Salmonella infection was made by positive stool culture and, in 5 cases, in combination with blood culture. Eleven patients were found to be infected with *Salmonella enterica* serovar Enteritidis, three with *S. enterica* serovar Typhimurium, three with *S. enterica* serovar Typhi, two with *S. enterica* serovar Paratyphi B, one with *S. enterica* serovar Thompson, one with *S. enterica* serovar Montevideo, one with *S. enterica* serovar Haifa, one with *S. enterica* serovar Infantis and five with other types (three with *Salmonella* O9, one with O4 and one with O7). All patients were immunocompetent and were successfully treated with antibiotics. All blood samples were obtained during the acute phase of *Salmonella* infection (median, 9th day of illness; range 4th–19th days).

Twenty-one patients with acute bacterial enterocolitis other than *Salmonella* infection (12 males and 9 females; median age, 10 years; range, 1.8–29 years) served as disease controls (*Campylobacter jejuni*, 14; pathogenic *Escherichia coli*, 6; *Shigella flexneri*, 1). All blood samples were obtained during the acute phase (median, 7th day of illness; range, 4th–12th day). Forty-four healthy subjects (24 males and 20 females; median age, 7.8 years; range, 1.0–38 years) served as normal controls. Informed consent was obtained from the subjects or their parents before the study. This study was approved by the Ethics Committee of Kyushu University.

2.2. Detection of cell-surface determinants by flow cytometry

Ethylenediaminetetraacetic acid blood samples were collected from both patients and controls. Fluorescein isothiocyannate (FITC)-conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-CD161 (NKRP1A), and phycoerythrin-cyanin 5.1 (PC5)-conjugated anti-TCRαβ and anti-TCRγδ antibodies (Beckman Coulter, Miami, FL, USA) were used as fluorochrome-conjugated monoclonal antibodies against the surface determinants. A three-color flow cytometric analysis was performed by using an EPICS XL instrument (Beckman Coulter). The analysis gate was set within the lymphocyte by using forward and side scatters as previously described [15]. Specificity of staining was assessed using fluorochrome-conjugated isotype-matched monoclonal antibodies. Each analysis was performed using at least 20,000 cells. Further analysis was conducted using FlowJo (version 7.6.5; Tree Star Inc.).

2.3. Intracellular cytokine detection by flow cytometory

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized peripheral blood samples via density-gradient centrifugation using LSM (Cappel-ICN Immunobiologicals, Costa Mesa, CA, USA) and were stimulated for 4 h at 37 °C and under 5% CO_2, with 25 ng/ml phorbol 12-myristate acetate (PMA; Sigma Chemical, St. Louis, MO, USA) plus 1 μg/ml ionomycin (Sigma Chemical) and 10 μg/ml brefeldin A in the culture medium (RPMI-1640 plus 10% fetal calf serum) containing gentamycin. After stimulation, 0.1 ml of the sample was stained with FITC-conjugated anti-CD161 (Medical & Biological laboratories, Nagoya, Japan) and PC5-conjugated anti-TCRαβ antibodies for IL-4 detection and PE-conjugated anti-CD161 and anti-TCRγδ antibodies for IFNγ detection, respectively. Erythrocytes were lysed for 10 min with 2 ml of 1X FACS lysing solution (Beckton Dickinson, San Jose, CA, USA). After washing, cells were treated with 500 μl of 1× FACS permeabilizing solution (Beckton Dickinson) for 10 min and washed again with PBS. Cells were incubated for 30 min with FITC-conjugated anti-IFNγ or PE-conjugated anti-IL-4 antibodies (Beckton Dickinson). Cells were washed and resuspended in PBS and analyzed by flow cytometry (EPICS XL, Beckman Coulter). Flow cytometric analysis was conducted by gating on lymphocytes and αβ T cells or γδ T cells. Specificity of staining was assessed using fluorochrome-matched isotype antibodies. Each analysis was performed using at least 20,000 cells. Further analysis was conducted using FlowJo (version 7.6.5; Tree Star Inc.).

2.4. Statistical analysis

The Kruskal-Wallis test followed by the Mann-Whitney U test with the Bonferroni correction was performed to analyze the differences in the proportion of αβ T cells or γδ T cells among patients with *Salmonella* infection, other bacterial infections and normal controls. The P values <0.017 (0.05/3) were considered to be statistically significant.

3. Result

The clinical features and laboratory data for the patients with *Salmonella* infections are shown in Table 1. Twenty-five patients had diarrhea, and 27 patients developed a fever over 38°C. Three patients had a fever without diarrhea. Two of them were infected with *S. enterica* serovar Typhi, and one with *S. enterica* serovar Thompson.

The number of peripheral γδ T cells in the patients with *Salmonella* infection (*Salmonella* group: median, 201 μl) was significantly higher than those in other two groups (disease controls: median, 104 μl, $P = 0.001$; normal controls: median, 133 μl, $P = 0.007$, Fig. 1A). On the other hand, no significant differences in the number of peripheral αβ T cells were seen among the three groups (*Salmonella* group: median, 1699 μl; disease controls: median, 1247 μl; normal controls: median, 1853 μl, Fig. 1B).

The proportion of $NKRP1A^+$ cells in the γδ T cell population (Fig. 2A) was significantly higher in the *Salmonella* group (median, 73.0%) than in the disease controls (median, 61.2%, $P = 0.008$) and normal controls (median, 57.3%, $P = 0.0004$) (Fig. 2B). The proportion of $NKRP1A^+$ γδ T cells producing IFNγ after stimulation with PMA plus ionomysin (Fig. 3A) was also significantly higher in the *Salmonella* group ($n = 10$, median, 58.3%) than in the disease controls ($n = 8$, median, 31.3%, P=0.003) and normal controls ($n = 10$, median, 20.1%, $P = 0.0008$) (Fig. 3B). On the other hand, no significant differences were seen in the proportion of $NKRP1A^-$ γδ T cells producing IFNγ after stimulation among the three groups (Salmonella group: median, 38.9%; disease controls: median, 21.1%; normal controls: median, 18.7%, Fig. 3C, D). Neither the proportion of IL-4-producing $NKRP1A^+$ nor $NKRP1A^-$ γδ T cells after stimulation was significantly different among the three groups (data not shown).

Within the αβ T cell population, the proportion of $NKRP1A^+$ cells (Fig. 4A) was significantly higher in the *Salmonella* group (median, 17.1 %) than in normal controls (median, 9.5%, $P < 0.0001$) but the difference between the *Salmonella* group and disease controls (median, 13.1%) was not significant (Fig. 4B). The proportion of $NKRP1A^+$ αβ T cells producing IL-4 (Fig. 5A) was significantly higher in the *Salmonella* group ($n = 10$, median, 11.7%) than in both the disease controls ($n = 8$, median, 4.6%, $P = 0.01$) and the normal controls ($n = 10$, median, 2.7%, $P = 0.001$) (Fig. 5B). Although the proportion of $NKRP1A^+$ cells in the αβ T cell population in the disease controls was also significantly higher than that in normal controls ($P = 0.0008$), the proportion of $NKRP1A^+$ αβ T cells producing IL-4 were not significantly different between the two groups. No significant differences were seen in the proportion of $NKRP1A^-$ αβ T cells producing IL-4 after stimulation among three groups (*Salmonella* group: median, 0.67%; disease controls: median, 0.99%; normal controls: median, 0.75%, Fig. 5C, D). Neither the proportion of IFNγ-producing $NKRP1A^+$ nor $NKRP1A^-$ αβ T cells after stimulation was significantly different among the three groups (data not shown).

Table 1
Clinical features and laboratory data of 28 patients with *Salmonella* infection.

Patient no.	Age (month)	Sex	Form of infection	Serotype	Fever	Diarrhea	WBC (/μl)	CRP (mg/L)
1	45	F	S	*S.* Typhi	(+)	(+)	6000	329.5
2	126	M	S	*S.* Typhi	(+)	(−)	2990	42
3	188	M	S	*Salmonella* O9	(+)	(+)	3800	284.2
4	283	F	S	*S.* Thompson	(+)	(−)	5800	259.8
5	398	F	S	*S.* Typhi	(+)	(−)	3400	50.8
6	12	F	G	*S.* Enteritidis	(+)	(+)	17300	44.6
7	13	M	G	*S.* Typhimurium	(+)	(+)	13800	126.4
8	21	F	G	*S.* Infantis	(+)	(+)	5910	77.6
9	35	M	G	*S.* Enteritidis	(+)	(+)	11310	119.1
10	35	M	G	*Salmonella* O7	(+)	(+)	10880	32.7
11	44	F	G	*S.* Haifa	(+)	(+)	7500	56.8
12	48	M	G	*S.* Enteritidis	(+)	(+)	6700	9.3
13	49	M	G	*S.* Enteritidis	(+)	(+)	3840	19.9
14	53	F	G	*S.* Paratyphi B	(+)	(+)	5950	60.8
15	64	F	G	*Salmonella* O9	(+)	(+)	5900	29
16	67	M	G	*S.* Enteritidis	(+)	(+)	3980	13.2
17	71	M	G	*Salmonella* O4	(+)	(+)	6850	41.9
18	74	M	G	*S.* Enteritidis	(+)	(+)	11420	21.6
19	78	F	G	*Salmonella* O9	(+)	(+)	10600	115.3
20	80	F	G	*S.* Typhimurium	(+)	(+)	6300	130.3
21	101	M	G	*S.* Enteritidis	(+)	(+)	6120	81.6
22	102	F	G	*S.* Typhimurium	(−)	(+)	6200	0.6
23	110	M	G	*S.* Enteritidis	(+)	(+)	7010	24
24	118	M	G	*S.* Montevideo	(+)	(+)	7720	35
25	132	F	G	*S.* Enteritidis	(+)	(+)	9520	270.6
26	138	F	G	*S.* Enteritidis	(+)	(+)	5300	6.7
27	153	F	G	*S.* Enteritidis	(+)	(+)	6660	54.5
28	182	M	G	*S.* Paratyphi B	(+)	(+)	3400	131.5

S: systemic infection, G: gastroenteritis. Systemic infection was defined that *Salmonella* species was isolated from blood culture.

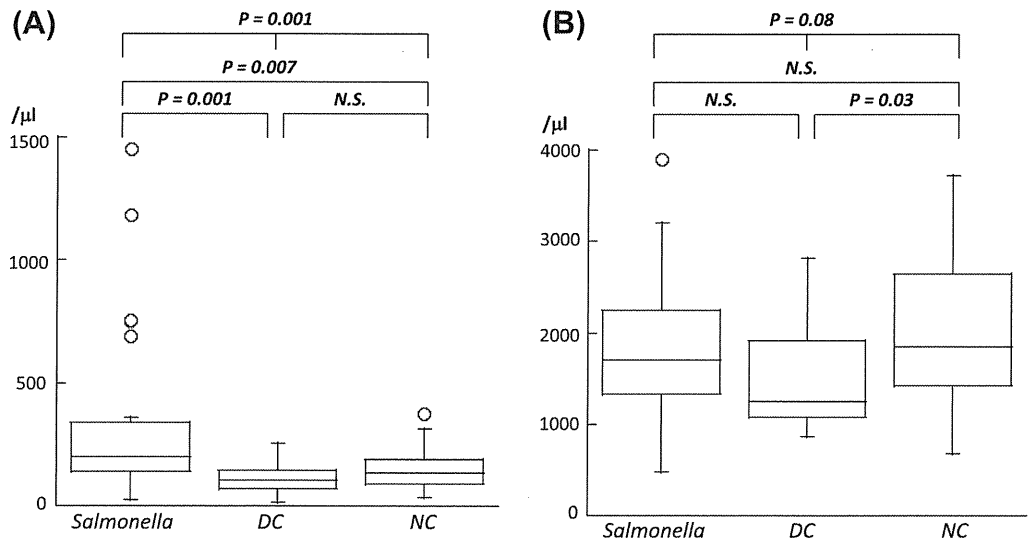

Fig. 1. The number of peripheral γδ T cells. (A) and αβ T cells (B) gated on lymphocytes in the patients with *Salmonella* infection, disease controls and normal controls by flow cytometic analysis. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents median, and whiskers indicate the values of the 10th and 90th percentiles, and open circles represent the outlier values beyond the 10th and 90th percentiles. DC: disease controls, NC: normal controls, NS: not significant.

4. Discussion

The present study showed that the proportion of NKRP1A$^+$ γδ T cells producing IFNγ was significantly higher in the patients with acute phase *Salmonella* infection than those of the patients with acute bacterial enterocolitis other than *Salmonella* infection and compared to healthy normal controls. In addition, the proportion of NKRP1A$^+$ αβ T cells producing IL-4 was significantly increased in the patients with an acute phase *Salmonella* infection. These data suggested that NKRP1A$^+$ T cells might play an important role during the early phase of *Salmonella* infection. To the best of our knowledge, the present study is the first to show that the proportion of NKRP1A$^+$ T cells was increased in human patients with *Salmonella* infection.

IFNγ plays an essential role in controlling bacterial replication in the course of a *Salmonella* infection [2,16]. In mice, neutralization of IFNγ with antibodies, or IFNγ knock-out results in increased bacterial numbers in the spleen and liver and decreases the survival of the host [17,18], whereas IFNγ treatment of infected mice results in the opposite outcome [19]. In humans, deficiencies of the IL-12/IL-23/IFNγ axis are associated with increased risks of recurrent *Salmonella* infection [20]. The main producers of IFNγ during

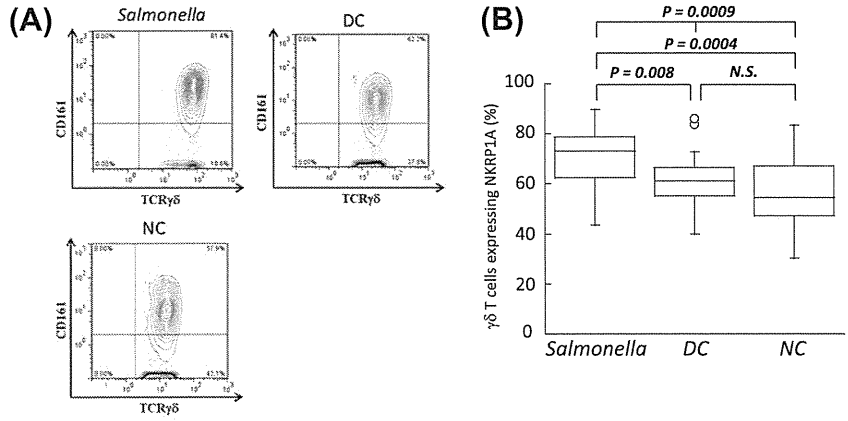

Fig. 2. Flow cytmetric analysis of NKRP1A$^+$ γδ T cell population. (A) A representative dot plot of CD161 (NKRP1A)$^+$ γδ$^+$ cells gated on CD3$^+$ lymphocytes in the patients with *Salmonella* infection, disease controls and normal controls. The y-axis of each plot represents specific fluorescence of CD161-PE; the x-axis represents specific fluorescence of TCR-γδ-PC5. (B) The proportions of γδ T cells expressing NKRP1A in the patients with *Salmonella* infection, disease controls and normal controls. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents median, and whiskers indicate the values of the 10th and 90th percentiles, and open circles represent the outlier values beyond the 10th and 90th percentiles. DC: disease controls, NC: normal controls, NS: not significant.

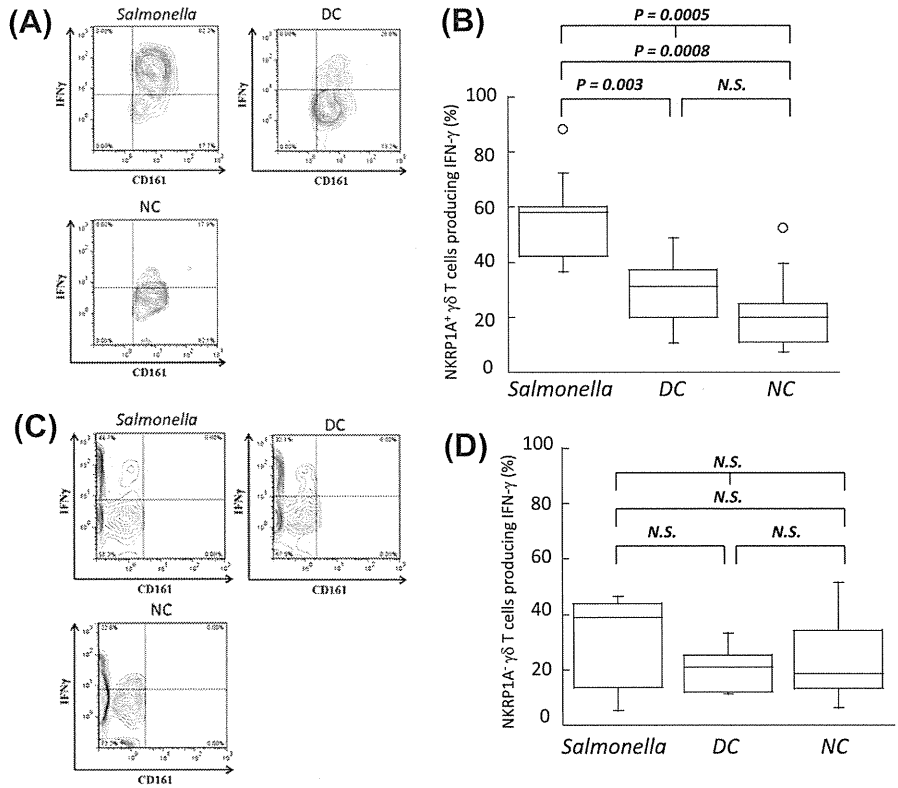

Fig. 3. Flow cytometric analysis of intracellular IFN-γ production of γδ T cells. (A and C) A representative dot plot of intracellular IFN-γ staining in CD161 (NKRP1A)$^+$ cells (A) and CD161$^-$ cells (C) gated on γδ T cells in the patients with *Salmonella* infection, disease controls and normal controls. The y-axis of each plot represents specific fluorescence of IFNγ-FITC; the x-axis represents specific fluorescence of CD161-PE. (B and D) The proportions of NKRP1A$^+$ γδ T cells (B) and NKRP1A$^-$ γδ T cells (D) producing IFN-γ in the patients with *Salmonella* infection, disease controls and normal controls. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents median, and whiskers indicate the values of the 10th and 90th percentiles, and open circles represent the outlier values beyond the 10th and 90th percentiles. DC: disease controls, NC: normal controls, NS: not significant.

the early phase of primary *Salmonella* infections appear to be macrophages, neutrophils, NK cells and NKT cells [1–3,21,22].

In our previous report, we found that γδ T cells were preferentially activated and expanded during a *Salmonella* infection, and this cell population expressed significantly higher level of IFNγ mRNA in patients during the acute phase of *Salmonella* infection than in healthy controls, suggesting that IFNγ -producing γδ T cells contribute to the early protection against *Salmonella* infection [23,24].

Recently, Pozo et al reported that NKRP1A crosslinking led to an enhanced IFNγ production through activation of acid sphingomyelinase and the resultant ceramide production [25]. In the present study, the proportion of NKRP1A$^+$ γδ T cells producing IFNγ was increased during the acute phase of *Salmonella* infection. It is possible that NKRP1A$^+$ γδ T cells might play an important role in the early defense against *Salmonella* infection as a main producer of IFNγ among the γδ T cells.

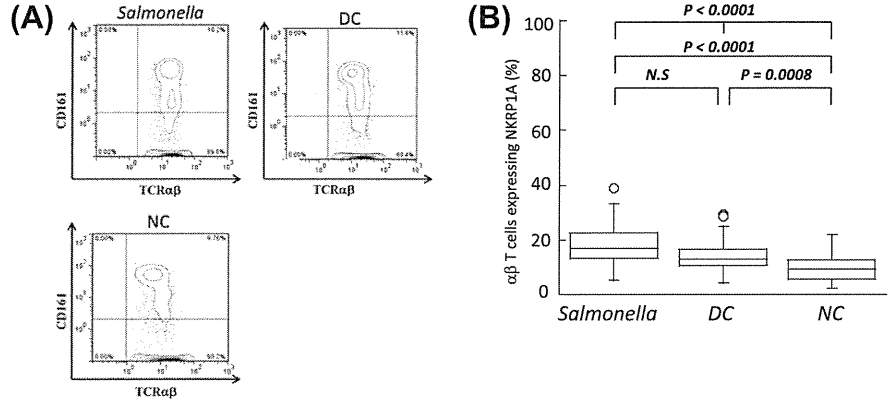

Fig. 4. Flow cytometric analysis of NKRP1A$^+$ αβ T cell population. (A) A representative dot plot of CD161 (NKRP1A)$^+$ αβ$^+$ cells gated on CD3$^+$ lymphocytes in the patients with *Salmonella* infection, disease controls and normal controls. The y-axis of each plot represents specific fluorescence of CD161-PE; the x-axis represents specific fluorescence of TCR-αβ-PC5. (B) The proportions of αβ T cells expressing NKRP1A in the patients with *Salmonella* infection, disease controls and normal controls. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents median, and whiskers indicate the values of the 10th and 90th percentiles, and open circles represent the outlier values beyond the 10th and 90th percentiles. DC: disease controls, NC: normal controls, NS: not significant.

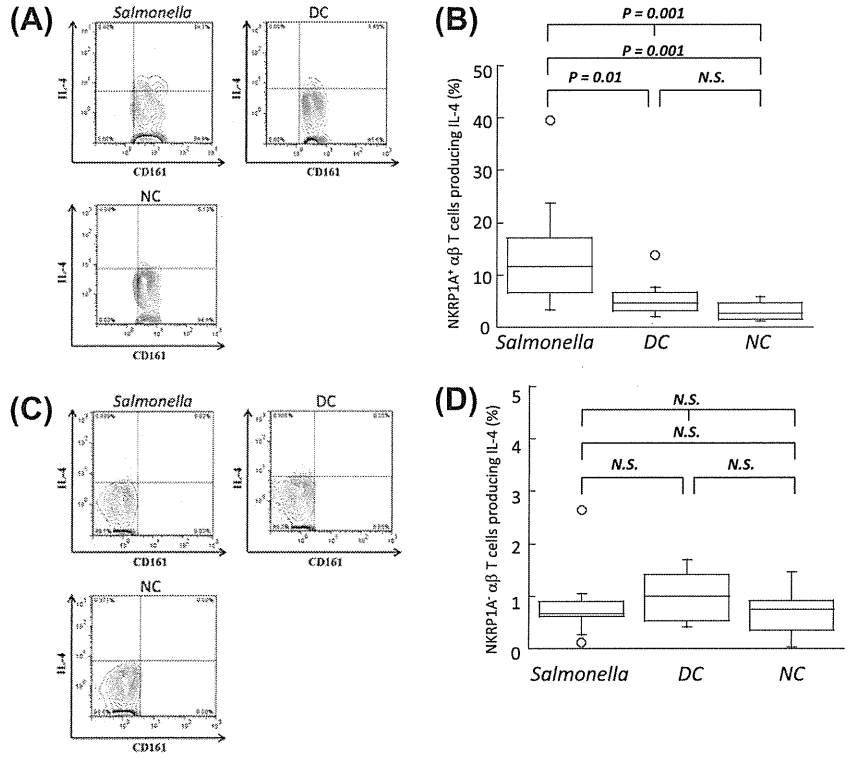

Fig. 5. Flow cytometric analysis of intracellular IL-4 production of αβ T cells. (A and C) A representative dot plot of intracellular IL-4 staining in CD161 (NKRP1A)$^+$ and CD161$^-$ cells (C) gated on αβ T cells in the patients with *Salmonella* infection, disease controls and normal controls. The y-axis of each plot represents specific fluorescence of IL-4-PE; the x-axis represents specific fluorescence of CD161-FITC. (B and D) The proportions of NKRP1A$^+$ αβ T cells (B) and NKRP1A$^-$ αβ T cells (D) producing IL-4 in the patients with *Salmonella* infection, disease controls and normal controls. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents median, and whiskers indicate the values of the 10th and 90th percentiles, and open circles represent the outlier values beyond the 10th and 90th percentiles. DC: disease controls, NC: normal controls, NS: not significant.

Similarly, Naiki et al. reported that NK1.1$^+$ αβ T cells were the main source of IL-4 production during the early phase of *Salmonella* infection in mice, and suggested that this cell population had an inhibitory function on the IL-12 production by macrophages and regulated the excessive inflammatory response [14]. As reduced IL-12-mediated signaling results in low IFNγ production, it was speculated that NKRP1A$^+$ αβ T cells and NKRP1A$^+$ γδ T cells, proportions of which were both increased during the acute phase of human *Salmonella* infection, might play a different role in supplementing IFNγ production.

In the present study, the proportion of NKRP1A$^+$ αβ T cells was also increased in the patients with acute bacterial enterocolitis other than *Salmonella* infection while the proportion of these cells producing IL-4 was not significantly increased, compared with those of healthy subjects. The NKRP1A molecule is also expressed on T-helper 17 cells which have been demonstrated to be responsible for the development of autoimmune diseases and allergic diseases [26]. Recent studies suggest that NKRP1A$^+$ αβ T cells can contribute to the mucosal response to pathogens by secreting a subset of cytokines such as IL-17 and IL-22

[27,28]. The increase in the proportion of NKRP1A$^+$ αβ T cells, which was commonly observed in *Salmonella* and non-*Salmonella* enterocolitis, may indicate that these cells play an important role in the gut mucosal barrier function.

In conclusion, the proportion of NKRP1A$^+$ T cells was elevated in the patients during the acute phase of a *Salmonella* infection, and it was suggested that this cell population might play an important role in the early defense against a *Salmonella* infection. Further investigations will be needed to elucidate the role and function of NKRP1A$^+$ T cells in humans during *Salmonella* and other infections.

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血液免疫系細胞分化障害による疾患の診断と治療に関する調査研究

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