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# Gr-1<sup>high</sup> Polymorphonuclear Leukocytes and NK Cells Act via IL-15 to Clear Intracellular *Haemophilus influenzae* in Experimental Murine Peritonitis and Pneumonia<sup>1</sup>

Shuichi Miyazaki,<sup>2\*</sup> Fumio Ishikawa,<sup>†</sup> Kayo Shimizu,<sup>‡</sup> Tsuneyuki Ubagai,<sup>§</sup> Paul H. Edelstein,<sup>¶</sup> and Keizo Yamaguchi\*

Polymorphonuclear leukocytes (PMNs) can be divided into Gr-1<sup>high</sup> and Gr-1<sup>low</sup> subpopulations, but the differences in the functions of these cells in the host are unknown. This study investigated the roles of these two cell populations in the clearance of an intracellular pathogen (*Haemophilus influenzae*) causing murine peritonitis and pneumonia. Microarray analysis and quantitative real-time PCR analysis of protease peptone-elicited peritoneal murine PMNs showed that *IL-15* mRNA levels were significantly higher in Gr-1<sup>high</sup> PMNs than in Gr-1<sup>low</sup> PMNs. In addition, IL-15 was produced only by Gr-1-positive PMNs, especially Gr-1<sup>high</sup> PMNs. IL-15 was required for efficient clearance of experimental murine *H. influenzae* pneumonia, as 4 days postinfection lungs from IL-15 knockout mice contained 50- to 100-fold more bacteria than did wild-type mouse lungs. Gr-1 PMN-depleted C57BL/6 mice were more susceptible to *H. influenzae* pneumonia than were Gr-1 PMN replete C57BL/6 mice or C57BL/6 nude mice, demonstrating that Gr-1 PMNs are important in the clearance of intracellular bacteria. IL-15-activated NK cells killed *H. influenzae* in PMNs. Flow cytometry confirmed the expression of CD69 on the cell membrane of IL-15-activated NK cells. Our results show that Gr-1<sup>high</sup> PMNs produce more IL-15 than Gr-1<sup>low</sup> PMNs, and that IL-15-activated NK cells protect against early infection by *H. influenzae*. *The Journal of Immunology*, 2007, 179: 5407–5414.

Polymorphonuclear leukocytes (PMNs)<sup>3</sup> migrate to sites of inflammation to phagocytose infective pathogens rapidly, and to induce antimicrobial activities involving reactive oxygen and reactive nitrogen intermediates for killing pathogens. These responses are directed primarily against extracellular bacteria and fungi (1, 2), whereas intracellular bacteria, viruses, and protozoa are usually protected from this killing system of PMNs and other immune responses (3–5). In the past two decades, evidence has accumulated on the mechanism for killing intracellular pathogens in innate immunity. Intracellular bacteria such as *Listeria monocytogenes* are first phagocytosed by phagocytes at site of infection (6). NK cells recognize and respond to intracellular pathogens; they are an important part of the initial defenses against intracellular pathogens (7). NK cells are activated by cytokines such as IL-12 and type 1 IFNs produced by activated macrophages and dendritic cells. Upon their activation, NK cells directly lyse

macrophages and other cells infected with intracellular pathogens by exocytosis and also secrete cytokines for killing intracellular pathogens (7).

Several lines of evidence demonstrate the involvement of IL-15 in clearing intracellular infection caused by *Salmonella choleraesuis* and other microorganisms (8–11). IL-15 is also crucial for the activation of a number of lymphocyte populations such as NK cells and NKT cells (12–16). Although *IL-15* mRNA is expressed in numerous normal tissues and cell types, IL-15 protein is secreted at levels that are barely detectable under physiological conditions (17, 18). *IL-15* gene expression and protein production are up-regulated under inflammatory conditions, as described for monocytes and macrophages exposed to IFN- $\gamma$  and LPS, or infected with bacteria or viruses (19–21).

Based on the findings that IL-15 plays an important role in initial innate immunity and that PMNs are usually the first immune cell that arrive at the site of infection, it is possible that PMNs produce this cytokine to help eliminate infective pathogens. PMNs can be divided into three subpopulations based on distinct patterns of cytokine and chemokine production, TLR expression, and surface CD49d and CD11b expression, and are thus classified into PMN-I, PMN-II, and PMN-N subsets (22). PMN-I cells were obtained from methicillin-resistant *Staphylococcus aureus* (MRSA)-resistant hosts, while the PMN-II subset was derived from MRSA-susceptible hosts, suggesting that PMN subsets differ in their effect on innate immunity against MRSA. Our group also reported that PMNs can be divided based on the expression level of PMN-specific Gr-1 Ag into Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs (23). Gr-1 PMN surface markers are atypical for PMN-I, PMN-II, and PMN-N cells and therefore Gr-1 PMNs cannot be classified as any of these three PMN subsets.

In this study, we tested the hypothesis that PMNs participate directly or indirectly in the initial host defenses against infection by intracellular pathogens, in addition to their role in killing

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<sup>3</sup> Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; MRSA, methicillin-resistant *Staphylococcus aureus*; KO, knockout; PEC, peritoneal exudate cell; PP, protease peptone; CBO, cell-bound organism.

extracellular microorganisms. To test our hypothesis, we analyzed the functional roles of Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs in mouse infections and inflammation.

## Materials and Methods

### Mice

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Toho University School of Medicine. Four- to 6-wk-old female C57BL/6 mice and C57BL/6 nude mice were obtained from Charles River Laboratories. Four-week-old female IL-15 knockout (KO) mice were purchased from Taconic Farms. Unless otherwise stated, each experimental group consisted of six mice.

### Preparation of murine PMNs

Murine peritoneal exudate cells (PEC) were harvested on 3 h after injecting mice i.p. with 3% proteose peptone (PP; 3 ml; Difco) under anesthesia, by peritoneal lavage using 3 ml of warm RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS. The cells washed by centrifugation were counted using a hemacytometer, and were also examined microscopically after staining with May-Grünwald-Giemsa stain. Granulocytes were differentiated from mononuclear cells in counts of 200 cells. PMNs comprised >90% of cells in the suspension. The number of PMNs was calculated by multiplying the total number of PECs by the proportion of PMNs. The proportion of Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs was determined by flow cytometry, as described below, and the absolute numbers of these cells in the washed and resuspended PEC was calculated by multiplying the total number of PMNs by the proportion of each PMN fraction.

### Peritoneal infection

Mice were injected with *Haemophilus influenzae* 770235<sup>b</sup> (24) to produce peritonitis (i.p., 0.5 ml, 1–2 × 10<sup>6</sup> CFU/ml). In this circumstance, *H. influenzae* is an intracellular organism (3, 25, 26). Ten minutes postinfection, peritoneal lavage (RPMI 1640, 3 ml) was performed in six mice to determine the baseline bacterial concentrations and cell counts. Thirty minutes, and 1 or 3 h postinfection, the PECs were harvested and enumerated as described for the preparation of PMNs; six mice each were sampled at each time point. The bacterial counts in PEC suspension from each sample were assayed by dilution and plating on chocolate agar plates.

### Preparation of NK cells

Spleen cells were isolated from C57BL/6 mice. Nonadherent cells were collected on a nylon fiber column, and NK cells were negatively sorted by using a mouse NK cell isolating kit and the protocol supplied by the manufacturer (Miltenyi Biotec). The purity of isolated NK cells was >95%, using anti CD49b mAb (DX5 clone; rat IgM; eBioscience).

### Flow cytometry

Freshly isolated PMNs from PECs were blocked using 2.4G2 hybridoma cell supernatant for 15 min at 4°C; clone 2.4G2 produces rat anti-mouse FcII/III (CD32) mAb (27). The PMNs were then stained with FITC-conjugated anti-Gr-1 mAb (clone: RB6-8C5; BD Pharmingen) for 30 min at 4°C (28). The stained cells were analyzed using FACSCalibur (BD Pharmingen) and were sorted according to the content of Gr-1 Ag using a BD FACSria Cell Sorter (BD Pharmingen). PMNs were defined as being Gr-1<sup>high</sup> or Gr-1<sup>low</sup> cells based on anti-Gr-1 fluorescence intensity staining, with Gr-1<sup>high</sup> cells having a fluorescence intensity of >600 intensity units and Gr-1<sup>low</sup> cells having a fluorescence intensity between 20 and 500 intensity units. The purity of PMNs was checked by May-Grünwald-Giemsa stain.

For intracellular IL-15 staining, PECs were separated by Lympholyte-M (Cedarlane Laboratories). The separated granulocytes were treated with LPS (1 µg/ml) and rGM-CSF (100 U/ml; PeproTech) for 20 h at 37°C; then, brefeldin A (5 µg/ml; Sigma-Aldrich) was added for an additional 4 h. Cells were stained with FITC-conjugated anti-Gr-1 mAb, and followed by 4% paraformaldehyde fixation and 0.1% saponin permeabilization. After blocking, the cells were stained with biotin-conjugated anti-IL-15 mAb (PeproTech) at 4°C for 30 min, followed by the addition of streptavidin-allophycocyanin (BD Pharmingen). Cell surface IL-15 expression was also detected in purified and LPS-stimulated PMNs. Samples were analyzed using CellQuest software on a FACSCalibur.

### Microarray production

A Mouse Cytokine Chemokine Chip version 1.0 was manufactured by Kaken Geneqs. In brief, 117 clones coding for most of the known cyto-

kines/chemokines and their receptors were included in this cDNA microarray. Sequence homology was confirmed by the National Center for Biotechnology Information sequence search tool (nucleotide-nucleotide basic local alignment search tool search). Housekeeping genes (*β-actin*, *GAPDH*, ribosomal protein L12, ribosomal protein S18, H1 histone family member 2, myosin Ib, ornithine decarboxylase structural 1, and transferrin receptor) were spotted as positive controls, and three mouse-unrelated reagents (tobacco chloroplast DNA, part of the cloning vector, and solvent for the cDNAs) were included as negative controls.

### RNA isolation and cDNA microarray hybridization

Murine Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMN fractions were obtained as described above. Total RNA was isolated from the two different PMN fractions using a RNeasy Plus Mini kit (Qiagen) and the procedure supplied by the manufacturer. The quantities and qualities of total RNA samples were determined with an Agilent 2100 Bioanalyzer (Yokogawa Analytical). RNA (3.1 µg) from Gr-1<sup>high</sup> PMNs was fluorescently labeled with cyanine 3, while the reference RNA (2.4 µg) derived from Gr-1<sup>low</sup> PMNs was labeled with cyanine 5 using a 3DNA Array Detection Array 350 kit (Genisphere), and the instructions provided by the manufacturer. Briefly, cDNA probes were tagged with the 3DNA capture sequence by reverse transcription using a special RT dT primer. Hybridization was conducted in a formamide-based hybridization buffer at 45°C for 16 h. 3DNA hybridization was then performed using the included 3DNA Array 350 Capture Reagent, which contained 375 fluorescent dyes per molecule and hybridized to the cDNA probe by virtue of a complementary sequence to the capture sequence. DNA hybridization was performed in formamide-based hybridization buffer containing antifade reagent at 50°C for 3 h. Following washing, the slide was immediately imaged using a GenePix 4000B microarray scanner and GenePix Pro 4.1 software (Axon Instruments). The criterion for selection of differentially expressed genes was that the change of gene expression was >3-fold on average. Four to five replicates of each condition were tested.

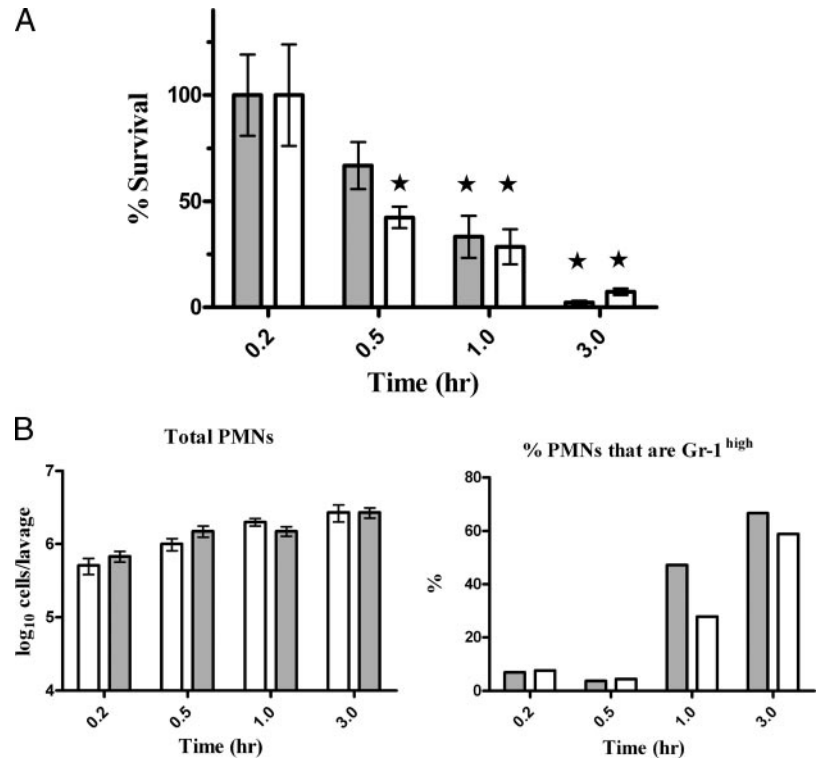
### Quantitative real-time PCR analysis

Gene expression analysis of PP-stimulated Gr-1-subtype PMNs was performed using identical total RNAs with cDNA microarray analysis. Total RNA was reverse-transcribed to cDNA using the SuperScript III First-Strand Synthesis SuperMix for RT-PCR kit (Invitrogen Life Technologies). Briefly, 1 µg of total RNA was incubated with 2.5 µM oligo(dT)<sub>20</sub> and 50 ng of random hexamers including 200 U of SuperScript III RT enzyme in a 40-µl reaction volume at 25°C for 10 min, then at 50°C for 20 min. The design and sequence of primers and probes for mouse *IL-15* was based on the previously reported oligonucleotide sequences with FAM as a reporter dye and TAMRA as a quencher dye (29). The gene expression levels of cytokine *IL-15* of mouse PMNs were quantified using the ABI7300 Real-Time PCR System (Applied Biosystems). cDNAs were amplified with TaqMan probes using Platinum Quantitative PCR SuperMix-UDG with the ROX kit (Invitrogen Life Technologies). The experimental conditions were described previously (29). *IL-15* mRNA expression levels of mouse PMNs were normalized relative to *β-actin* gene expression levels. Fold differences in *IL-15* mRNA levels between Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs were determined using Sequence Detection System software (Applied Biosystems).

### Experimental bronchopneumonia caused by *H. influenzae*

The difference in severity of bronchopneumonia caused by *H. influenzae* among C57BL/6, C57BL/6 nude, and IL-15 KO mice (12 animals/group) was examined using the partially modified mouse pneumonia model of cell-bound organisms (CBOs) (3). Cell-bound organisms are prepared by adding *H. influenzae* to murine fetal lung cells in tissue culture; the bacteria are internalized by the fetal lung cells, but are not killed by them, and are protected from the bactericidal action of serum factors (3). This study differed from the previous report in that C57BL/6, C57BL/6 nude, and IL-15 KO mice were used rather than Slc/ICR mice and the animals were not pretreated with formalin. Briefly, CBOs are instilled intranasally into anesthetized mice and, 4 days later, the mice are euthanized to determine lung bacterial load (3). The delivered and retained bacterial doses were ~1 × 10<sup>6</sup> and ~1 × 10<sup>5</sup> CFU/animal, respectively; the retained dose is substantially lower than the retained dose because not all the inoculum enters the lung. Four mice from each group were used to determine the proportion of Gr-1<sup>high</sup> to Gr-1<sup>low</sup> PMNs in the abdominal cavity by flow cytometry as described above. The remaining eight mice from each group were used for counting viable organisms in the infected tissues. The lower limit of detection of *H. influenzae* in lung was 100 CFU/animal, due to the need to dilute growth-inhibitory tissues before plating.

**FIGURE 1.** Survival of i.p. *H. influenzae* and cellular response to infection. **A**, Percent survival (mean and SE) of peritoneal *H. influenzae*. Baseline concentrations for *H. influenzae* were  $2.5 \times 10^5$  CFU/ml. \*,  $p < 0.05$ , compared with initial value at 0.2 h. ■, C57BL/6 nude mice; □, C57BL/6 mice;  $n = 6$  for each group. **B**, PMN and Gr-1 PMN concentrations in peritoneal exudate after *H. influenzae* infection. Bar heights, Mean values and error bars SE;  $n = 6$  for each data point;  $p < 0.05$  for comparison of 0.2-h baseline vs 3-h counts for the each of the two different mouse strains;  $p < 0.01$  for comparison of data at 1 and 3 h vs baseline value for Gr-1<sup>high</sup> percentage. ■, C57BL/6 nude mice; □, C57BL/6 mice.



Gr-1-positive PMNs were depleted in some mice by the administration of rat anti-mouse Gr-1 mAb (R&D Systems) or normal rat IgG (Sigma-Aldrich) to C57BL/6 mice (0.2 mg, i.p.) 1 day before infection; the dose was selected based on a previous report (30). The normal rat IgG possessed no *H. influenzae*-agglutinating Abs. Immune reconstitution of some IL-15 KO mice was performed by injecting them with PECs (i.p.,  $2 \times 10^6$  cells) from C57BL/6 mice or IL-15 KO mice 3 h before infection.

#### Bacterial killing activity by NK cells following treatment with IL-15

To determine the ability of NK cells to kill *H. influenzae* phagocytosed by PMNs, NK cells were added to the phagocytosed bacteria in quintuplicate and viable bacterial counts were determined at selected times. PECs were obtained from C57BL/6 mice as described above, and then resuspended in RPMI 1640. The PECs were incubated (35°C in 5% CO<sub>2</sub> for 1 h) with an equal volume of bacteria. Gentamicin (100 mg/L) was added for 30 min to kill extracellular bacteria, then removed by washing three times with RPMI 1640. The phagocytosed bacteria were dispensed into 12-well tissue-culture trays at a final bacterial concentration of  $5 \times 10^6$  CFU/ml, and a final PEC concentration of  $4 \times 10^5$  cells/ml, in a 1-ml volume. NK cells ( $5 \times 10^5$  cells/ml final concentration), IL-15 (20 ng/ml final concentration), both NK cells and IL-15 or medium only, were added to the wells containing phagocytosed bacteria. Anti-IFN- $\gamma$  mAb (20  $\mu$ g/ml final concentration, clone: R4-6A2; eBioscience) was added as needed to some wells containing NK cells, IL-15, and phagocytosed bacteria. The chambers were incubated at 35°C and 5% CO<sub>2</sub>. Viable counts were assayed by plating samples on chocolate agar plates at the desired times.

#### CD69 induction assay by PMNs

To determine whether NK cells were activated by Gr-1 PMN subtypes, flow cytometry analysis was conducted of anti-CD69-labeled NK cells after incubating them with Gr-1 PMN subtypes. Sorted Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs ( $1 \times 10^6$  cells/ml) were cultured with 0.1  $\mu$ g/ml LPS in the presence of 100 U/ml  $\gamma$ GM-CSF (PeproTech) for 24 h. Cells were cocultured with MACS-purified NK cells at a ratio of 10:1. After 24 h, CD69 expression on NK cells was analyzed with a FACSCalibur.

#### Statistical analysis

All parametric data were expressed as mean  $\pm$  SD. Differences between mean in vitro and in vivo number of PMNs and viable organisms were examined for statistical significance using one-way ANOVA and the Tukey correction when making multiple comparisons, and a two-tailed *t*

test for comparisons of two mean values (Prism version 5.0; GraphPad Software); in some cases, log-transformed values were compared to allow comparisons with similar variances. Lung bacterial concentrations below the detection limit of 100 CFU were excluded from analysis of mean values; a two-tailed  $\chi^2$  test was used to compare groups having no detectable lung bacteria with those with detectable bacteria. For graphical simplicity, bacterial counts below the detection limit are graphed as 50 CFU/animal lung. A *p* value of  $<0.05$  indicated a statistically significant difference.

## Results

### Relationship between viable organism counts and altered Gr-1<sup>high</sup>:Gr-1<sup>low</sup> ratio of PMNs following infection with *H. influenzae*

To determine the role of Gr-1-positive PMNs in bacterial infection, we examined bacterial clearance, and the number of Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs, in the abdominal lavage fluid of mice with experimental bacterial peritonitis. The average number of viable *H. influenzae* organisms in C57BL/6 mouse peritoneal lavage fluid decreased to 28 and 7%, 1 and 3 h postinfection, respectively, of the 10-min postinfection count ( $4.8 \times 10^5 \pm 2.9 \times 10^5$  CFU/animal) (Fig. 1A). C57BL/6 nude mice were able to clear i.p. *H. influenzae* as well as C57BL/6 mice; 1 and 3 h after infection, the average peritoneal lavage fluid bacterial counts were 33 and 2%, respectively, of the 10-min postinfection count ( $6.5 \times 10^5 \pm 3.05 \times 10^5$  CFU/animal).

The total number of peritoneal PMNs increased ~4-fold from 10 min to 3 h following infection with *H. influenzae* to maximum average counts of  $2.3 \times 10^6$  cells/lavage ( $p < 0.05$ , 10 min baseline vs 3 h counts for each of the two different mouse strains) (Fig. 1B). The bulk of the recruited PMNs were Gr-1 PMNs, with an average of 80% of PMNs being Gr-1 cells 10 min after infection, and 99% being Gr-1 PMN cells 3 h after infection. Over the 3-h observation period, the proportion of PMNs that were Gr-1<sup>high</sup> cells increased markedly; ~10% of PMNs were Gr-1<sup>high</sup> cells at baseline, whereas at 3 h, this proportion increased to 59–66% ( $p < 0.01$ , Fisher exact test). The numbers of Gr-1<sup>low</sup> PMNs in

Table I. Cytokine and cytokine receptor gene expression in *Gr-1<sup>high</sup>* and *Gr-1<sup>low</sup>* PMNs<sup>a</sup>

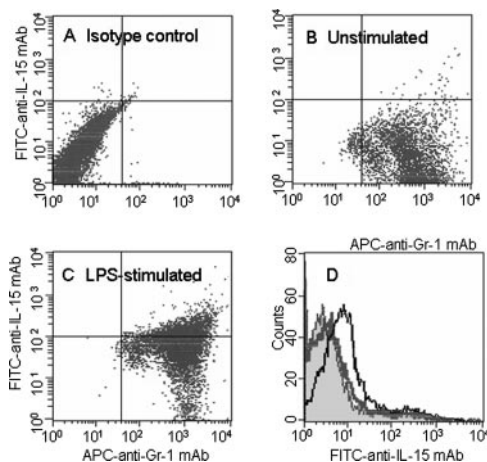
| Cytokine                | <i>Gr-1<sup>high</sup></i> : <i>Gr-1<sup>low</sup></i> PMN expression |
|-------------------------|---|
| IL-1 $\alpha$           | 0.80 $\pm$ 0.22   |
| IL-1 $\beta$            | 3.05 $\pm$ 0.47   |
| IL-2                    | 1.87 $\pm$ 0.27   |
| IL-4                    | 0.85 $\pm$ 1.49   |
| IL-5                    | 0.88 $\pm$ 0.33   |
| IL-6                    | 0.82 $\pm$ 0.74   |
| IL-7                    | 0.38 $\pm$ 0.28   |
| IL-9                    | 0.98 $\pm$ 0.75   |
| IL-10                   | 0.47 $\pm$ 0.21   |
| IL-11                   | 0.34 $\pm$ 0.42   |
| IL-12                   | 0.55 $\pm$ 0.06   |
| IL-15                   | 14.73 $\pm$ 4.09  |
| IL-16                   | 0.25 $\pm$ 0.09   |
| IL-17                   | 0.39 $\pm$ 0.25   |
| IL-18                   | 1.34 $\pm$ 1.40   |
| CCL2                    | 0.20 $\pm$ 0.04   |
| CCL3                    | 1.23 $\pm$ 0.32   |
| IL-2 $\beta$ receptor   | 0.83 $\pm$ 0.39   |
| IL-15 $\alpha$ receptor | 0.30 $\pm$ 0.25   |

<sup>a</sup> Data are mean  $\pm$  SD; *n* = 4–5 in each group.

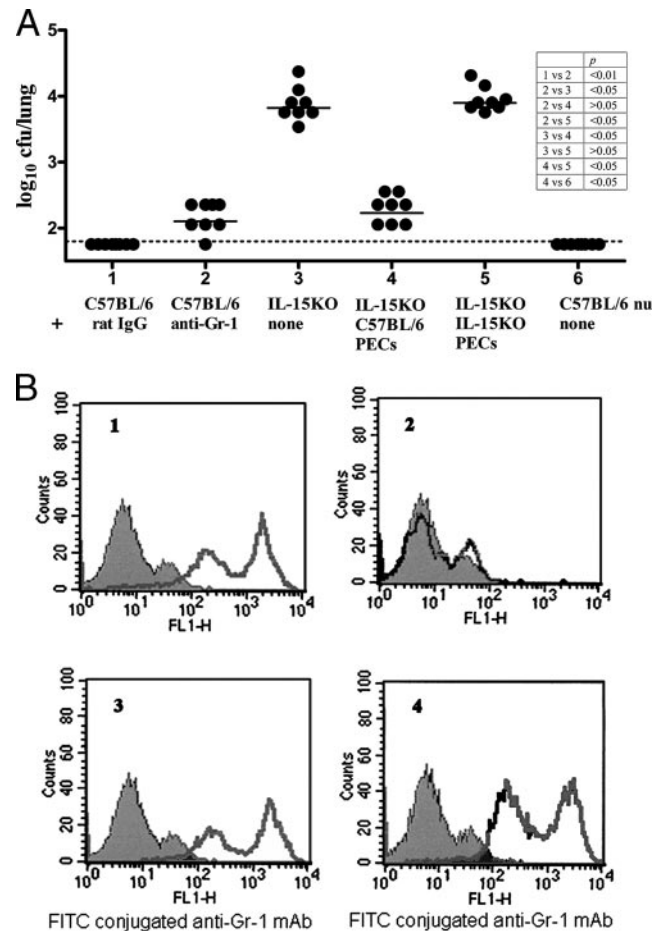
mice infected with *H. influenzae* did not change significantly after inoculation (*p* > 0.05).

#### Gene expression profiling with cDNA microarray hybridization and quantitative real-time PCR

To investigate the mechanism of action of *Gr-1<sup>high</sup>* PMNs against intracellular organisms, microarray analysis was used to determine the gene expression of various cytokines in PP-elicited peritoneal *Gr-1<sup>high</sup>* and *Gr-1<sup>low</sup>* PMNs. Using May-Grünwald-Giemsa staining, 99% of the flow-cytometry purified *Gr-1<sup>high</sup>* PMNs were PMNs, while the *Gr-1<sup>low</sup>* PMN cell fraction was composed of 90% PMNs and 10% monocytes. The ratios of *Gr-1<sup>high</sup>*:*Gr-1<sup>low</sup>* PMN mRNA levels for various cytokines is shown in Table I. *IL-15* mRNA levels were 14.73 (95% confidence interval = 8.2 to 21.2) times greater in *Gr-1<sup>high</sup>* PMNs than in *Gr-1<sup>low</sup>* PMNs. *IL-1 $\beta$*  was the only other measured cytokine that was expressed significantly more in *Gr-1<sup>high</sup>* PMNs than in *Gr-1<sup>low</sup>* PMNs, with a 3.05-fold



**FIGURE 2.** Intracellular and cell surface-bound IL-15 expression as measured by flow cytometry of PP-elicited peritoneal PMNs. Cells were stained with FITC anti-IL-15 mAb (A–C), and with isotype control Ab (A) or allophycocyanin-anti-Gr-1 mAb (B–D). A, Isotype control; B, unstimulated, intracellular IL-15 staining; C, LPS-stimulated, intracellular IL-15 staining; D, LPS stimulated (thin line) or unstimulated (thick line) cells, surface-bound IL-15; gray shading represents isotype control staining.

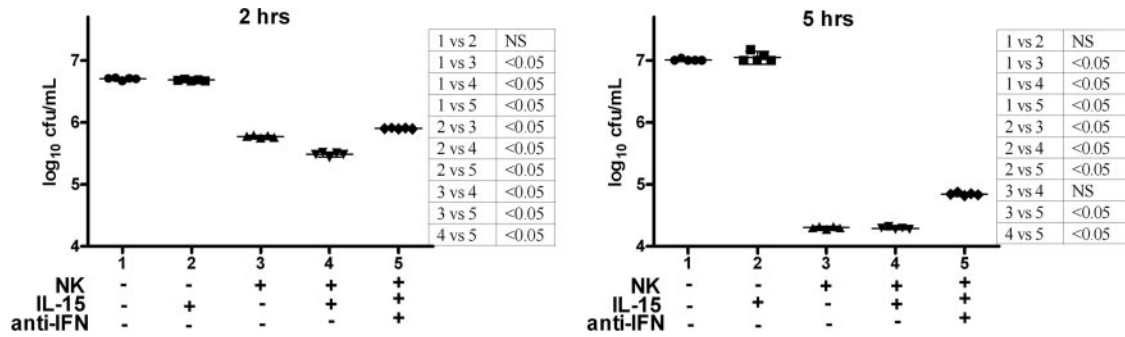


**FIGURE 3.** Relation between murine susceptibility to *H. influenzae* infection and pattern of *Gr-1<sup>high</sup>*/*Gr-1<sup>low</sup>* PMN ratio. A, Murine susceptibility to *H. influenzae* infection. Mice were infected with phagocytosed *H. influenzae* (CBO) after treatment with the indicated Abs or cells. The *H. influenzae* concentrations in both lungs of each of eight mice 4 days postinfection are shown. Data points shown below the horizontal dotted line had no detectable bacteria; horizontal bars represent the geometric means for data above the detection limit. The inserted table gives the results of statistical testing for each group comparison; the top and bottom comparisons were performed by  $\chi^2$  testing and the other comparisons by one-way ANOVA. B, Flow cytometry of peritoneal murine *Gr-1<sup>high</sup>* and *Gr-1<sup>low</sup>* PMNs elicited by i.p. 3% proteose peptone 3 h before peritoneal lavage. Panel 1, C57BL/6 mice untreated with Ab or cells. Panel 2, C57BL/6 mice pretreated 1 day before with anti-Gr-1 mAb (i.p., 0.2 mg). Panel 3, Untreated IL-15 KO mice. Panel 4, IL-15 KO mice treated with i.p. C57BL/6 PECs, 6 h before peritoneal lavage, and 3 h after i.p. PP injection. Solid histogram, Isotype control; *n* = 4 animals/group.

(95% confidence interval = 2.3 to 3.8) mRNA level difference detected. Thus, we were interested in the *IL-15* gene expression ratio between *Gr-1<sup>high</sup>* and *Gr-1<sup>low</sup>* PMNs. Quantitative real-time PCR showed that the mRNA expression level of *IL-15* in *Gr-1<sup>high</sup>* PMNs was ~28-fold higher than that of *Gr-1<sup>low</sup>* PMNs, which were expressed at basal levels, thus confirming the results of microarray analysis.

#### Detection of intracellular production of IL-15 and cell surface-bound IL-15

We next investigated whether and which *Gr-1* PMNs produce IL-15, using flow cytometry and staining of PP-elicited PECs for the cellular presence of IL-15 and *Gr-1* Ags. *Gr-1* PMNs cultured without LPS produced little IL-15, with only 0.26% of *Gr-1* cells



**FIGURE 4.** NK cell killing of phagocytosed *H. influenzae*. NK cells were added to *H. influenzae* phagocytosed by PECs in the presence of IL-15, IFN- $\gamma$  Ab, or no additives, for 2 or 5 h. Controls included phagocytosed bacteria incubated with just IL-15 or tissue-culture medium alone;  $n = 5$  for each point. The group mean and value for each data point is shown. The table shows the results of one-way ANOVA analysis of the data, with Tukey post-hoc comparisons.

positive for IL-15, with a geometric mean value of 705 fluorescence intensity units in those PMNs (Fig. 2B). In contrast, LPS stimulation of PMNs increased the proportion of IL-15-positive Gr-1-positive PMNs to 26.8% with a geometric mean value of 738 fluorescence intensity units (Fig. 2C). In the stimulated and non-stimulated PMNs, 89 and 92% of IL-15-positive Gr-1 PMNs were Gr-1<sup>high</sup> cells, respectively, and the level of IL-15 production correlated with the number of Gr-1<sup>high</sup> PMNs. Furthermore, flow cytometry with staining for cell surface IL-15 showed that resting PMNs did not secrete IL-15. In contrast, LPS-activated PMNs were strongly positive for surface-expressed IL-15 (Fig. 2D).

#### Role of IL-15 and Gr-1 PMNs in experimental *H. influenzae* murine pneumonia

To determine the role of IL-15 and Gr-1 PMNs in host defenses against *H. influenzae*, we determined the pulmonary clearance of *H. influenzae* 4 days after experimental induction of *H. influenzae* pneumonia in mice with and without functional IL-15 or Gr-1 PMNs (Fig. 3A).

C57BL/6 mice and C57BL/6 nude mice were resistant to *H. influenzae* infection, based on the lack of detectable infective organisms in the lungs of all eight mice. *H. influenzae* infection of C57BL/6 mice that were not pretreated with normal rat IgG gave results identical with the IgG-pretreated mice (data not shown). In contrast, seven of eight C57BL/6 mice depleted of circulating Gr-1 PMNs failed to clear lung *H. influenzae*, with average bacterial counts of 157 CFU/lung, demonstrating that Gr-1 PMNs play a role in clearance of the bacterium from the lungs ( $p < 0.01$ ,  $\chi^2$  test, for comparison of Gr-1-depleted vs non-Gr-1-depleted C57BL/6, or C57BL/6 nude, mice).

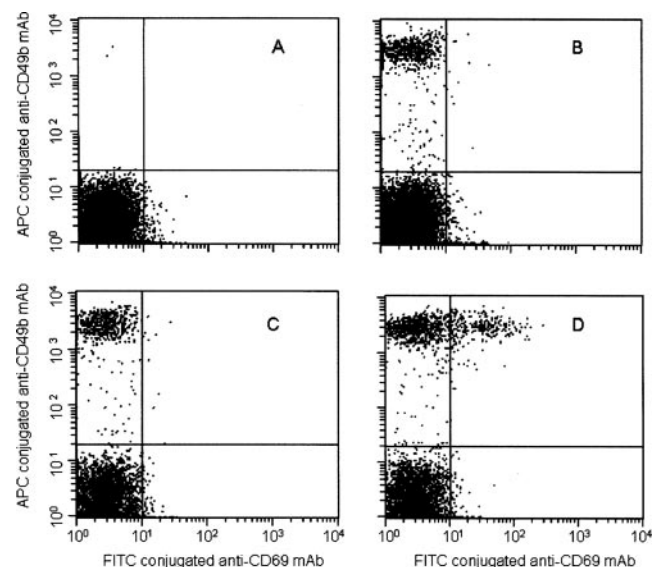
IL-15 KO mice had a severe immune defect and were unable to clear *H. influenzae* from the lungs; mean bacterial counts were  $8 \times 10^3$  CFU/lung (Fig. 3A). This immune defect was rescued by the i.p. administration of PP-elicited PECs obtained from C57BL/6 mice; mean lung bacterial counts were 197 CFU/lung ( $p < 0.05 \times$  one-way ANOVA, Tukey comparison test, vs IL-15 KO mice). IL-15 KO mice injected with homologous PECs failed to clear lung bacteria.

Flow cytometric analysis of PP-elicited PECs obtained from C57BL/6 mice 1 day after administration of anti-mouse Gr-1 mAb confirmed the absence of Gr-1 PMNs in the PECs (Fig. 3B). The relative ratios of Gr-1<sup>high</sup> to Gr-1<sup>low</sup> in PP-elicited PECs of IL-15 KO mice were similar to those found in C57BL/6 mice. The ratios of Gr-1<sup>high</sup>:Gr-1<sup>low</sup> PMNs in C57BL/6 and IL-15KO mice were 1.18 and 1.40, respectively. As expected, administration of i.p. PECs to IL-15 KO mice increased the numbers of i.p. Gr-1 cells of both types. The ratio of Gr-1<sup>high</sup>:Gr-1<sup>low</sup> PMNs in the PEC-res-

cued IL-15 KO mice was 0.97. The relative populations of Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PECs in C57BL/6 nude mice were similar to those in the wild-type background C57BL/6 mice strain (data not shown).

#### Effect of exogenous IL-15 on NK cell killing of phagocytosed *H. influenzae*

To determine whether exogenous IL-15 promotes NK cell-mediated killing of phagocytosed *H. influenzae* in excess of the IL-15 produced by the phagocytic cells, the effect of exogenous IL-15 on bacterial killing was determined. Also, to determine whether the NK cell bactericidal effect was IFN- $\gamma$  dependent, the effect of IFN- $\gamma$  Ab addition on the NK bactericidal effect was examined. Incubation of the phagocytosed bacteria with RPMI 1640 alone, or the culture medium with IL-15, had no significant effect on bacterial concentrations (Fig. 4). In contrast, addition of NK cells to the phagocytosed bacteria resulted in a 1–3 log<sub>10</sub> decrease in bacterial concentration over 2–5 h ( $p < 0.001$ ). Addition of IL-15 to the NK cells reduced the bacterial concentration by half



**FIGURE 5.** Expression of CD69 on the membrane of NK cells cocultured with Gr-1<sup>high</sup> or Gr-1<sup>low</sup> PMNs. NK cells were stained with FITC-conjugated anti-CD69 mAb (eBioscience) and allophycocyanin-conjugated anti-CD49b (eBioscience). A, Isotype control containing Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMN cells. B, NK cells cocultured with Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs, without stimulation of the PMNs with LPS. C, NK cells cocultured with Gr-1<sup>low</sup> PMNs stimulated with LPS. D, NK cells cocultured with Gr-1<sup>high</sup> PMNs stimulated with LPS.

of that observed for the NK cells alone at 2 h ( $p < 0.05$ ), but after a 5-h incubation no added benefit of IL-15 was apparent. Further addition of anti-IFN- $\gamma$  to the IL-15 and NK cells resulted in decreased bacterial killing over that seen with combined IL-15 and NK cells ( $p < 0.05$  at 2 and 5 h). These results suggest that the phagocytic cells provide sufficient IL-15 to mediate NK cell killing of *H. influenzae*, and that this process is to some degree IFN- $\gamma$  dependent.

#### Day 69 expression on NK cells by stimulation of PMNs

To confirm that NK cells are activated by Gr-1<sup>high</sup> cells, NK cells were cocultured with Gr-1-positive PMNs purified to subtype fractions by flow cytometry. NK cell activation was detected by flow cytometric analysis of CD69 expression. As shown in Fig. 5D, the percentage of CD69-expressing NK cells cocultured with LPS-stimulated Gr-1<sup>high</sup> PMNs was 22.8%, but only 0.2% when the NK cells were cocultured with LPS-stimulated Gr-1<sup>low</sup> PMNs (Fig. 5C). The latter value was the same for NK cells that were cocultured with non-LPS-stimulated Gr-1<sup>high</sup> and Gr-1<sup>low</sup> cells (Fig. 5B). These results confirm that stimulated Gr-1<sup>high</sup>, but not Gr-1<sup>low</sup>, PMNs activate NK cells.

## Discussion

We conducted a series of experiments using PMNs harvested from the peritoneum of mice inoculated i.p. with bacteria, as a model of PMN migration to sites of infection. Using this model, we classified PMNs into two groups (Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs) based on the quantity of Gr-1 translated (23). It is well-known that anti-Gr-1 mAb strongly reacts with Ly-6G, and this mAb also reacts with another Ly-6 family molecule, Ly-6C (31). Ly-6G is expressed on PMNs while Ly-6C is expressed on PMNs, monocytes, and other cells (23, 32). In the present study, the purity of Gr-1<sup>high</sup> PMNs was very high while that of Gr-1<sup>low</sup> PMNs was lower than Gr-1<sup>high</sup> PMNs. Combined together, these data indicate that the Gr-1<sup>high</sup> PMN preparation contained no other cells, while Gr-1<sup>low</sup> PMN preparations contained monocytes. Under these conditions, we investigated the functional differences between Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs.

In response to i.p. infection, Gr-1<sup>high</sup> cells rapidly became the predominant type of PMNs in the peritoneal exudate, as has been previously shown for the response to i.p. LPS injection (23). These cells, and presumably other immune effectors, were able to control *H. influenzae* peritonitis. The rate of fall of *H. influenzae* bacterial counts in C57BL/6 nude mice was the same as in C57BL/6 mice, and in addition the migrating numbers of total PMNs, Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs were not apparently different between the two strains of mice. Combined together, these results suggest that Gr-1<sup>high</sup> cells might have other role(s) apart from the phagocytosis of intracellular bacteria.

As this killing of intracellular bacteria may involve IL-15, we determined whether Gr-1 subtype PMNs make IL-15, and whether the IL-15 is surface expressed. First, we compared mRNA content in Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs by microarray analysis. The results showed that *IL-15* gene expression in Gr-1<sup>high</sup> PMNs was significantly greater than in Gr-1<sup>low</sup> PMNs. Moreover, quantitative real-time PCR confirmed that mRNA content in Gr-1<sup>high</sup> PMNs was significantly higher than in Gr-1<sup>low</sup> PMNs. Furthermore, both intracellular and surface IL-15 protein was detected mainly in Gr-1<sup>high</sup> PMN by flow cytometry. Taken together, the results indicate that Gr-1<sup>high</sup> PMNs produce IL-15, and that such production is higher than in Gr-1<sup>low</sup> PMNs.

The contribution of Gr-1 PMNs to bacterial killing was determined in a murine pneumonia model. These studies showed that Gr-1 cells played an important role in bacterial killing, based on

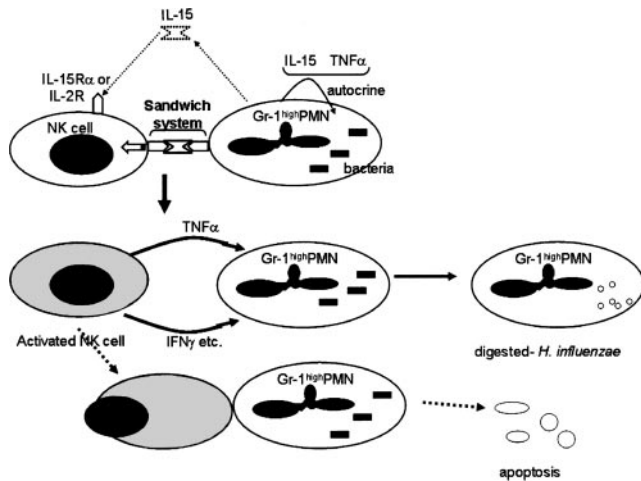
the decreased lung clearance of the bacteria in mice depleted of Gr-1 PMNs. Furthermore, these studies showed that IL-15 plays a dominant role in the control of this pneumonia, based on the high bacterial load in the lungs of IL-15 KO mice, and the clearance of these bacteria when the IL-15 deficit was rescued by C57BL/6 PECs. Because IL-15 is made by activated Gr-1<sup>high</sup> cells, it is likely that Gr-1<sup>high</sup> cells modulate intracellular bacterial killing via the production of IL-15, and perhaps other cytokines. A previous report described that IL-15 KO mice are susceptible to vaccinia virus (33). Taken together, these data indicate that PMNs among the PECs transplanted from C57BL/6 mice can produce IL-15, which enhances NK-induced killing activity in IL-15 KO mice and thereby host defenses against *H. influenzae*. These observations provide good evidence that PMNs, which produce IL-15, play an important role against intracellular pathogen by activating NK cells to enhance their killing activity.

We did not determine whether the C57/BL6 PECs used to rescue the *H. influenzae*-infected IL-15 KO mice acted by migration from the peritoneum to the lung. We do know that after i.p. administration of LPS, peritoneal Gr-1 PMNs migrate from the peritoneum to the mesenteric lymph nodes (23), which establishes the possibility of further migration of these cells to the inflamed lung via the lymphatic system. Until this question is studied directly, the mechanism by which peritoneal PMNs rescued the infected IL-15 KO mice is uncertain.

NK cells are essential players in host defenses against intracellular pathogens (8, 34), and were required for the in vitro killing of intracellular *H. influenzae* in our experiments. We believe that it is highly likely that NK cells play a similar role in vivo, in both the lung and peritoneum, but only experiments with *H. influenzae*-infected NK cell-deficient mice would answer this question definitively.

The relative role of NK cells and IL-15 in killing intracellular *H. influenzae* was determined by in vitro experiments (Fig. 4). Addition of IL-15 to PECs containing the bacteria had no effect on bacterial killing, whereas the addition of NK cells to the PECs resulted in substantial bacterial killing. Addition of IL-15 to the NK cells had a minor effect on the bacterial killing produced by the NK cells alone, significant only at 2 h postincubation, but not at the 5-h time point. These paradoxical findings are best explained by sufficient IL-15 production by the Gr-1<sup>high</sup> cells present in the PECs used to ingest the bacteria.

Previous studies reported the up-regulation of *IL-15* mRNA in mouse cells at 3 h postinfection (35, 36). Taken together, these findings indicate that PMNs have already started producing IL-15 when PMNs and native NK cells were mixed. In this regard, there are two isoforms of IL-15; one is the secretable isoform and the other is the nonsecretable one (36). The former isoform exists on the cell surface and exhibits various biological effects. The latter IL-15 is expressed in the nucleus and regulates IL-15 production by posttranscriptional control (37). Another report also described IL-15 binding to IL-15R $\alpha$  on the cell surface (38). In our study, *IL-15R $\alpha$*  mRNA was expressed in both Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs by microarray analysis. With regard to the receptor, we cannot exclude the possible involvement of the IL-2R in this process because *IL-2R* mRNA was detected by microarray analysis and previous reports described IL-15 binding to not only IL-15R $\alpha$  but also IL-2R (39). Moreover, the present study could not detect any secreted IL-15 by LPS-stimulated PECs by ELISA. The instructions provided by the manufacturer stipulated that the detection limit of this method is 100 pg/ml. It seems that most of the secreted IL-15 binds to IL-15R $\alpha$  (or IL-2R) on the surface of PMNs that phagocytose *H. influenzae* organisms, resulting in activation of native NK cells.



**FIGURE 6.** Schematic diagram of the “positive feedback loop” for the interaction of PMNs and NK cells. See text for more details.

The *in vitro* bacterial killing experiments did not directly determine whether isolated Gr-1<sup>high</sup> cells were the sole contributors to *H. influenzae* killing by NK cells, or whether both Gr-1<sup>high</sup> and Gr-1<sup>low</sup> PMNs are important for this effect. Unfortunately, we were unable to purify sufficient numbers of functional Gr-1 PMN subfractions to determine this directly, as the cells purified by flow cytometry were poorly functional. However, indirect evidence exists that NK-directed killing of intracellular *H. influenzae* is dependent on Gr-1<sup>high</sup>, but not Gr-1<sup>low</sup>, PMNs. This indirect evidence includes the fact that IL-15 is expressed almost exclusively by Gr-1<sup>high</sup>, but not Gr-1<sup>low</sup>, PMNs, and that NK cells were activated by Gr-1<sup>high</sup>, but not Gr-1<sup>low</sup>, PMNs. Taken together, these data provide strong support for the primary role of Gr-1<sup>high</sup>, but not Gr-1<sup>low</sup>, PMNs in the innate immune response against intracellular bacteria.

Our results demonstrate that intracellular *H. influenzae* is killed by interaction of IL-15-activated NK cells and PMNs (3, 25, 26). With regard to the mechanism involved in killing of intracellular *H. influenzae*, anti-IFN- $\gamma$  mAb only partially protected the organisms from the bactericidal activity of IFN- $\gamma$ , although IFN- $\gamma$  plays a significant role against intracellular pathogen infection. Previous work demonstrated that human PMNs stimulated by a combination of LPS, IL-12, and TNF- $\alpha$  secrete IFN- $\gamma$  (40). These results suggest that it is possible that IFN- $\gamma$  had already been produced and/or expressed before addition of anti-IFN- $\gamma$  mAb. It has been reported that the production of this cytokine from NK cells is enhanced by synergistic actions of IL-15 and IL-12 (39). The killing action of NK cells against intracellular pathogens is not mediated through only IFN- $\gamma$  but also other cytokines like TNF- $\alpha$  (41, 42). These results suggest that a cytokine(s) other than IFN- $\gamma$  is also intimately associated with killing of intracellular *H. influenzae* in PMNs.

The results of our *in vitro* and *in vivo* studies thus allow us to propose the following schema on the killing mechanism of Gr-1<sup>high</sup> PMNs (Fig. 6). IL-15 KO mice are susceptible to *H. influenzae* infection, although the wild-type C57BL/6 mice are resistant. Thus, IL-15 enhances PMN activity via the autocrine system and activates NK cells by binding to IL-15R $\alpha$  and/or IL-2R on the surface of NK cells and Gr-1<sup>high</sup> PMNs. As discussed above, IL-15 plays a pivotal role in the activation and survival of NK cells via the sandwich system because most IL-15 was sandwiched between Gr-1<sup>high</sup> PMNs and NK cells. Secretion of IL-15 by Gr-1<sup>high</sup> PMNs results in further activation of NK cells. Activated NK cells

also produce IFN- $\gamma$ , and other cytokines like TNF- $\alpha$  are also involved in killing intracellular pathogens. We propose the term “positive feedback loop” for this overall bactericidal system. Finally, PMNs, which form the first line of defense in the inflammatory response against invading pathogens, play the most important role in innate immunity against infections of both extracellular and intracellular pathogens.

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## Disclosures

The authors have no financial conflict of interest.

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