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Inefficient phagosome maturation in infant macrophages

Fumiji Saito [a,d], Hirotaka Kuwata [d], Eiji Oiki [b], Masato Koike [c], Yasuo Uchiyama [c], Kenya Honda [a,e,*], Kiyoshi Takeda [a,d,e]

[a] Laboratory of Immune Regulation, Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Center for Medical Research and Education, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan
[c] Department of Cell Biology and Neuroscience, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan
[d] Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan
[e] WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan

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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

* Corresponding author. Address: Laboratory of Immune Regulation, Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 3982.
E-mail address: honda@ongene.med.osaka-u.ac.jp (K. Honda).

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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