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Characterization of Interferons Induced by Bacteria and Interferon-Producing Leukocytes in Human Peripheral Blood

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All of 23 different preparations of formaldehyde-fixed and heat-killed bacteria induced the appearance of high levels of interferon (IFN) in cultures of human peripheral blood mononuclear leukocytes. Some bacteria induced peak IFN titers after 24 h of culture, whereas other bacteria showed maximal titers on culture days 2 to 3. The IFN displayed various properties. One type, which appeared early during the cultures, had characteristics of IFN-α, being resistant to pH 2 treatment but neutralized by antibodies to IFN-α. A second type, which appeared later, on culture days 2 to 3, resembled IFN-γ in being sensitive to pH 2 treatment but resistant to anti-IFN-α antibodies. A third type, which appeared to be sensitive to both pH 2 and antibody treatment, was interpreted as atypical IFN-α. The application of cell fractionation procedures indicated that nonadherent, predominantly Fc receptor-bearing, non-T, non-B cells were producers of IFN-α as defined by its antigenic properties. They copurified approximately with cells carrying natural killer activity toward human erythroid leukemia K562 cells. Some bacteria apparently also stimulated T lymphocytes to produce material with properties of IFN-γ.

Human peripheral blood mononuclear leukocytes (PBLs) produce interferon (IFN) in response to a variety of stimuli. The classic IFN inducers are viruses, but mitogens, various microorganisms, and many synthetic substances can also initiate IFN production (see reference 26 for a review). Recent reports indicate that the bacteria *Corynebacterium parvum* (12, 21), *Listeria monocytogenes* (17), *Staphylococcus aureus* Cowan I (14), and *Chlamydia trachomatis* (5) induce IFN production in human PBLs. Also, *Mycoplasma* spp., which contaminate many in vitro cell lines, induce IFN responses in PBL cultures (2, 3).

It is possible that bacteria in general may induce IFNs in certain types of human PBLs. In the present investigation, therefore, we tested the IFN-inducing ability of a large number of different bacteria. We found that they all were potent inducers of IFN in cultures of PBLs. For some bacteria the types of IFN induced as well as the IFN-producing cell types were studied. Both IFN-α and IFN-γ were produced by the induced lymphocytes, and several different cell types were involved in this production.

MATERIALS AND METHODS

Cell preparation. Human PBLs were prepared by Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation of buffy coats obtained from (citrate-phosphate-dextrose)-adenine containing blood of normal donors (4). The cells were washed thrice and suspended in RPMI 1640 tissue culture medium containing HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (10 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), fresh L-glutamine (2 mM), and either 5% fetal calf serum (Flow Laboratories, Inc., Irvine, United Kingdom) or 10% human blood group AB serum.

Plastic-adherent cells were prepared by incubating 10^8 cells in 10 ml of medium containing 5% fetal calf serum in plastic petri dishes (diameter, 9 cm) for 45 to 60 min at 37°C. Nonadherent cells were collected, and the remaining adherent cells were washed and scraped off with a rubber policeman and kept at 4°C. They contained approximately 95% monocytes as determined by esterase staining (6, 16).

Plastic-nonadherent cells, when indicated after a second depletion of plastic-adherent cells, were passed through a nylon wool column (13) to remove B lymphocytes and residual monocytes and macrophages. The cell yield after plastic adherence and nylon wool column passage ranged between 45 and 52%. Contamination by esterase-positive monocytes was <1%, and contamination by surface immunoglobulin positive B lymphocytes was 1%.

The nylon wool-nonadherent cells were further fractionated by rosette formation with 2-amino-ethyl-isothiouronium, bromide-treated sheep erythrocytes (20). Cells (4×10^6/ml) were mixed with equal volumes of 1% (vol/vol) sheep erythrocytes in medium with 50% fetal calf serum for 10 min at room temperature, centrifuged for 5 min at 200 × *g*, and then incubated in

pellet form for 60 min at 4°C. The pellet was gently resuspended, and the cells were centrifuged on Ficoll-Paque for 20 min at 800 × *g* and 4°C. Gradient interphase cells were washed twice and designated E^-. Pelleted rosette-forming leukocytes were washed once, and the sheep erythrocytes were lysed by distilled-water hypotonic shock. The freed leukocytes were washed and designated E^+. The total yield after the rosette procedure was approximately 80%, and 65 to 88% of the recovered cells were in the E^+ fraction.

Nylon wool-nonadherent cells were also passed through a column of glass beads coated with rabbit anti-human immunoglobulin complexes to remove B lymphocytes and Fc receptor-bearing cells (28). The yield after the immunoglobulin-anti-immunoglobulin passage ranged between 40 and 77% of the input cell number.

Cell culture conditions. The various cell preparations were cultured in RPMI 1640 medium with human AB serum. Cell concentrations of 1×10^6 or 2×10^6/ml were used, with at least duplicate 0.2-ml cultures in flat-bottomed tissue culture microtiter plates (Nunclon; Nunc, Roskilde, Denmark) for each treatment. The cultures were maintained at 37°C in 5% CO_2 in air with or without IFN inducers. Culture supernatants excluding cells were harvested without centrifugation at the indicated times and stored at −70°C until assayed for IFN.

Bacteria and control IFN inducers. The bacteria used (see Table 1) were clinical isolates, with the exception of the *S. aureus* Cowan I. The bacteria were killed by incubation in 0.5% formaldehyde for 3 h at room temperature and further heat treated for 5 min at 80°C. They were washed thrice, packed by centrifugation, and diluted in serum-free medium at the desired concentration (vol/vol) and stored at −20°C. All bacteria were tested for IFN induction at various final dilutions in the cell cultures, and levels of IFN were constant over a wide range (1/250 to 1/4,000). Dilutions of 1/500 to 1/1,000 (vol/vol) were used if not otherwise indicated.

Concanavalin A was purchased from Pharmacia Fine Chemicals and used at a final concentration of 40 μg/ml. Sendai virus, originally obtained from Kari Cantell, was grown in eggs, and the allantoic fluids were collected and clarified by low-speed centrifugation. An optimal final dilution of 1/10 (ca. 600 hemagglutinating units per ml) in the cell culture medium was used. The medium of virus-stimulated PBL cultures was harvested on culture day 1 and dialyzed at pH 2 for 24 h and at pH 7 for a further 24 h to inactivate the virus.

All inducers were added at the initiation of the cultures and were present throughout the culture period. Control experiments showed that none of the various tested IFN inducers or pH 2-inactivated Sendai virus protected human amnion cells against vesicular stomatitis virus challenge when directly added in the IFN assay.

IFN assay and characterization. The antiviral activity in culture supernatants (designated IFN) was determined by a conventional cytopathic effect inhibition assay performed in microtiter plates with adherent WISH human amnion cells (American Type Culture Collection, Bethesda, Md.) as the indicator cells and vesicular stomatitis virus (Indiana strain) as the challenging virus (19). It should be noted that WISH cells may actually be HeLa cells (18). The National Institutes of Health reference leukocyte IFN (G-023-901-527) was used to calibrate the assay, and the IFN concentrations were expressed as reference units per milliliter.

The pH stability of IFN in culture supernatants was tested by dialysis against 0.05 M HCl-KCl buffer (pH 2.0) for 24 h and then against phosphate-buffered saline (pH 7.2) for 24 h. Compared with untreated controls, the pH 7.2 dialysis alone never reduced the antiviral activity.

A sheep antiserum against Sendai virus-induced human leukocyte IFN was kindly provided by Kari Cantell and used to characterize the antigenic properties of antiviral activity in PBL culture supernatants. It contained 450,000 neutralizing units per ml against leukocyte IFN and 3,000 against fibroblast IFN. Equal volumes of antiserum diluted 1/100 in Dulbecco modified Eagle medium containing 5% fetal calf serum and test supernatant were mixed and incubated for 2 h at 37°C. The samples were then assayed for residual IFN activity as described above.

Cytotoxicity assay. The cytotoxic activity of cell preparations was determined in a 4-h ^{51}Cr release assay with 5×10^3 ^{51}Cr-labeled K562 erythroleukemia target cells per V-shaped microtiter well as previously described (25). Twofold dilutions of the effector cells, starting at an effector/target ratio of 100:1, were used. Specific lysis of target cells was calculated according to the following formula:

$$\text{Percent specific lysis} = \frac{(\text{test release}) - (\text{spontaneous release})}{(\text{maximal release}) - (\text{spontaneous release})} \times 100,$$

where all release values are expressed as counts per minute. The cytotoxic activity of the various cell fractions was expressed in lytic units by multiplying by 100 the inverted ratio of effector to target cells that gave 30% specific lysis.

RESULTS

IFN-inducing capacity of bacteria. The bacteria listed in Table 1 were tested for their ability to induce IFN production in cultures of human PBLs. They all induced significant but markedly varying titers of antiviral activity (Table 1), expressed as reference IFN units per milliliter of culture medium. Two complete experiments were carried out with each strain, with similar results.

Time course studies were also performed for several of the bacterial strains. Three experiments were carried out with the bacteria listed under experiment 1 in Table 1, and representative results are given in Table 2. In one type of response, maximal IFN titers were already seen on culture day 1, as exemplified by *Escherichia coli*. All *E. coli* strains displayed this pattern. In the other type of response, represented by that induced by the beta-hemo-

TABLE 1. Induction of antiviral activity in PBL cultures by bacteria

Bacteria[a]	IFN titer[b]
Experiment 1	
Streptococcus spp. (strains A324, A559, A1509, G313, G1492)	5,180–15,550
B. catarrhalis (strains 5, 685, 807, 8193)	1,730–15,550
E. coli (strains PW, 14, 604, 2596, Ser)	2,020–15,550
S. aureus Cowan I	2,020
Experiment 2	
Sarcina sp.	10,130
L. monocytogenes	380
Klebsiella sp.	3,380
Proteus vulgaris	380
Haemophilus influenzae	1,130
Streptococcus pneumoniae	2,250
Staphyloccus epidermidis (strains 1, 2)	3,380, 1,130

[a] Final bacteria dilutions of 1/125 to 1/4,000 (vol/vol) in the tissue cultures were tested, but the dilution was not critical in most cases. IFN concentrations were always maximal at a dilution of 1/500 to 1/1,000, and these values are given in the table. Cultures in triplicate were used at each dilution and pooled for IFN assay.

[b] Maximal IFN concentrations in the supernatant medium (reference IFN units per milliliter) on culture day 3. Each experiment was performed with PBLs from one donor and replicated once.

lytic streptococcal group G, the IFN titers increased beyond day 1 and attained maximal levels on culture day 3. Other streptococci (*Streptococcus pneumoniae* was not tested) behaved similarly, and so did the *Branhamellae* strains and *S. aureus* Cowan I.

Characterization of antiviral activities. The antiviral activity induced by bacteria was characterized with regard to sensitivity to anti-IFN-α antibodies and pH 2 treatment. Supernatants obtained from cultures on day 3, using four different bacteria as stimulators, were largely (75 to 95%) inactivated at pH 2 (Table 3). Antibodies to IFN-α completely neutralized the pH 2-resistant residue. In contrast, such antibodies did not significantly impair the IFN induced by three of the four different bacterial strains used in experiment 1 of Table 3. However, the *E. coli*-induced IFN was completely neutralized by the antibodies, despite the recorded sensitivity to pH 2 treatment.

The IFN on culture day 1 of supernatants from PBLs stimulated by *Streptococcus* sp. was resistant to pH 2 and sensitive to anti-IFN-α antibodies (Table 3), as would be expected from IFN-α. However, when supernatants from the same streptococcus-stimulated cultures were collected on culture days 3 to 4, the IFN was relatively sensitive to pH 2 treatment (fourfold reduction) but less so to anti-IFN-α antibodies (twofold reduction).

The IFN present in the supernatants of *E. coli*-stimulated cultures was again completely abolished by anti-IFN-α antibodies both early (day 1) and later (day 3 to 4) in the culture period. Also, here the residual pH 2-resistant IFN was sensitive to anti-IFN-α antibodies.

Characterization of IFN-producing cells. To define the types of cells responsible for the production of IFN, human PBLs were fractionated and the IFN responses of the various cell fractions were assessed, using as IFN inducers four different bacteria (*E. coli*, beta-hemolytic streptococcal group G, *Branhamella catarrhalis* and *S. aureus* Cowan I), Sendai virus, and the T-cell mitogen concanavalin A. The natural killer (NK) activity of the various cell fractions against ^{51}Cr-labeled erythroleukemia K562 cells was also assessed.

The sequential removal of plastic-adherent cells, nylon-wool adherent cells, and E-rosette-forming cells had little or no negative impact on the IFN response to the bacterial inducers (Table 4). An increase in the IFN responses was instead seen; this varied from experiment to experiment, but usually was in the range of 200 to 300% in the E-rosette-depleted cell fraction when compared with the original unseparated cells. The exception was *B. catarrhalis*, which induced less IFN in the various cell fractions (50 to 70%) when compared with unseparated PBLs. The plastic-adherent cells and the E-rosette-forming cells displayed essentially no responses to the bacteria. Passage of nylon wool-nonadherent cells through immunoglobulin-anti-immunoglobulin columns reproducibly decreased the IFN responses of passed cells to bacteria, although with regard to both *B. catarrhalis* and, particularly, *E. coli*, significant IFN responses (56% for the latter inducer) were retained.

With regard to the control inducers, the T-cell mitogen concanavalin A stimulated IFN responses in the fractionated cells in a manner

TABLE 2. IFN concentrations in the medium of PBL cultures on days 1 to 4[a]

Inducer	Concn[b] on culture day:			
	1	2	3	4
E. coli PW	800	800	800	800
Streptococcus sp. strain G313	800	800	3,200	3,200

[a] All bacteria listed under experiment 1 in Table 1 were tested in three time course experiments.

[b] Expressed in reference IFN-α units per milliliter of medium.

TABLE 3. Effect of anti-IFN-α antibodies and pH 2 treatment on IFN induced by bacteria

Expt[a]	Bacterium	IFN concn[b] after the following treatment:			
		pH 7	pH 7 + anti-IFN-α	pH 2	pH 2 + anti-IFN-α
1	*Streptococcus* sp. strain G313	100	100	13	<3
	B. catarrhalis 807	100	100	3	<3
	E. coli PW	100	<6	25	<6
	S. aureus Cowan I	100	100	13	<1
2	*Streptococcus* sp. strain G313				
	Day 1	100	6	100	<6
	Day 4	100	50	25	<2
	E. coli PW				
	Day 1	100	<13	25	<13
	Day 4	100	<13	25	<13

[a] The culture supernatants of experiment 1 were harvested on day 3. The results of each experiment were obtained with PBL from a different blood donor.

[b] Residual IFN concentration, expressed as the percentage of that of control (pH 7 treated) culture supernatants.

which would be expected if this response was due to T lymphocytes aided by adherent cells (Table 4). Sendai virus, in contrast, induced IFN responses in plastic-adherent cells. The responses were reduced after passage of cells through a nylon wool column, but again increased relatively in E-rosette-depleted cells. Passage of nylon wool-nonadherent cells through an immunoglob-

TABLE 4. IFN-producing capacity and NK activity in different fractions of human PBLs

Cell fraction	IFN concn[a] with the following IFN inducer:[b]						NK activity[c]
	Streptococcus sp. strain G313	*S. aureus* Cowan I	*B. catarrhalis* 807	*E. coli* PW	ConA	Sendai virus	
Unseparated	100	100	100	100	100	100	7.8
Plastic adherent	9	0	0	1	3	200	1.4
Plastic nonadherent	141	168	71	100	84	84	13
Plastic nonadherent, nylon wool passed	84	200	50	100	18	35	11
Plastic nonadherent, nylon wool passed, immunoglobulin-anti-immunoglobulin passed	1	1	10	56	8	17	0.4
Plastic nonadherent, nylon wool passed, E-rosette positive	0	1	0	1	7	1	0.8
Plastic nonadherent, nylon wool passed, E-rosette negative	200	238	50	283	11	141	21.1

[a] Geometric mean of the IFN concentrations in culture supernatants, expressed as the percentage of that obtained with unseparated cells. Four experiments were performed (three with immunoglobulin-anti-immunoglobulin passage), except for *B. catarrhalis* and *E. coli*, where only two experiments were done (one with immunoglobulin-anti-immunoglobulin passage).

[b] The following final concentrations were used: *Streptococcus* sp. strain G313, 1/1,000; *S. aureus* Cowan I, 1/1,000 to 1/2,000; *B. catarrhalis* 807, 1/500; *E. coli* PW, 1/1,000; concanavalin A (ConA), 40 μg/ml; Sendai virus, 600 hemagglutinating units per ml. Supernatants from bacterially stimulated cultures were harvested after 2 to 3 days.

[c] Average lytic units (see text) from two experiments. The cytotoxicity assay was performed on cells immediately after fractionation.

ulin-anti-immunoglobulin column caused a 50% reduction of the responses.

The NK activity of the various cell fractions towards K562 cells was distributed approximately as were the IFN responses, especially to the *Streptococcus* and *Staphylococcus* strains, i.e., the NK activity was not altered or increased after plastic adherence and nylon wool column passage and increased after removal of E-rosette-forming cells (Table 4). The NK activity was abolished after immunoglobulin-anti-immunoglobulin column passage.

Table 5 demonstrates the properties of the antiviral activity appearing in the medium of bacterium-stimulated cultures of unfractionated PBLs and nonadherent, E-rosette-depleted (E^-) PBLs, the latter consisting mainly of "null" cells. A similarly complex pattern of sensitivity to pH 2 treatment and to anti-IFN-α antibodies as shown in Table 3 was found. *E. coli*, in both unfractionated PBLs and E^- cells, induced IFN which was resistant to pH 2 but sensitive to anti-IFN-α antibodies, suggesting typical IFN-α. On the other hand, *S. aureus* induced IFN in unfractionated PBLs which was completely destroyed at pH 2 and partially (75%) neutralized by antibodies, but the IFN induced in E^- cells was similar to that induced by *E. coli*. With regard to *B. catarrhalis*, most of the activity was neutralized by both antibodies and pH 2 treatment. *Streptococcus* sp. induced antiviral activity in cultures of unfractionated PBLs which was largely destroyed by pH 2 treatment and neutralized by antibodies (75 and 87%, respectively). With regard to the IFN produced by the E^- cells, 50% was neutralized by antibodies and 50% was destroyed at pH 2.

These results, obtained with the four different types of bacteria on unfractionated PBLs (Table 5), differ in several respects from those given in Table 3. Further characterization experiments (data not shown) suggest a similar variation with regard to IFN properties.

Common to all four bacterial inducers was that the pH 2-resistant IFN always was completely neutralized by the anti-IFN-α antibodies.

DISCUSSION

Our results and those of others (2, 3, 5, 12, 14, 17, 21) indicate that a large number of different bacterial strains induce the appearance of material with antiviral activity in cultures of human PBLs. Although it has not been proven in each instance, the antiviral activity may be mediated by one or several different IFNs. In fact, the IFN induced by the bacteria in our study showed three major patterns. First, the IFN appeared to be resistant to pH 2 treatment but sensitive to anti-IFN-α antibodies. Second, the IFN produced late in the culture period could be sensitive to pH 2 treatment but resistant to the anti-IFN-α antibodies. Third, in some instances the major part of the IFN was sensitive to both pH 2 treatment and the antibodies. The first and second patterns resemble those of classic IFN-α and IFN-γ, respectively. We interpret the third type of sensitivity pattern as IFN-α with anomalous behaviour with regard to pH 2 stability. The existence of such pH 2-labile IFN-α has also been proposed by others (10, 22). We consider it less likely that the third pattern represents simultaneously present and synergizing IFN-α and IFN-γ (7), because no such synergism was demonstrated in our IFN assay by deliberately mixing these IFNs (Rönnblom and Alm, unpublished data).

Thus, different types of IFNs were induced with a restricted number of bacteria (*E. coli*,

TABLE 5. Characterization of IFNs produced by bacterium-stimulated unfractionated PBLs and nonadherent, E-rosette-negative PBLs

Inducer	Cell type[a]	Activity[b] after the following treatment:			
		pH 7	pH 7 + anti-IFN-α	pH 2	pH 2 + anti-IFN-α
Streptococcus sp. strain G313	PBL	100	13	25	<3
	E^-	100	50	50	<6
B. catarrhalis 807	PBL	100	13	6	<6
	E^-	100	<6	25	<6
E. coli PW	PBL	100	<6	100	<6
	E^-	100	<3	100	<3
S. aureus Cowan I	PBL	100	25	<6	<6
	E^-	100	<3	100	<3

[a] PBL, unfractionated human PBLs; E^-, plastic-nonadherent, nylon wool-nonadherent cells depleted of E-rosette-forming cells. Cell cultures were established and stimulated by 1/500 to 1/1,000 (vol/vol) final dilutions of the indicated bacterium, and culture supernatants were harvested on day 3.

[b] Percent residual antiviral activity of culture supernatants.

streptococci, *B. catarrhalis*, and staphylococci). Even the same type of bacterium, e.g., *E. coli*, could in different experiments induce either pH 2-stabile or pH 2-labile IFN-α (cf. Tables 3 and 5). Conceivably, PBLs from different donors display genetic or acquired variations with regard to the spectrum of IFN-α subtypes induced by one type of bacterium, and this problem will be addressed in subsequent studies with purified PBL subpopulations. We do have evidence that different bacteria tested on PBLs from one blood donor induce IFN-α with different properties with regard to pH sensitivity (the present study) and antigenicity (Rönnblom and Alm, unpublished data).

One other obvious cause of the different IFNs induced by the various bacteria is that different cell types in the PBL population are engaged in the IFN responses. Results of cell-mixing experiments (Rönnblom and Alm, unpublished data) as well as the results of others (17, 29) demonstrate that bacteria or bacterial products induce IFN-γ production in T lymphocytes aided by macrophages. Such IFN-γ gradually accumulates during the culture period, with peak concentrations attained on culture days 3 to 4. This would explain the preponderance of IFN-γ-like antiviral activity found for some bacterium-stimulated PBL cultures in the present study late in the culture period. Different bacteria as well as individual blood donors varied considerably with regard to the magnitudes of the IFN responses, and this could depend on prior immunization of the PBL donors to relevant bacteria. Donors with cell-mediated (T cell) immunity would mount more vigorous IFN-γ responses, but we have no information regarding cellular immunity to the various bacteria or presence of antibacterial antibodies in serum. Other causes for such variations are therefore conceivable.

All bacteria rapidly (within 24 h) induced production of IFN-α in PBL cultures. We were able to demonstrate the major importance of nonadherent, E-rosette-negative null cells in the IFN-α responses to at least streptococci, staphylococci, and *E. coli*. The cells responding to the first two types of bacteria clearly expressed Fc receptors and were eliminated after passage through an immunoglobulin-anti-immunoglobulin column. In contrast, half of the IFN response to *E. coli* remained after such column passage. This suggests a heterogeneity with regard to cell types involved in IFN-α responses to bacteria. Interestingly, macrophages that actively interact with bacteria (9) and are major IFN-α producers when challenged with Sendai virus produce little or no IFN after stimulation by bacteria.

We did not here formally eliminate the possibility that B lymphocytes were producers of IFN-α. This would be one possibility because several bacteria have been reported to be polyclonal B-cell activators (1, 23, 24). Although these cells were not positively enriched in any of the cell fractions, they were largely eliminated after nylon wool column passage without reduction of the IFN production in the passing nonadherent cells. Furthermore, B cells purified by being panned on anti-immunoglobulin-coated plastic dishes did not produce IFN when cultured with bacteria (Rönnblom and Alm, unpublished data). Therefore, such cells are probably not major IFN producers.

Cells with NK activity towards K562 erythroleukemia cells copurified approximately with the cells producing IFN-α, especially in response to streptococci and staphylococci. Whether the NK cells are identical to such IFN-producing cells remains to be determined, but is a distinct possibility. We have in another study demonstrated that cells with similar null-cell characteristics are stimulated to IFN production by *Plasmodium falciparum* parasites (L. Rönnblom, E. A. Ojo-Amaize, L. Franzén, H. Wigzell, and G. V. Alm, Parasite Immunol., in press).

A wide variety of nonviral infectious microorganisms, therefore, can rapidly induce IFN-α in peripheral blood null cells, at least some of which are NK-like. Such IFN may in several ways rapidly activate defense mechanisms (27), e.g., phagocytic cells (11), NK cells (8), and T-cell-mediated immunity (15). In a sense the IFNs may then be considered as stress hormones which enhance the functions of the immune system. It will therefore be important to further define the cells engaged in early IFN responses, the spectrum and functions of IFNs produced after contact with bacteria and various parasites, and the possible physiological relevance of these IFNs.

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