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Inefficient phagosome maturation in infant macrophages

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ABSTRACT

The quantitative and qualitative differences between the immune systems of infants and adults have been extensively investigated in the context of adaptive immunity. Here, we demonstrate that the infantile innate immune system is immature and weak against bacterial infections. Upon infection by *Escherichia coli*, macrophages from infantile mice showed a lower performance in killing the bacteria. In infant macrophages, bacteria were taken up relatively normally and delivered into lysosomal compartments, but not efficiently digested. The inefficient bacterial killing in infant macrophages was correlated with impaired acidification of the lysosomal compartments and reduced lysosomal recruitment of Rab7, an essential component of the acidification process. The acidification defect was not intrinsic to the cells, and was rescued by pretreatment with interferon-γ. Thus, we propose that the limited capacity of phagosome maturation is one of the major causes of the high sensitivity to infectious microorganisms during infancy and that the specific cytokine milieu shapes the nature of infantile innate immunity.

Neonates and infants generally show higher sensitivities to infectious diseases than adults, and are especially sensitive to infections by intracellular pathogens [1,2]. This phenomenon has been believed to be mostly due to the immature state of the adaptive immune system. Neonates are born with an adaptive immune system that is less efficient in the production of T helper type 1 (TH1)-cell associated cytokines, including interferon (IFN)-γ, thus favoring the provision of an overall cytokine milieu dominated by T helper type 2 (TH2) cytokines, which is inefficient in the elimination of intracellular pathogens. Accumulating evidence suggests that the weaknesses of the neonatal and infantile immune systems are not solely due to the intrinsic properties of T cells. Indeed, it has been shown that under some circumstances, neonatal T cells are competent in developing mature TH1 cell responses [1,2]. For example, when stimulated with agents that promote strong TH1 cell responses, such as DNA vaccines or oligonucleotides containing unmethylated CpG motifs, adult-like TH1 responses occur [3,4]. Thus, it is highly likely that non-T cells, i.e. innate immune cells, and/or the local environment are also immature and responsible for the vulnerability to intracellular pathogens during infancy.

Since Toll-like receptors (TLRs) are central to the innate immune responses against pathogens, functional studies of neonatal and infantile innate immunity have mainly been conducted in the context of TLR responses [5]. For example, newborn monocyte-derived dendritic cells exhibit impaired expression of interleukin (IL)-12 in response to TLR3 and TLR4 ligands [6]. Thus, the weak TLR-mediated responses could represent a reason why neonates and infants are susceptible to intracellular pathogens. However, it is obvious that the susceptibilities of neonates and infants to intracellular pathogens cannot be explained simply by these partial defects in TLR signaling.

Phagocytosis is another key element of innate immunity against invading pathogens. Phagosomes undergo gradual, yet profound, acidification during the course of maturation, and this acidification process is essential for limiting the replication of and, in many cases, the survival of internalized microorganisms [7–9]. However, the phagocytic capacity of infant immune cells is less well characterized than their TLR-mediated cytokine responses. In the present study, we analyzed phagosome maturation in resident peritoneal macrophages from 2-week-old infant mice during *Escherichia coli* infection and compared them with the responses in cells from 8-week-old adult mice. We demonstrate that phagosome maturation is severely impaired in infant

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macrophages, leading to inefficient killing of internalized bacteria in these macrophages.

Material and methods

Mice and isolation of peritoneal macrophages. 2- and 8-week-old Balb/c mice were obtained from CLEA Japan Inc. Resident peritoneal macrophages were obtained by peritoneal lavage. To examine the effects of IFN-γ, the macrophages were pretreated with 20 ng/ml IFN-γ (Genzyme) for 3 h before *E. coli* (DH5α) infection.

Escherichia coli killing assay. Isolated resident peritoneal macrophages were infected with *E. coli* at a ratio of 1:10 (macrophages: *E. coli*) at 37 °C for 30 min, and then treated with 10 μg/ml gentamicin for 1–3 h. Intracellular *E. coli* were counted by plating onto LB agar plates after solubilization of the infected macrophages in 0.01% Triton X-100 in sterile water.

Electron microscopy. Isolated resident peritoneal macrophages were infected with *E. coli* for 30 min. After washout of the *E. coli*, the cells were incubated for a further 1 h and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4 °C for 1 h. The cells were postfixed with 1% OsO_4 in the same buffer at 4 °C for 1 h, dehydrated in a graded series of ethanol and embedded in Quetol 812 (Nissin EM). Silver sections were cut with an ultramicrotome, stained with lead citrate and uranyl acetate, and observed with an H-7650 electron microscope (Hitachi).

Immunostaining. Macrophages were plated on 35 mm glass-bottom dishes (Matsunami Glass) and infected with *E. coli* labeled with 2 μM 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) for 30 min followed by a 1-h chase. The cells were then fixed with 4% PFA, permeabilized with 0.3% Triton X-100, and incubated with anti-Lamp1 (clone 1D4B; BD) and/or anti-Rab7 (Cell Signaling Technology) antibodies followed by Alexa Fluor 350- and/or Alexa Fluor 594-labeled secondary antibodies (Molecular Probes). The immunostained cells were analyzed using an IX71 fluorescence microscope (Olympus). The colocalization rates of *E. coli*-positive and Rab7- or Lamp1-positive vesicles were calculated automatically using the MetaMorph Image processing system.

Acridine orange and LysoTracker Red labeling. For acridine orange labeling, macrophages plated on glass-bottom dishes were incubated with unlabeled *E. coli* for 0.5–3 h, and 6 μM acridine orange (Invitrogen) was added for the last 15 min of incubation. The labeled cells were visualized using an IX71 fluorescence microscope (Olympus). The green emission (passed through a 530/30 nm band pass filter) and red emission (passed through a 610 nm long pass filter) were collected and the total integrated red fluorescence intensity within each cell was determined using the MetaMorph Image processing system. For LysoTracker Red labeling, macrophages were incubated with 1 μM LysoTracker Red (Invitrogen) for 3 h at 37 °C, and then infected with CFSE-labeled *E. coli*.

pH measurement. Macrophages were incubated with FITC-conjugated *E. coli* bioparticles (Invitrogen) for 30 min, washed and incubated for a further 3 h. The cells were analyzed under the IX71 fluorescence microscope using 488/360 nm excitation and 530/450 nm emission filters. To measure the lysosomal pH values at steady state, macrophages were incubated with FITC-labeled dextran (Sigma) for 3 h. To establish a pH calibration curve after recording, the cells were equilibrated with a calibration buffer (5 mM NaCl, 115 mM KCl, 1.2 mM $MgSO_4$, and 25 mM MES buffer titrated between pH 4.5 and 7.0) containing 20 μM monensin and 10 μM nigericin, and examined under the fluorescence microscope. The 530/450 nm ratio of phagosomal vesicles was calculated using the MetaMorph Image processing system.

Real-time RT-PCR. Real-time PCR was performed with an ABI 7300 real-time PCR system using Power SYBR Green PCR Master Mix (Applied Biosystems). The values were normalized by the amount of GAPDH in each sample. The following primer sets were used: guanylate-binding protein (GBP) 1, 5′-CCT GGA GAC AGG AAG CAA CTT T-3′ and 5′-AGG TCT GCA CCA GGC TTT TTA G-3′; GBP2, 5′-ATC AGC TCG TTG CTC AGA CTT G-3′ and 5′-GCT GCC TCT GTG AGT GAC TGA T-3′; GBP3, 5′-GGT GGT CAC CAT AGA GGA AAG G-3′ and 5′-TAG CCC AGC TCA ATC TTC TTC C-3′; IFN-induced protein with tetratricopeptide repeats 1 (Ifit1), 5′-CTT GCC AAA GCT ATG TCA TTC G-3′ and 5′-CTT GCC AAA GCT ATG TCA TTC G-3′.

Statistical analysis. Differences between control and experimental groups were evaluated using Student's *t*-test.

Results

Impaired killing of internalized bacteria in infant macrophages

First, we assessed the bacterial uptake abilities of infant innate immune cells. Isolated resident peritoneal macrophages from 2- and 8-week-old Balb/c mice (2w Mφ and 8w Mφ, respectively) were incubated with CFSE-labeled *E. coli* (DH5α) for 60 min at 37 °C, and their capacities to take up the bacteria were examined by flowcytometry. 2w Mφ showed almost comparable capacity to take up bacteria compared with 8w Mφ (Fig. 1A).

Next, we analyzed the killing of internalized bacteria in 2w Mφ and 8w Mφ using a bacterial survival assay [7]. Macrophages were allowed to ingest *E. coli* for 30 min at 37 °C, and then treated with gentamicin for the times indicated in Fig. 1B. The survival of *E. coli* engulfed by macrophages was analyzed by colony-forming unit (CFU) enumeration. The number of surviving bacteria was significantly higher in 2w Mφ than in 8w Mφ (Fig. 1B). Consistent with this result, transmission electron microscopy revealed that the internalized *E. coli* in 2w Mφ and 8w Mφ were morphologically different. Specifically, most of the internalized *E. coli* were undergoing degradation in 8w Mφ, whereas intact bacteria were often seen in 2w Mφ (Fig. 1C). These results demonstrate that infant macrophages are less efficient at killing internalized bacteria than adult macrophages.

Impaired phagosome maturation in infant macrophages

The different bactericidal capacities between 2w Mφ and 8w Mφ noted above might be due to differences in the intracellular delivery of the engulfed bacteria. To test this, macrophages were incubated with CFSE-labeled *E. coli* for 30 min followed by a 1 h chase, fixed and stained with an anti-Lamp1 antibody that stains lysosome-related organelles [10]. There was no significant difference between 2w Mφ and 8w Mφ in the localization of internalized *E. coli*, which were mainly localized within Lamp1-positive lysosomal compartments (Fig. 2A and B), suggesting that the lysosomal delivery of bacteria is not disturbed in infant macrophages.

The above results prompted us to examine phagosomal maturation in infant macrophages. To this end, we first used acridine orange (AO), a frequently used fluorescent probe to examine the acidification of organelles. When accumulated in acidic compartments, the emission of AO shifts from green to red [11]. Macrophages were incubated with *E. coli* for 0.5–3 h, and 6 μM AO was added for the last 15 min of incubation. The stained macrophages were then examined under a fluorescence microscope, and the green and red emissions were collected. In 8w Mφ, there was marked red fluorescence in discrete cytoplasmic organelles containing engulfed *E. coli* (Fig. 2C and D). In contrast, 2w Mφ contained fewer compartments exhibiting red fluorescence

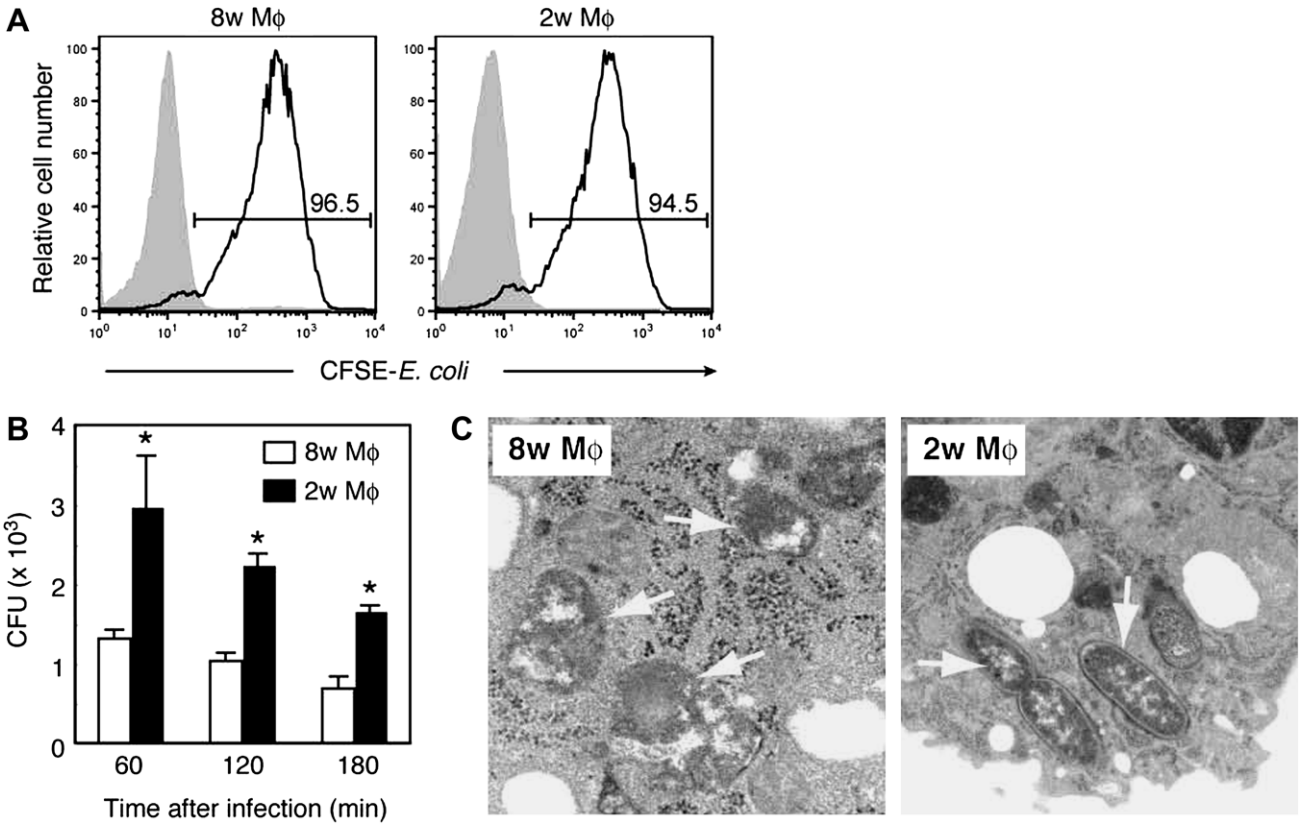

Fig. 1. Survival of internalized *E. coli* in infant macrophages. (A) 2w Mφ and 8w Mφ were incubated with or without CFSE-labeled *E. coli* for 1 h, fixed, stained with a PE-conjugated anti-CD11b antibody and analyzed by FACS. Representative histograms gated on $CD11b^+$ cells are shown. The solid lines and gray histograms represent cells incubated with and without *E. coli*, respectively. (B) Intracellular bacterial survival assays. After incubation of 2w Mφ and 8w Mφ with *E. coli*, the attached bacteria were killed by incubation with gentamicin for the indicated periods, and the macrophages were lysed. Live bacteria in the lysates were counted after inoculation on LB agar plates. $^*p < 0.01$ vs 8w Mφ. (C) 2w Mφ and 8w Mφ were infected with *E. coli* and analyzed by electron microscopy. Arrows indicate internalized *E. coli*. For all panels, the data are representative of at least three independent experiments with similar results.

(Fig. 2C and D), indicating weak acidity in the lysosomal compartments of infant macrophages. We also examined phagosomal acidification in infant macrophages using the acidotrophic fluorescent probe LysoTracker Red. Macrophages were incubated with 1 μM LysoTracker Red, infected with CFSE-labeled *E. coli* and examined under a fluorescence microscope for 3 h after the infection. The lysosomal compartments of 2w Mφ exhibited weaker fluorescent signals of LysoTracker Red than 8w Mφ both at steady state and during incubation with *E. coli* (Fig. 2E), further indicating that infant macrophages exhibit impaired phagosomal maturation.

Next, we measured the pH of phagosomes in 2w Mφ and 8w Mφ. For this purpose, macrophages were loaded with FITC-conjugated *E. coli* bioparticles. Since FITC fluorescence excited at 488 nm and emitted at 530 nm increases with pH, while fluorescence excited at 360 nm and emitted at 450 nm is nearly insensitive to pH, the pH can be measured by calculation of the 530/450 nm ratio of FITC fluorescence [12]. As shown in Fig. 2F, intracellular compartments containing *E. coli* bioparticles were less efficiently acidified in 2w Mφ than in 8w Mφ. Notably, the pH values of the endosomal/phagosomal/lysosomal compartments of 2w Mφ under steady-state conditions, which were measured using FITC-labeled dextran, were also higher than those in 8w Mφ, suggesting that the impaired phagosomal maturation in infant macrophages is not due to a simple defect in the response to *E. coli*-associated stimuli, but more likely arises from a defect in the machinery of phagosomal acidification.

Reduced recruitment of Rab7 to lysosomes in infant macrophages

It has been shown that lysosomal recruitment of Rab7, a small GTPase, is an essential step for complete acidification of lysosomes [10,13]. Therefore, we examined the subcellular localization of Rab7 in 2w Mφ and 8w Mφ at 1 h after *E. coli* infection by immunocytochemical analysis. In 8w Mφ, about 50% of the vesicles containing internalized CFSE-labeled *E. coli* were Rab7-positive (Fig. 3A and B). In contrast, although CFSE-labeled *E. coli* were frequently observed in Lamp1-positive compartments in 2w Mφ (Fig. 2A and B), the overlaps between CFSE-labeled *E. coli* and Rab7 were less frequent in 2w Mφ (<20%) than in 8w Mφ (Fig. 3A and B). These results suggest that reduced recruitment of Rab7 to lysosomal compartments may account for the impairment of phagosomal acidification in infantile macrophages.

Effects of IFN-γ on phagosomal maturation and killing of E. coli in infant macrophages

The impairment of phagosomal maturation observed in 2w Mφ is quite similar to that reported for macrophages from mice defective in IFN-γ signaling pathways [14]. In addition, it has been shown that the infant immune system is less efficient in the production of TH1-cell associated cytokines, including IFN-γ [1,2]. Indeed, IFN-γ-inducible genes, such as GBP1, GBP2, GBP3, and Ifit1, were expressed at lower levels in 2w Mφ than in 8w Mφ (Fig. 4A). To test whether addition of IFN-γ could rescue the above-described defects in 2w Mφ, we

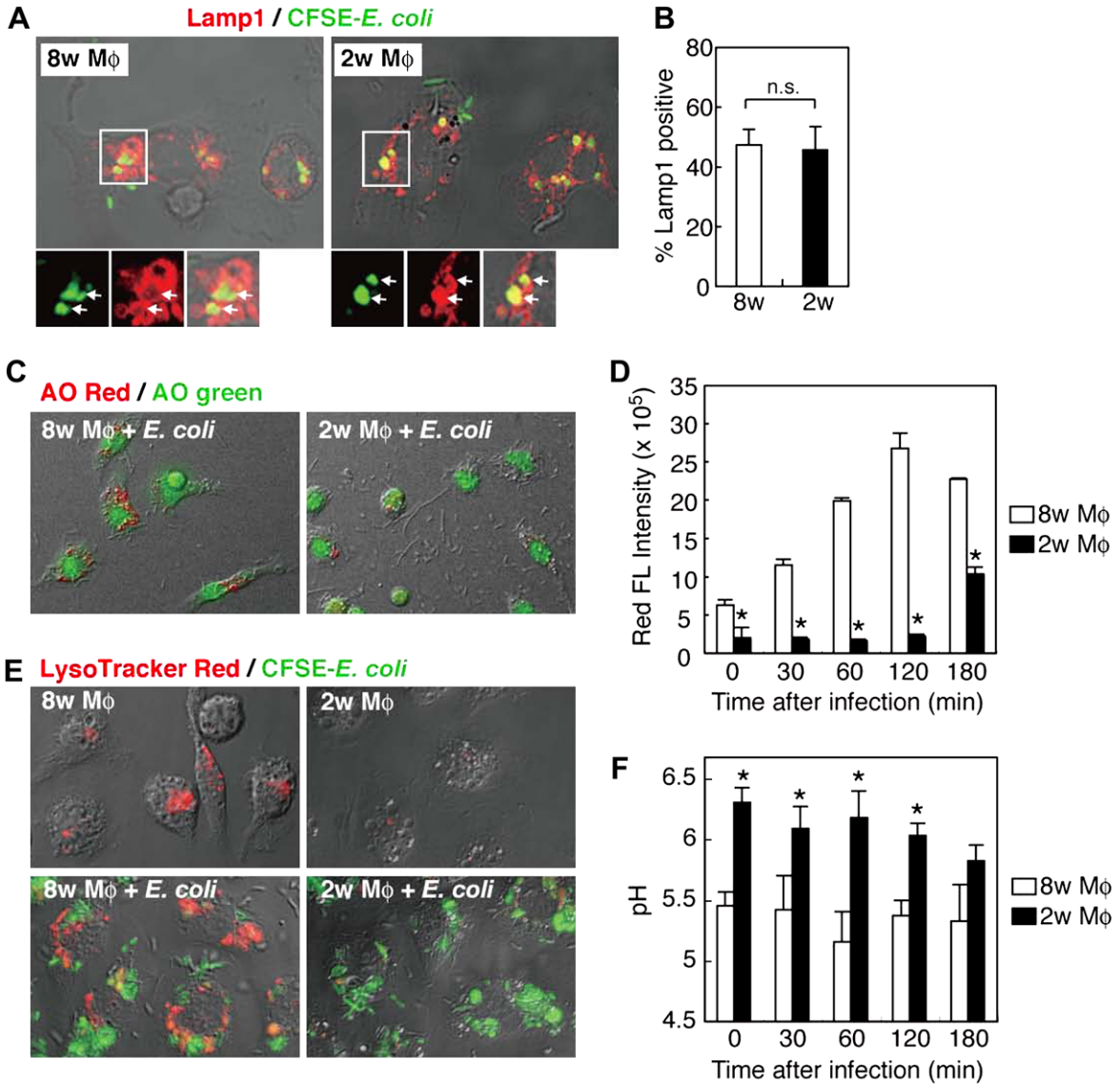

Fig. 2. Phagosome maturation in infant macrophages. (A,b) 2w Mφ and 8w Mφ were infected with CFSE-labeled *E. coli* and stained with an anti-Lamp1 antibody. Representative images are shown in (A), and the percentages (means + SD; $n > 100$) of Lamp1-positive *E. coli*-containing vesicles are shown in (B). (C,D) 2w Mφ and 8w Mφ were incubated with *E. coli* for the indicated times, and acridine orange (AO) was added for the last 15 min of incubation. The stained cells were then examined under a fluorescence microscope, and the green and red emissions were collected. Representative images of cells incubated with *E. coli* for 1 h are shown in (C). The total integrated fluorescence intensity for red emission of acridine orange within each cell was determined and is shown as the mean + SD ($n > 50$) in (D). (E) 2w Mφ and 8w Mφ were incubated with LysoTracker Red, and then infected with CFSE-labeled *E. coli*. Representative images of cells at 0 h (upper panels) and 2 h (lower panels) after the infection are shown. (F) 2w Mφ and 8w Mφ were loaded with FITC-conjugated *E. coli* bioparticles, and their phagosome pHs were determined. For all panels, data are representative of at least three independent experiments with similar results. $^{*}p < 0.01$ vs 8w Mφ. n.s., not significant.

pretreated 2w Mφ with IFN-γ for 3 h followed by infection with *E. coli*. IFN-γ pretreatment restored the reduced recruitment of Rab7 to lysosomal compartments (Fig. 3B and C) and the impairment of phagosomal acidification in 2w Mφ (Fig. 4B). Furthermore, the efficiency of internalized *E. coli* killing was improved by IFN-γ pretreatment, and 2w Mφ exhibited comparable bactericidal capacity to 8w Mφ (Fig. 4C). Taken together, these results indicate that the defects in 2w Mφ are not intrinsic to the cells but presumably due to the infancy-specific cytokine milieu.

Discussion

The results of the present study illustrate a previously ill-defined aspect of infantile immunity, namely a defect in phagosome maturation. Infant macrophages, a representative of innate immune cells, have a limited capacity for phagosome maturation and therefore have a lower activity to kill internalized bacteria. Since IFN-γ pretreatment rescued these defects, the local and systemic cytokine milieu specific to the infantile period probably leads to the weak bactericidal character of infant phagocytes.

We observed that internalized *E. coli* were delivered into Lamp1-positive compartments in infant macrophages, but Rab7 was not recruited to these vesicles. Therefore, the maturation defect in infant macrophages is likely to be due, at least in part, to inadequate recruitment of Rab7 to lysosomal compartments. Recently, the following series of successive fusion events for phagosome maturation has been proposed: (1) phagosome maturation begins with fusion to early endosomes that coincides with acquisition of Rab5; (2) subsequent interactions with late endosomes containing Lamp1 and Lamp2 occur; (3) the Lamps then mediate fusion with traditional late endosomes carrying Rab7; and (4)

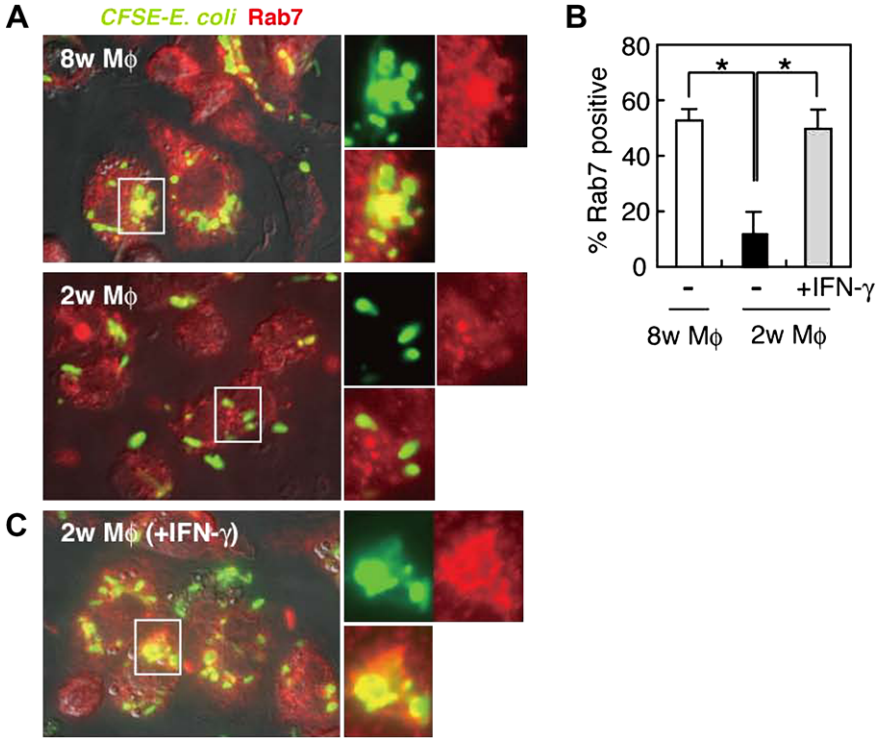

Fig. 3. Recruitment of Rab7 to phagosomes. (A–C) 2w Mφ and 8w Mφ were preincubated with or without IFN-γ, and then infected with CFSE-labeled *E. coli*. The cells were then stained with an anti-Rab7 antibody and analyzed using a confocal microscope. Representative images without IFN-γ pretreatment (A) and with IFN-γ pretreatment (C) are shown. The percentages of *E. coli*-containing vesicles positive for Rab7 are shown as means + SD ($n > 150$) in (B). $^{*}p < 0.01$. Data are representative of at least three independent experiments with similar results.

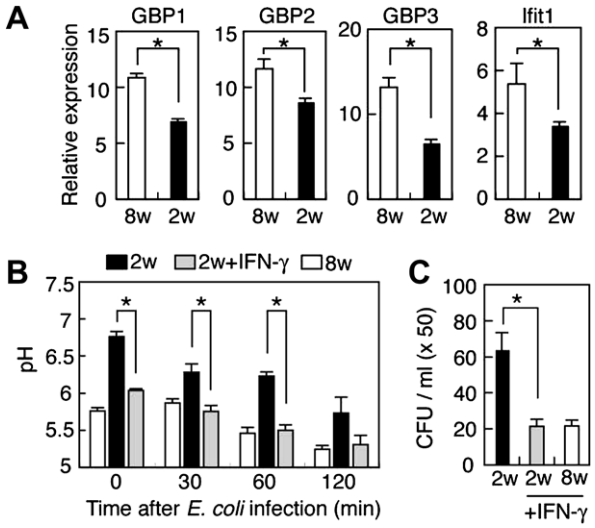

Fig. 4. Effects of IFN-γ on phagosome maturation and killing of *E. coli* in infant macrophages. (A) Real-time RT-PCR analyses of GBP1, GBP2, GBP3, and Ifit1 mRNA levels in 2w Mφ and 8w Mφ under steady-state conditions. (B) After pretreatment with IFN-γ, 2w Mφ were loaded with FITC-conjugated *E. coli* bioparticles for 30 min, chased for the indicated periods and analyzed for their phagosome pHs. (C) 2w Mφ and 8w Mφ were preincubated with or without IFN-γ, and then subjected to intracellular bacterial survival assays as described in the legend for Fig. 1B. For all panels, the data are representative of at least three independent experiments with similar results. $^{*}p < 0.01$.

maturation culminates with phagosome–lysosome fusion, forming a microbicidal organelle [10]. Our present data lend support to this model, since Lamp1 and Rab7 can be localized in separate vesicles, and Rab7 recruitment probably occurs after Lamp1 recruitment and is a critical step to create a fully acidified phagosome. Recent studies have shown that cytokines can modulate phagosome maturation. For example, TNFα, IL-6, IL-12, and IFN-γ have been shown to modify phagosome maturation [14,15]. In particular, it has been shown that treatment of macrophages with IFN-γ increases the cellular content of Rab5 [16]. Our results indicate that IFN-γ also regulates Rab7. However, in this case, IFN-γ does not enhance the expression of Rab7 (data not shown), but rather regulates trafficking of Rab7-positive vesicles to phagosomal compartments, thereby accomplishing their maturation. Although the mechanism underlying IFN-γ-mediated recruitment of Rab7 to lysosomes remains unclear, IFN-inducible molecules, such as dynamin-like GBP family members, may regulate the intracellular trafficking of Rab7-positive vesicles. This aspect needs further investigations.

The innate immune system is mediated by germline-encoded pattern recognition receptors, and has thus been considered to be fully active even during infancy. However, recent accumulating evidence and the present study suggest that the innate immune system in infants is immature and different from that in adults. The question then arises as to why the infant innate immune system is immature. One possibility is that the infantile immune system lacks the IL-12-IFN-γ-positive feedback circuit. IL-12 signaling strongly activates IFN-γ production, and IFN-γ in turn positively modulates the IL-12 production machinery [6,17]. Although it remains unknown which cytokine production is primarily defective in infant cells, it is possible that this feedback circuit only becomes operational after interaction of the immune system with environmental factors, such as symbiotic bacteria or other factors. Another possibility is that the immature adaptive immune system drives the immature innate immune system. Indeed, there is a report that infection with herpes viruses, which first occurs during childhood and latently persists for the life of the host, maintains prolonged production of IFN-γ by adaptive

immune cells, resulting in systemic activation of macrophages and a basal activation state of innate immunity against subsequent bacterial infections [18]. Thus, the innate and adaptive immune systems seem to be mutually dependent on each other and become mature in a concerted fashion. Although further studies are required to clarify how the innate and adaptive immune systems become educated, our present study provides information about a previously unidentified and important character of the infant immune system against invading microorganisms.

Acknowledgments

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