^+IFN\gamma^+$ cells remained high until day 14 pi, the Th2 response was clearly developed at this time of infection [21], suggesting that NK cells were not efficient at triggering the Th1 response.

The absence of NK cells in and around the granuloma suggests that these cells were not implied in the early phase of granuloma

Table I: Changes (± SEM) in the absolute numbers and percentage of hepatic $CD161^+$ cells and their cytokine production during the early phase of infection with *F. hepatica* in rat.

	No. of	$CD161^+$ (%)				
	$CD161^+$ ($\times 10^6$)	$CD161^+$	$IL2^+$	IFN-γ^+	$IL4^+$	$IL10^+$
Control	2.26 ± 0.57	37.12 ± 0.99	15.40 ± 0.42	16.90 ± 0.50	24.87 ± 0.70	15.20 ± 0.40
7 dpi infected	*3.84 ± 0.28	40.45 ± 5.62	*19.42 ± 2.70	*21.80 ± 0.30	*34.78 ± 4.80	17.00 ± 2.30
14 dpi infected	*5.09 ± 0.92	51.44 ± 2.51	10.00 ± 0.50	*22.12 ± 1.10	27.78 ± 1.40	15.43 ± 0.80

Each group contained at least four animals. The results are representative of three separate experiments. * Indicates $P < 0.05$ compared to values in control.

formation. However, in immunocompetent mice experimentally infected with *Schistosoma mansoni,* the NK cell depletion leads to an increase in hepatic collagen deposition in the late stages of granuloma formation [2]. This suggests that NK cells might have a regulatory function later on during *F. hepatica* infection.

This preliminary study needs to be completed by in vivo depletion of NK cells during early and chronic phase of fascioliasis in rat which should help us to elucidate the role of these cells in Th cytokine polarisation, their participation in the granuloma formation and in the development of the inflammatory response.

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