Original article

Interferon-alpha-producing cells are localized in gut-associated lymphoid tissues in transmissible gastroenteritis virus (TGEV) infected piglets

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Abstract – Transmissible gastroenteritis virus (TGEV) infection of piglets results in a very rapid and massive release of IFN-α in serum and secretions. The objective of this work was to characterize the IFN-α-producing cells (IPC) in tissues of TGEV-infected piglets. Caesarean-derived colostrum-deprived piglets were infected orally with the TGEV virulent Miller strain and IPC were characterized in situ by immunohistochemistry, using a rabbit anti-pig IFN-α antiserum. IPC were almost exclusively detected in intestinal tissues and mesenteric lymph nodes (MLN), as early as 6 h post inoculation (p.i.), with a peak at 12–18 h. They disappeared by 24 h. IPC were localized between enterocytes in the small intestine epithelial layer, in the lamina propria, around the Peyer's patches and, at highest frequency, in MLN. Very few IPC were present in the spleen and popliteal lymph nodes of infected piglets. Double immunohistochemical staining for IFN-α and leukocyte markers on MLN cryosections showed that IPC were mainly Swine Leukocyte Antigen (SLA) class II positive, and were not stained by an anti-macrophage (SWC3a) MAb. In addition, double staining with anti-TGEV and anti- IFN-α MAbs showed that viral antigens were present in MLN, close to IPC. These results show for the first time the presence of IPC in gut mucosa and gut-associated lymphoid tissues in response to an enteropathogenic virus. Moreover, this work shows that IFN-α released in serum is likely to originate almost exclusively from gut IPC triggered locally by viral antigens to produce IFN-α, since there were very few IPC in spleen or peripheral lymph nodes. MHC class II molecule expression by gut-associated IPC suggests that these cells may be the in vivo mucosal counterparts of the dendritic cells recently shown to produce IFN-α after in vitro viral induction.

interferon / transmissible gastroenteritis virus (TGEV) / pig / gut / lymphoid tissue

Résumé – Les cellules productrices d'interféron alpha chez les porcelets infectés par le virus GET sont localisées dans les tissus lymphoïdes associés à l'intestin. La gastroentérite transmissible

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(GET) du porcelet est une infection virale qui entraîne une production précoce et intense d'interféron alpha (IFN-α), tant au niveau du sérum que des sécrétions des animaux infectés. Le but de ce travail a été de caractériser les cellules productrices d'IFN-α dans les tissus des porcelets infectés par le virus GET. Pour cela, des porcelets obtenus par hystérotomie et privés de colostrum ont été infectés par voie orale par la souche virulente Miller du virus GET, et les cellules productrices d'IFN-α ont été caractérisées in situ par immunohistochimie, à l'aide d'un antisérum de lapin anti-IFN-α de porc. Les cellules productrices d'IFN-α ont été presque exclusivement retrouvées dans l'intestin et le ganglion mésentérique, dès 6 h après infection, avec un maximum à 12–18 h, et n'étaient plus présentes à 24 h. Ces cellules étaient localisées dans l'épithélium de l'intestin grêle, dans la lamina propria et autour des plaques de Peyer, ainsi que, en plus grand nombre, dans le ganglion mésentérique. À l'inverse, très peu de cellules productrices d'IFN-α se trouvaient dans la rate ou les ganglions poplités des porcelets infectés. Par un double marquage immunohistochimique de l'IFN-α et de marqueurs leucocytaires sur coupes à congélation de ganglions mésentériques, nous avons montré que les cellules productrices d'IFN-α exprimaient les molécules SLA (Swine Leukocyte Antigen) de classe II, mais n'étaient pas reconnues par des anticorps anti-macrophage (SWC3a). De plus, le double marquage à l'aide d'anticorps anti-IFN-α et d'anticorps anti-virus GET a permis de montrer que des antigènes viraux étaient présents dans les ganglions mésentériques, à proximité des cellules productrices d'IFN-α. Ces résultats montrent pour la première fois que les cellules productrices d'IFN-α en réponse à un virus entéropathogène sont présentes dans la muqueuse intestinale et dans les tissus lymphoïdes associés à l'intestin. De plus, l'absence de cellules productrices d'IFN-α dans la rate et les ganglions périphériques des porcelets infectés suggère que l'IFN-α sérique présent chez ces animaux provient pour l'essentiel de cellules productrices d'IFN-α intestinales, stimulées localement par les antigènes viraux. Enfin, le fait que ces cellules expriment les molécules CMH de classe II peut indiquer qu'elles seraient, in vivo, la contrepartie muqueuse des cellules dendritiques dont la capacité à sécréter l'IFN-α après contact in vitro avec un inducteur viral a été récemment démontrée.

interféron / virus de la gastroentérite transmissible (VGET) / porc / intestin / tissu lymphoïde

1. INTRODUCTION

Type I interferons (IFN-α/β) constitute an early host response to viral infections, exhibiting both antiviral and immunomodulatory properties [3, 30]. The biological relevance of IFN-α/β to antiviral defenses was clearly established by data showing that mice in which the type I IFN receptor gene was deleted by homologous recombination, were much more sensitive to several virus infections [21]. Several cell types, belonging to the lymphohematopoietic lineage, were shown to secrete IFN-α/β, by in vitro studies. Monocytes/macrophages produce IFN-α/β when infected by viruses such as the Sendai virus, whereas an unfrequent leukocyte population, referred to as natural interferon-producing cells (NIPC) were shown to secrete high amounts of IFN-α following a short contact with non infectious viral structures (reviewed in [11]).

Blood NIPC belong to a very rare subset of leukocytes distinct from T cells, B cells, NK cells or monocytes and express several markers of dendritic cells [6, 25, 28].

The viral infection in which we studied the IFN-α/β response in detail is the transmissible gastroenteritis infection of piglets. This acute infection is due to the coronavirus transmissible gastroenteritis virus (TGEV) which replicates in enterocytes and has been shown to induce high amounts of IFN-α in infected newborn piglets [15]. In vitro and in vivo studies on the mechanisms of IFN-α induction by TGEV have shown that non infectious TGEV preparations could induce IFN-α production and suggested the crucial role played by the external viral glycoprotein M in IFN-α induction [2, 17, 24]. We have also analyzed IFN-α-producing cells ex vivo by immunohistochemistry after an intravenous injection of UV-inactivated TGEV in newborn piglets. We found that

an infrequent population of IFN-α-producing cells, sharing several properties with in vitro described NIPC, were present in the spleen but not in the circulation of injected animals [24]. In the case of natural TGEV infection, however, virus replication takes place primarily in the intestinal tract, and IFN is present both in serum and intestinal fluids [15]. The aim of the present study was, therefore, to analyze by immunohistochemistry the presence of IFN-α-producing cells in TGEV infected piglets, with a special emphasis on the intestinal tract.

2. MATERIALS AND METHODS

2.1. Pigs and experimental design

A total of 7 Caesarean-derived colostrum-deprived piglets were used in these experiments and kept in individual Horsefall-type isolation units. At 4 days of age, 5 animals were inoculated orally with 10^6 PID50 of TGEV, and 2 animals served as non infected controls. Pigs were monitored clinically by visual examination and euthanized by pentobarbital injection at 6 ($n = 1$), 12 ($n = 1$), 18 ($n = 2$) or 24 ($n = 1$) h post inoculation (h p.i.). At necropsy, serum samples and intestinal fluids were collected for IFN-α immunoassays. Pieces of spleen, popliteal and mesenteric lymph nodes, and segments of duodenum, jejunum and ileum were collected for immunohistochemistry. Tissues were either fixed in 10% neutral buffered formalin, stored at 4 °C, dehydrated and embedded in paraffin, or frozen in embedding medium and stored at –80 °C for cryosections.

2.2. Virus preparation

The virulent Miller strain of transmissible gastroenteritis virus (TGEV) was used for this study. The virus preparation was the fourth pig passage material prepared by homogenization of the small intestines of pigs killed 18 hours after inoculation. A 20% tissue suspension was prepared in phosphate buffered saline, centrifuged ($1\,000 \times g$, 20 min, 4 °C), and the supernatant was collected and frozen at –70 °C as the stock virus. The infectivity titre of the virus stock was 10^6 PID50 (pig infectious dose 50%) per mL.

2.3. Immunohistochemistry

Both cryosections and paraffin sections were treated with rabbit anti-porcine IFN-α antiserum, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG, as described previously by Riffault et al. [24]. IFN-α-producing cells were visualized as red cells after addition of the Fast Red substrate. For double staining, cryosections of mesenteric lymph nodes were treated as described by Riffault et al. [24]. After IFN-α staining, the sections were incubated with MAb, then with biotin-conjugated anti-mouse immunoglobulin, peroxidase-streptavidin and 4-chloro-1-naphtol. Labelled cells were visualized by a dark blue staining. The MAb used in this study included anti-swine leukocyte Ab (MSA3: anti-SLA class II and 74-22-15: anti-porcine macrophage marker SWC3a) [19] or a mixture of 3 anti-TGEV MAb (51.13 and 20.5: anti-S; 5.1: anti-N) [16].

2.4. IFN-α immunoassay

A specific ELISA for porcine IFN-α was performed as already described [8, 26], by using MAb K9 for coating, and peroxidase-conjugated F17 MAb as a second Ab. In each assay, our internal standard of recombinant porcine IFN−α was included.

3. RESULTS

3.1. Clinical symptoms

The first clinical signs observed in TGEV-infected piglets were loss of appetite

and vomiting starting at 12 h p.i. Diarrhea appeared at 18 h p.i. Control piglets remained healthy.

3.2. IFN-α levels in serum and intestinal fluids of TGEV-infected piglets

IFN-α was detected by ELISA in the serum and intestinal fluid of TGEV-infected piglets, but not in control animals (Tab. I). Intestinal and serum IFN-α were first detected at 6 and 12 h p.i., respectively. Peak levels occurred at 12–18 h p.i. for serum and at 18 h p.i. for intestinal fluid. Low amounts of serum IFN-α were still detected at 24 h p.i. (Tab. I).

Table I. IFN-α concentration (U·mL^{-1}) in the serum and intestinal fluid of TGEV-infected piglets, measured by ELISA.

Time post-inoculation (h)	IFN-α titre (U·mL^{-1}) in	
	serum	intestinal fluid
6	0	300
12	2700	0
18	1600	1500
18	3000	400
24	530	0

One animal at each time p.i., except at 18 h p.i. ($n = 2$). No IFN-α was detected in control piglets.

3.3. Tissue distribution of IFN-α producing cells

IFN-α-producing cells were studied using immunohistochemistry applied to the spleen, lymph nodes and gut collected from control animals and piglets euthanized at 6, 12, 18 and 24 h p.i. Positive IFN-α-producing cells were detected in paraffin sections of mesenteric lymph nodes (MLN), duodenum, jejunum and ileum of TGEV-infected piglets (Tab. II, Fig. 1). No staining was observed in the absence of anti-IFN-α antiserum or with sections from control animals (except for a few positive cells in control MLN: Tab. II). A few rare positive cells were first detected at 6 h p.i. in the jejunum, ileum, mesenteric and popliteal lymph nodes and spleen. At 12 and 18 h p.i., more positive cells were seen, mainly in MLN, and to a lesser extent in the gut mucosa, whereas very few IFN-α-producing cells were present in the spleen and popliteal lymph nodes (Fig. 1, Tab. II). At 24 h p.i., IFN-α-producing cells were almost undetectable. The kinetics of the intracellular expression of IFN-α protein and its secretion in serum and intestinal fluids were therefore comparable, with an onset at 6 h, a peak at 12–18 h and a decline before 24 h p.i.

In the small intestinal mucosa, a few IFN-α-producing cells were present between enterocytes (Figs. 1A and 1B) but they were more frequently detected in the lamina propria of the villi (Fig. 1C) and around the Peyer's patches (not shown). Most
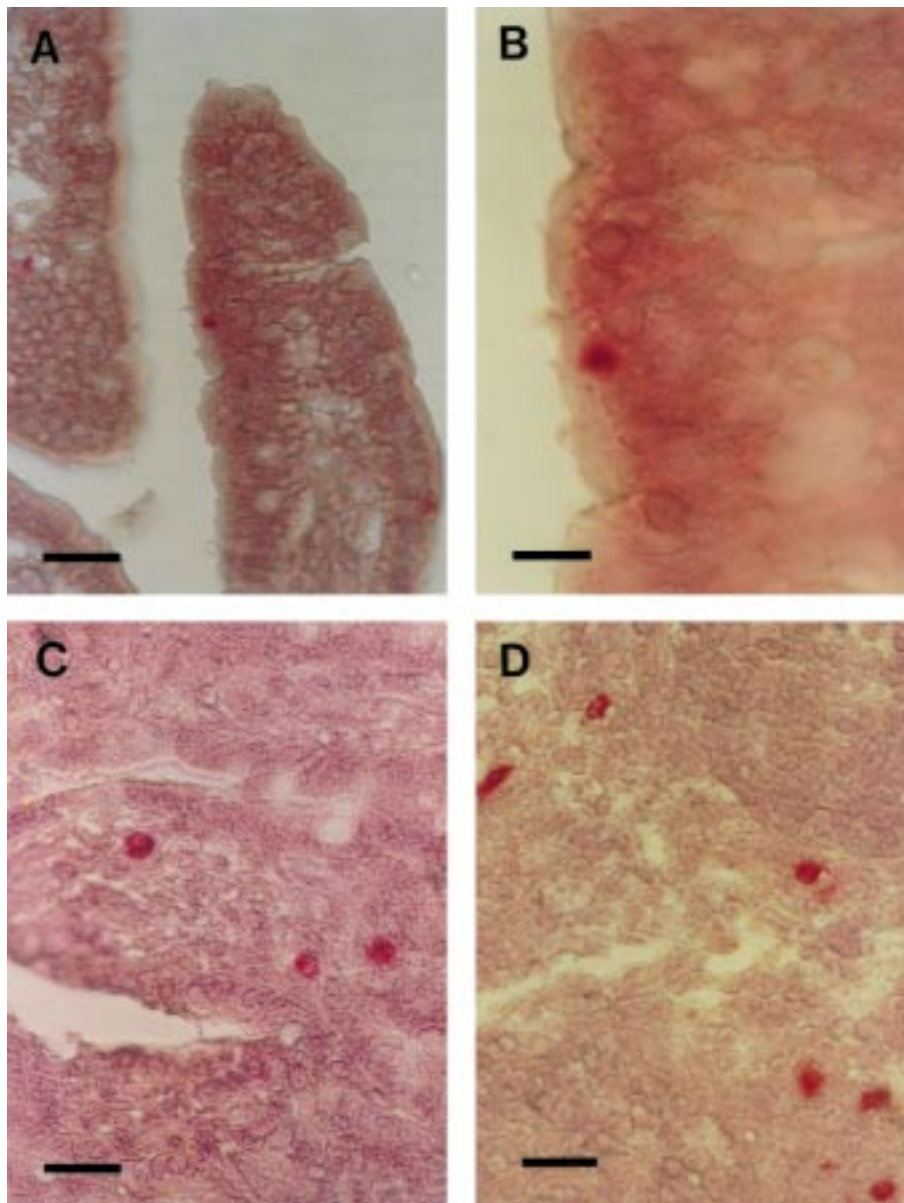
Table II. Semi-quantitative analysis of IFN-α-producing cells detected by immunohistochemistry on intestinal and lymphoid tissues collected at different times after TGEV infection.

Hours p.i.	Duodenum	Jejunum	Ileum	Mesenteric LN	Spleen	Popliteal LN
control	–	–	–	+/–	–	–
6	–	+ (IE)	+ (IE, LP)	+/–	+/–	+
12	–	+ (LP)	+ (IE, LP)	+++	+	+/–
18	++ (LP)	++ (LP)	++ (LP, PP)	+++	–	+/–
24	–	–	ND	+	–	–

One animal at each time p.i.
LN: lymph node. IE: intraepithelial. LP: lamina propria. PP: around the Peyer's patches.
+/–: less than 1 positive cells per tissue section. +: less than 5 positive cells per tissue section. ++: less than 15 positive cells per field. +++: between 15 and 30 positive cells per field.

Figure 1. Localization of IFN-α-producing cells in the gut and mesenteric lymph node of TGEV-infected piglets. Immunohistochemical staining was made on paraffin sections using anti-porcine IFN-α antibody. (**A** and **B**) IFN-α-producing cells (red cells) in the jejunum at 6 h p.i.: intraepithelial localization. (**C**) IFN-α-producing cells in ileal villi at 18 h p.i.: localization in the lamina propria. (**D**) IFN-α-producing cells in mesenteric LN at 18 h p.i.: localization between follicular areas. Original magnifications: × 132, bar = 76 μm (A, C, D) and × 330, bar = 30 μm (B).
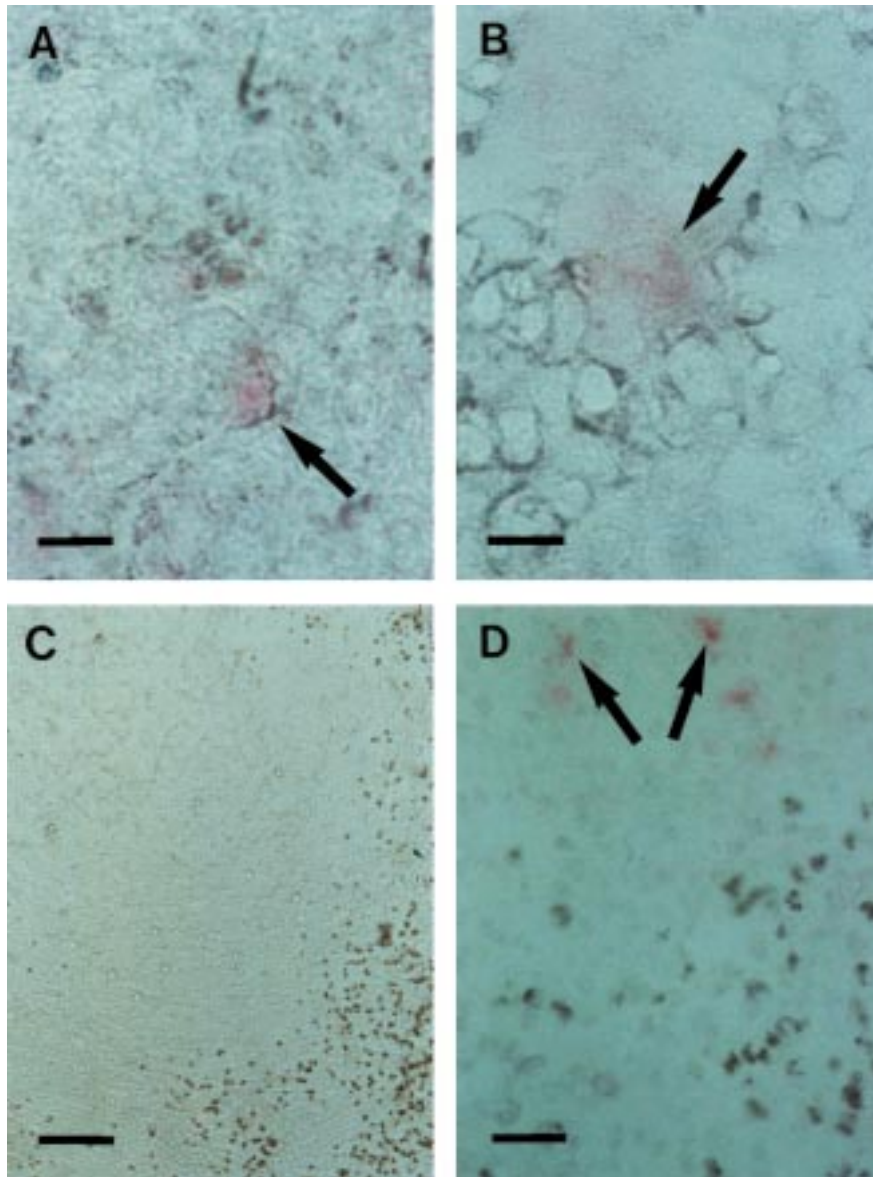
Figure 2. Phenotype of IFN-α-producing cells and localization of TGEV antigens in mesenteric lymph nodes (MLN) 12 h after TGEV infection. Double staining of MLN cryosections was performed with anti-porcine IFN-α antibody (in red) and either anti-leukocyte MAb (**A** and **B**) or anti-TGEV MAb (**D**) (in dark blue). (**A**) Staining with anti-SLA class II (MSA3) MAb, showing an IFN-α-producing cell (red) with surface staining (dark blue). (**B**) Staining with anti-SWC3a (74-22-15) MAb, showing an IFN-α-producing cell negative for SWC3a, and several SWC3a single positive cells. (**C**) Single staining with anti-TGEV MAb (see Methods), showing numerous TGEV positive cells (dark blue) in parafollicular areas. (**D**) Double staining with anti-TGEV MAb, showing single positive cells for IFN-α (red) distinct from single positive cells for TGEV (dark blue). Original magnifications: × 330, bar = 30 μm (A, B); × 33, bar = 300 μm (C) and × 132, bar = 76 μm (D). Arrows indicate IFN-α-producing cells.

IFN-α-producing cells were found in MLN, around follicular areas (Fig. 1D).

3.4. In situ analysis of MLN IFN-α producing cells

A phenotypic analysis of MLN IFN-α-producing cells was made by double staining on cryosections, with antibody to IFN-α and to leukocyte markers. Because previous experiments had shown that spleen IFN-α-producing cells in response to inactivated TGEV were largely SLA class II positive and to a lesser extent SWC3a positive, these two markers were used on MLN sections. Results in Figure 2A show IFN-α-producing cells in MLN from infected piglets expressing SLA class II antigens, while we could not find double staining for IFN-α and the porcine macrophage marker SWC3a (Fig. 2B).

To determine if the presence of IFN-α-producing cells was correlated with the presence of TGEV, immunohistochemical analysis of TGEV antigens was performed on cryosections of gut mucosa and MLN. Viral antigens were abundant at 12–18 h p.i. in the small intestine epithelium, as expected (data not shown), and in parafollicular areas of MLN (Fig. 2C). Tissue sections from non-infected piglets were negative. Double staining with Ab to IFN-α and to TGEV on MLN showed that IFN-α-producing cells were localized close to TGEV antigens, but there were no double positive cells (Fig. 2D).

4. DISCUSSION

We found that the majority of IFN-α-producing cells after TGEV infection of newborn piglets were located in the small intestine and mesenteric lymph nodes, whereas very few positive cells, if any, were present in the spleen or popliteal lymph nodes. As high amounts of IFN-α are present in serum early after TGEV infection ([15] and Tab. I), it must be concluded that most, if not all, circulating IFN-α in infected

animals originate from IFN-α-producing cells restricted to the gut and MLN localizations. The observation that the kinetics of serum IFN-α and of intestinal IFN-α-producing cells were parallel (from 6 to 24 h p.i., with a peak at 12–18 h: Tabs. I and II) also supports the hypothesis of the intestinal origin of circulating IFN-α. Using a different experimental model, in which pigs were injected intravenously with inactivated TGEV, IFN-α-producing cells were also present in only one organ, the spleen, and peripheral blood leukocytes did not produce IFN-α, indicating that spleen IFN-α-producing cells accounted for circulating IFN-α [24]. Following in utero TGEV infection, IFN-α-producing cells were also found in foetal lymphoid organs [27]. Other studies showed that circulating IFN-α in response to non infectious virus injection in pigs and in mice was likely to originate from IFN-α-producing cells in lymphoid tissues such as draining lymph nodes [1, 23] and the spleen [9, 10]. Therefore, although numerous in vitro studies looked at the nature of IFN-α-producing cells from peripheral blood lymphocyte preparations [6, 25, 28], and reviewed in [11], they may be not fully relevant to the in vivo situation.

We have analyzed the tissue distribution of mucosal IFN-α-producing cells in tissue sections from TGEV-infected piglets. These cells were present in the jejunum and ileum and, in higher numbers, in MLN (Tab. II, Fig. 1). Interestingly, IFN-α-producing cells were present between enterocytes and may, therefore, be described as intra-epithelial IFN-α-producing cells. The lamina propria was found to contain a larger population of positive cells, which were also present in the vicinity of the Peyer's patches. More IFN-α-producing cells were seen in MLN interfollicular areas. Thus, our data describe for the first time the existence of a mucosal cell population specialized in the early production of IFN-α in response to an enteropathogenic virus, co-localized with mucosal lymphoid cells in the so-called gut-associated lymphoid tissue [29]. Their distribution pattern

(between enterocytes, in the lamina propria, surrounding the Peyer's patches, and in MLN) suggests that they can migrate from the intestinal epithelium, where TGEV replicates [22] down to the mucosa and to the draining lymph nodes. Alternatively, IFN-α-producing cells may be induced locally by viruses and remain localized in tissues where IFN-α will be produced. This latter hypothesis is supported by our findings that TGEV was present both in the gut epithelium, as originally described [22], and in draining MLN. However, we could not detect cells double stained for IFN-α and for TGEV antigens (Fig. 2D), which strongly indicates that IFN-α-producing cells are stimulated by viruses without being infected. These in vivo data confirm earlier in vitro experiments in which a subpopulation of leukocytes, referred to as natural interferon-producing cells (NIPC, reviewed in [11]), were induced to produce IFN-α by a non infectious process: either by inactivated virus or by virus-infected glutaraldehyde-fixed cells [5, 7, 18].

The results of double staining of IFN-α-producing cells in MLN with MAb to SLA class II antigens, are in agreement with our previous studies in pigs showing that a majority of spleen IFN-α-producing cells were SLA class II positive [24]. We did not detect MLN IFN-α-producing cells stained by anti-macrophage MAb, in contrast to what was observed for pig or mouse spleen IFN-α-producing cells [9, 24]. This could argue for a possible heterogeneity of the IFN-α-producing cell population, depending on the tissue or on the IFN-α-induction signal [12, 13]. However, the low number of cells stained by anti- IFN-α Ab on crysections did not allow us to provide a quantitative analysis of the percentage of double stained cells, and it is still possible that a low number of IFN-α-producing cells stained by anti-macrophage MAb could not be detected. Our finding that MLN IFN-α-producing cells were MHC class II positive is compatible with the current hypothesis that IFN-α-producing cells belong to the dendritic cell lineage [6, 14, 25, 28]. Indeed

dendritic cells have been described in the gut (reviewed in [20]), with an anatomical distribution compatible with the locations at which we found gut IFN-α-producing cells, namely the intestinal epithelium, the lamina propria, the Peyer's patches and draining lymph nodes. Our results suggest, therefore, that a specialized population of mucosal dendritic cells may produce high amounts of IFN-α, after local induction by enteropathogenic viruses, while presenting viral antigens to the local immune system. Since IFN-α is able to modulate adaptative immune responses, including differentiating T helper cells into Th1 cells (reviewed in [3, 4]), we suggest that mucosal IFN-α-producing cells may play two major roles in immune responses to enteropathogenic viruses: direct IFN-α-mediated inhibition of viral replication, and triggering of effective local anti-viral T cell responses.

ACKNOWLEDGEMENTS

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