Characterization and functional properties of gastric tissue-resident memory T cells from children, adults, and the elderly

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INTRODUCTION

In human, peripheral blood memory T (Tm) cells are commonly grouped into two major subsets based on their functional status and expression of defined homing receptors (e.g., CD62L, CCR7, and CD45RA) (1): central memory T (Tcm) and effector memory T (Tem) cells. While Tcm cells express the lymph node-targeting molecules CD62L and CCR7, Tem cells largely lack these receptors, and typically express defined homing molecules that endows them with the ability to migrate to peripheral non-lymphoid tissues (1, 2). Recently, a novel population of T cells known as tissue-resident memory CD8+ T (Trm) cells has been described. These Trm cells have the ability to remain for long periods of time in peripheral tissues (e.g., intestinal and vaginal mucosa, skin, brain, and salivary glands) after pathogenic clearance. These cells have been shown to be antigen-specific, express markers of CD8+ Tem cells, and their survival appears to be antigen-independent (3–6). Studies in epithelial and neuronal tissues have shown that Trm are characterized by their expression of high levels of CD103 (the α-chain of the integrin αEβ7) and CD69 (a surface molecule typically found on recently activated T cells) (7, 8). While initially described in mice, these cells have been recently also identified in human tissues (9).

The stomach’s primary function is to digest food. With its low pH environment, the stomach has a secondary function in limiting the number of microorganisms that enter the intestinal tract. However, some microorganisms such as Helicobacter pylori (H. pylori) can cause significant pathogenesis and have a niche in this harsh environment. Various immune cells have been identified in stomach biopsies obtained during esophagogastroduodenoscopy (EGD) procedures. Immune populations described in gastric lamina propria mononuclear cells (LPMC) include γδT cells (10), CD13+ macrophages (Mφ) (M1 and M2) (11), dendritic cells (DC) (12), natural killer (NK) (13), NK-T (14), neutrophils, B cells, and varying levels of CD8+ T cells, and the elderly

Keywords: LPMC, stomach, gastric tissue-resident/memory T cells, multifunctionality

References:

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Volunteers were recruited from the Baltimore–Washington area and University of Maryland Medical Center. The indications for EGD included abdominal pain, heartburn, GERD, dysphagia, and acute gastritis. Diagnostic pathology reports showed that the stomach’s antrum mucosa was either normal (n = 12) or exhibited mild inflammation (n = 45). No concurrent GI disease/disorders or other illnesses that may affect the GI tract were present. Additionally, all volunteers were confirmed to be H. pylori negative as determined by culture and rapid Urease test (CLO test). Tissue samples collected during EGD were transported to the laboratory facilities in a tube containing RPMI 1640 (Gibco, Carlsbad, CA, USA) with antibiotics/antifungal (Penicillin/Streptomycin/Amphotericin B; Gibco) and processed immediately after collection as shown in Figure 1 and Figure S1 in Supplementary Material. We first compared two methods the isolation of gastric LPMC: (i) a conventional method (CM) and (ii) bullet blender (BB) method. The CM method consisted of three steps: (a) removal of intraepithelial lymphocytes (IEL) [HBSS + EDTA (1 mM)], (b) digestion of the resulting tissues (collagenase D/DNase I), and (c) disaggregation of the tissues (by teasing of the tissues between the frosty ends of two microscope glass slides). The BB method also consisted of three steps. The first two steps were similar to the CM whist the last step consisted of homogenizing the gastric biopsy tissues using a BB (Next Advance, Averill Park, NY, USA) (Figure 1). To perform these methods, media was removed from the biopsies by using a 70-µm cell strainer (BD Falcon, Franklin Lakes, NJ, USA) and dried through the filter using sterile gauze. The tissue was then transferred to a pre-weighted 1.5 ml centrifuge tube and the net weight measured. Biopsies were then rapidly transferred to a 50 ml conical tube containing 10 ml of HBSS without CaCl$_2$, MgCl$_2$, MgSO$_4$ (Gibco) with antibiotics/antifungal mix (Gibco) and EDTA (1 mM) and incubated at 37°C for 30 min while shaking. The tissues were washed with 10 ml of HBSS buffer (with CaCl$_2$, MgCl$_2$ (Gibco) without EDTA and incubated for 10 min at room temperature (RT) while shaking. The tissues were then enzymatically digested either in six well plates (CM method) or 1.5 ml sterile screw-top polypropylene microcentrifuge tubes (BB method) containing 1 ml of digestion solution. Tubes used for the BB method also contained two stainless steel beads (3.2 mm in diameter each) used for mechanical digestion. To confirm that the cell type(s) present in the sample were gastric LPMC, the tissue was digested enzymatically in both the CM and BB methods. The homogenized gastric LPMC were then manually processed through a 70-µm strainer and separated into mononuclear cells (MNC) and remaining tissue fragments (T cell–free). The MNC were then washed and processed for flow cytometry analysis.

MATERIALS AND METHODS

VOLUNTEERS AND ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Volunteers were recruited from the Baltimore–Washington area and University of Maryland, Baltimore campus. Written informed consent was obtained and all procedures were approved by the University of Maryland, Baltimore IRB. Immediately after blood draws, PBMC were isolated by density gradient centrifugation and used freshly for stimulation and characterization. Blood and gastric biopsies were collected at the same time. A total of 57 volunteers (aged 7–85 years) were evaluated.

ISOLATION OF LAMINA PROPRIA MONONUCLEAR CELLS

Gastric biopsies were collected from volunteers (7–85 years-old) referred for outpatient diagnostic upper endoscopy (EGD) at the University of Maryland Medical Center. The indications for EGD included abdominal pain, heartburn, GERD, dysphagia, and acute gastritis. Diagnostic pathology reports showed that the stomach's...
After the second digestion by either method, cells were collected and washed with PBS and resuspended in RPMI 1640 medium containing 10% FBS, antibiotics, and antifungal mix (Gibco), 10 μM of 2.5 M CaCl₂, 10 μL of Collagenase D (100 μg/ml; Roche, Indianapolis, IN, USA), and 1 μL DNase 1 (10 μg/ml; Affymetrix, Cleveland, OH, USA). The biopsies (20 mg maximum per tube) were digested for 45 min at 37°C with intermittent pipetting (CM method) or shaking (BB method). Following 45 min incubation, the tissues were disaggregated using the frosty ends of glass slides (CM method). In the case of the BB method, following the 45 min digestion the tube was placed in a BB homogenizer (Next Advance Inc., NY, USA) and the tissue homogenized for 30 s (speed 1). Tissues were further incubated for 15 min (37°C).

After the second digestion by either method, cells were collected in a 50 mL tube through a 70 μm cell strainer and centrifuged at 1400 rpm. Cells were then washed and re-suspended in complete RPMI (Heat inactivated FBS (10%), 1-glutamine (2 mM), non-essential Amino acids (1×) (Gibco 11140), HEPES buffer (10 mM) (Gibco 15630-080), Sodium pyruvate (2.5 mM) (Lonza 13-155E), Penicillin/streptomycin (100 U/ml–100 μg/ml) (Sigma P0781), Gentamicin (50 μg/ml) (Gibco 15750-060)), and counted using Kova Glastic Slides (Hycor Biomedical, CA, USA). Cells were either stained immediately for immunophenotyping by flow cytometry or stimulated with mitogens overnight before staining (see below). To evaluate whether the enzymatic digestion resulted in loss of surface receptors, PBMC were either treated or untreated with the same digestion mix and processed as detailed above and assessed for surface markers (Figures S1C,D in Supplementary Material). To determine the effect of collagenase D in the digestion mix on surface marker expression the above procedure and assessed for surface markers (Figures S1C,D in Supplementary Material). T o determine the effect of collagenase D in the digestion mix on surface marker expression the above procedure was followed using an enzymatic mix in which collagenase D was replaced with dispase (1 μg/ml) (Figure S1C in Supplementary Material).

FLOW CYTOMETRY PROCEDURES AND STAINING

Ex vivo stimulation

Freshly isolated cells from PBMC and gastric biopsies (LPMC) were re-suspended in complete media and stimulated with medium, staphylococcal enterotoxin B (SEB) (10 μg/ml; Sigma) or Dynabeads Human T activator CD3/CD28 (4 × 10⁴ beads/ml) (Invitrogen Dynal, Oslo, Norway). For each treatment, 1 × 10⁵ LPMC and 1 × 10⁶ PBMC were cultured in 200 μl and 1 ml total volumes, respectively, and incubated at 37°C in 5% CO₂. In some experiments, cells were stained with CD107a-FITC at the time of stimulation. After 2 h, GolgiStop (Monensin, BD) and GolgiPlug (Brefeldin A, BD) were added at concentrations of 0.5 μl/ml and cultures continued overnight at 37°C in 5% CO₂.

Surface and intracellular staining

Following stimulation, PBMC and LPMC were plated in 96-well V-bottom plates for staining. Cells were washed twice with phosphate buffered saline (PBS) and stained for live/dead discrimination using Invitrogen LIVE/DEAD fixable yellow dead cell stain kit (YEVID) (Invitrogen, Carlsbad, CA, USA). Blocking of Fc receptors was performed using human immunoglobulin (3 μg/ml; Sigma) and was followed by surface staining, performed as previously described (21). Briefly, cells were stained with fluorescently labeled monoclonal antibodies (mAbs) directed to CD14-BV570 (clone M5E2, Biolegend, San Diego, CA, USA) and CD19-BV570 (clone HIB19, Biolegend), CD3-BV650 (clone OKT3, Biolegend), CD4-PE-Cy5 (clone RPA-T4, BD), CD8-PerCP-Cy5.5 (clone SK-1, Becton–Dickinson, BD), CD45RA-biotin (clone HI100, BD), integrin αβ7-Alexa Fluor 647 (clone Act-1, Leukosite, Cambridge, MA, USA), and CD62L-Alexa Fluor 780 (clone DREG-5, eBioscience, San Diego, CA, USA) at 4°C for 30 min. Staining with streptavidin–Qdot800 (Invitrogen) was performed for panels that included biotin-conjugated mAbs for 30 min at 4°C. The cells were then fixed and permeabilized using IC fixation and permeabilization buffers (eBioscience) according to the manufacturer’s recommendations. Intracellular staining with mAbs to IL-17A-BV421 (clone BL168, Biolegend), IL-2-BV605 (clone MQ1-17H12, Biolegend), IFN-γ-PE-Cy7 (clone B27, BD), TNF-α-Alexa 700 (clone MAb11, BD), MIP-1β-PE (clone 24006, R&D Systems, Minneapolis, MN, USA), and CD69-ECD (clone TP1.55.3, Beckman Coulter, Danvers, MA, USA) was performed at 4°C overnight. After staining, cells were fixed in 1% paraformaldehyde and stored at 4°C until data collection. Data were collected using a customized LSRII flow cytometer (BD) and then analyzed using WinList version 7 (Verity Software House, Topsham, ME, USA) software package. Graphs were generated using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA).

In experiments designed to characterize TREG cells, the staining panels were modified as follows. LPMC and PBMC were stained with mAbs directed to CD103-Alexa Fluor 488 (clone Ber-ACT8, Biolegend), CD14-BV570 (clone M5E2, Biolegend, San Diego, CA, USA), CD13-Pacific Orange (clone WM-15 eBioscience, San Diego, CA, USA conjugated to Pacific Orange in-house), CD19-BV570 (clone HIB19, Biolegend), CD3-BV650 (OKT3, Biolegend), CD4-PE-Cy5 (clone RPA-T4, BD), CD8-PerCP-Cy5.5 (clone SK-1, BD), CD45RA-biotin (clone HI100, BD), integrin αβ7-Alexa Fluor 647 (clone Act-1, Leukosite, Cambridge, MA, USA), and CD62L-Alexa Fluor 780 (clone DREG-5, eBioscience, San Diego, CA, USA) and intracellularly with mAbs to IL-17A-BV421 (clone BL168, Biolegend), IL-2-BV605 (clone MQ1-17H12, Biolegend), IFN-γ-PE-Cy7 (clone B27, BD), TNF-α-Alexa 700 (clone MAb11, BD), MIP-1β-PE (clone 24006, R&D Systems, Minneapolis, MN, USA), and CD69-ECD (clone TP1.55.3, Beckman Coulter, Danvers, MA, USA).

FCM ANALYSIS FOR MULTIFUNCTIONALITY

FCOM (Verity Software House, Topsham, ME, USA) is an analytical tool that is used to classify events based on combinations of selected gates. FCOM reduces multiparameter data to a series of multiple acquisition gates, one for every possible combination. FCOM was employed to determine the subsets of CD4⁺ and CD8⁺ producing multiple cytokines and/or expressing CD107a expression (i.e., multifunctionality).

STATISTICAL ANALYSIS

Data were analyzed using GRAPHPAD PRISM™ 5.03 statistical software (Graphpad, San Diego, CA, USA). Statistical differences in median values between two groups were determined using Mann–Whitney tests. Statistical differences between multiple
groups (more than two) were determined by Kruskal–Wallis tests and the Dunn’s post-test was used to compare selected group pairs. Values of *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ were considered significant.

**RESULTS**

**GASTRIC LPMC ISOLATION AND CELL YIELDS FROM CHILDREN, ADULTS, AND ELDERLY VOLUNTEERS**

Several methodologies to isolate gastric leukocytes from human stomach biopsies have been reported; however, there is a lack of consensus in the type of digestion enzymes to use, their concentration, digestion periods and whether or not to use mechanical dissociation techniques. Therefore, we optimized a protocol for isolation of gastric LPMC. We first compared two methods: (i) a conventional method (CM) and (ii) a blender method (BB) (described in detail in Section “Materials and Methods”). In the BB method, we optimized the homogenization step regarding the speed, time, and number of beads needed for a gentle dissociation of the cells from the gastric tissues. We found that homogenizing the tissue for 30 s at a speed of 1 and using 2 beads (stainless steel; 3.2 mm diameter) resulted in optimal cell yields (Figure S1A in Supplementary Material). This optimized BB method yielded superior cell numbers ($1.1 \times 10^4$/mg of tissue) from human biopsies compared to the CM method ($0.6 \times 10^4$/mg of tissue) (Figure S1B in Supplementary Material). Two digestion enzymes (collagenase D and dispase) were then compared by substituting each one using the optimized BB method. We observed that collagenase D treatment resulted in better cell yields and cell surface marker preservation than dispase, which had a marked effect on the expression of cell surface markers as shown by lower MFI for CD4+ and CD8+ T cells in both PBMC and LPMC isolated cells (Figure S1C in Supplementary Material). We further evaluated the effect of collagenase D on tissues by using PBMC treated in similar fashion as biopsies with the BB method. The results showed no significant differences in the expression levels of CD3, CD4, CD8, CD45RA, CD62L, and integrin α4β7 surface markers between PBMC treated with or without collagenase D (Figure S1D in Supplementary Material).

Stomach biopsies (antrum) obtained from *H. pylori* negative (CLO test negative) adult (18–64 years), children (7–17 years), and elderly (65–85 years) volunteers were processed as described in Section “Materials and Methods” and Figure 1. Gastric LPMC were isolated and enumerated from five biopsy samples from each adult and elderly volunteer and three biopsy samples from each child (Figure 2A). The viable cell yields in biopsies from adult and the elderly ranged from 230,000 to 2,300,000 (median 634,000) and 240,000 to 1,300,000 (median 605,000) cells, respectively; whereas in children’s biopsies cell yields ranged from 320,000 to 734,000 (median: 492,000) cells (Figure 2A). The total viable cell yields in the children group was significantly lower ($p < 0.05$) than in the adult group. However, the weight of biopsies varied between age groups as samples from children were significantly ($p < 0.05$) smaller in size and weight (8.8–33.4 mg) than samples from adults (20.5–76 mg) and the elderly (31.7–51.9 mg) (Figure 2A). To compare the viable cell yields among age groups we calculated the number of viable cells per milligram of tissue. The results showed that the cell yields obtained from biopsies of children (13,000–49,000 viable cells/mg of tissue, median: 21,000) were significantly higher ($p < 0.05$) than those obtained from biopsies of the elderly (6000–49,000, median: 15,500) (Figures 2A,B).
Finally, using Pearson’s regression analysis, we observed a significant inverse correlation ($r = -0.3, p = 0.021$) between the number of isolated cells per milligram of tissue and age (Figure 2C).

CHARACTERIZATION OF T CELL SUBSETS IN LPMC AND PBMC

Human gastric T lymphocytes have been shown to display a Th1 type response (IFN-γ, TNF-α, and IL-2 secretion) toward pathogens, such as H. pylori (15). However, the $T_M$ subset(s) that secrete(s) these cytokines and differences among the various age groups have not been explored. To address these shortcomings, the presence of T cells (CD3$^+$) groups have not been explored. To address these shortcomings, the presence of T cells (CD3$^+$) in LPMC and PBMC was assessed (Figure 3A). The frequency of these cells was significantly lower in LPMC than in PBMC from adult volunteers (median: 13.7 vs. 74.7%; $p < 0.0005$) (Figures 3A,B). Similar findings were observed in children and the elderly (Figure 3B). When CD3$^+$ T cells were divided into CD4$^+$ (CD3$^+$CD4$^+$) and CD8$^+$ (CD3$^+$CD8$^+$) T cells, the latter were more abundant in LPMC than in PBMC (Figures 3A,B). Therefore, there was an inversion in the CD4/CD8 ratio in LPMC (~1:3) compared to PBMC (~3:1). These results confirmed and extended those reported by others (16, 19, 20) by showing that these differences were observed in all age groups (Figures 3B). When CD4$^+$ T cells in LPMC of children and the elderly were compared to adults, children showed a much lower percentage of CD4$^+$ T cells than in PBMC ($*** p < 0.0005$) (Figure 3A). Similar results were observed in all the volunteers tested (Figure 3D). On the other hand, CD103 and CD69 were virtually absent from CD8$^+$ T cells in LPMC and PBMC. We observed that most CD8$^+$ T cells in LPMC co-expressed CD103 and CD69 (mean: 81.1%; median: 80.5%), while a much lower percentage of CD4$^+$ T cells in LPMC co-expressed these markers (mean: 35%; median: 28.8%) (Figures 3C,D). Similiar results were observed in all the volunteers tested (Figure 3D). On the other hand, CD103 and CD69 were virtually absent from CD4$^+$ T cells in LPMC and PBMC. We observed that most CD8$^+$ T cells in LPMC co-expressed CD103 and CD69 (mean: 81.1%; median: 80.5%), while a much lower percentage of CD4$^+$ T cells in LPMC co-expressed these markers (mean: 35%; median: 28.8%) (Figures 3C,D). Similarly, even though only ~30% of CD4$^+$ T cells in LPMC co-expressed CD103 and CD69, expression...
of CD69 or CD103 alone was found in ~35 and ~10% of these cells, respectively. In sum, the majority of the CD4+ and CD8+ T cells in LPMC expressed molecules compatible with those reported for T_{RM} cells. In contrast, the expression of CD103 and CD69 was virtually absent from PBMC CD4+ and CD8+ T cells (Figures 3C,D).

Expression of the homing marker integrin α4β7 was assessed in PBMC and LPMC in the subsets showing the T_{EM} phenotype (T_{RM} in LPMC and T_{EM} in PBMC) (Figure 4A). Gastric CD8+ T_{RM} and CD4+ T_{RM} showed a significantly lower level of expression of integrin α4β7 (p < 0.05) compared to CD8+ T_{EM} and CD4+ T_{EM} (Figures 4A,B). Interestingly, there were no significant differences in integrin α4β7 expression among gastric LPMC CD8+ T_{RM} or CD4+ T_{RM} between adult, children, and the elderly (Figure 4C).

**MITOGEN ACTIVATION OF LPMC AND PBMC**

We next examined whether isolated gastric LPMC CD8+ or CD4+ T_{RM} cells were functionally active by exploring whether they responded to mitogen stimulation by producing cytokines and up-regulating the expression of CD107a, a marker of degranulation associated with cytotoxic activity (23). Furthermore, we explored whether there were any differences in the responses between the different age groups. Gastric LPMC and PBMC were stimulated with various T cell stimulants including: (i) SEB (superantigen) and (ii) α-CD3/CD28 coated beads (TCR stimulation). As negative control, cells were incubated in media alone. The concomitant production of multiple cytokines (IFN-γ, TNF-α, IL-2, IL-17A, and MIP-1β) and up-regulation of CD107a were determined in CD107a that were determined in LPMC (Figures 5A,B) and PBMC in all three age groups. Up-regulation of CD69 as a cell activation marker was only considered for PBMC since in LPMC (T_{RM}) this marker is highly expressed regardless of stimulation (Figures 3C,D). In PBMC, the main T_{EM} subsets responding to the stimulations were CD8+ and CD4+ T_{EM} and T_{EM+RM} and these results were consistent with previous reports from our group, as well as others (6, 24). The percentages of gastric CD8+ T_{RM} and CD4+ T_{RM} producing cytokines and up-regulating CD107a following stimulation were compared to CD8+ T_{EM} and CD4+ T_{EM} (PBMC) and the results in all age groups are summarized in Tables 1–4.

Interestingly, control media gastric CD8+ and CD4+ T_{RM} cells from adult volunteers showed higher percentages of cells producing cytokines and up-regulating expression of CD107a than media only CD8+ T_{EM} and CD4+ T_{EM} (PBMC) cells (Figures 5C,D). This difference in baseline cytokine production was statistically significant (p < 0.05) only in CD8+ T cells (Figures 5C,D). Of note, in children, while higher CD8+ T_{RM} cells producing cytokines and up-regulating the expression of CD107a were observed, no statistically significant differences were found compared to CD8+ T_{EM} cells in media controls (Table 1). In the elderly group, a percentage of cells producing significant higher baseline levels of IL-2 and MIP-1β in gastric CD8+ T_{RM} cells were identified. Concerning the other cytokines investigated, as well as CD107a, although a similar trend to the other age groups was observed, the differences did not reach statistical significance, likely due to high subject-to-subject variability (Table 1). Additionally,
the diagnostic pathology reports allowed us to explore whether the
higher baseline cytokines levels of CD8⁺ and CD4⁺ T RM cells were
due to extrinsic factors causing inflammation of the antral mucosa.
Volunteers were classified as having either normal or mildly
inflamed (mild diffuse erythema, mild diffuse inflammation, reactive
tissue-resident memory T (T RM) cells in gastric LPMC. Representative plot of the activation of
gastric LPMC CD8⁺ (A) or CD4⁺ (B) T RM (CD62L⁺CD45RA⁺CD69⁺
CD103⁺) by two stimulants: (1) staphylococcal enterotoxin B (SEB;
10 µg/ml) and (2) anti-CD3/CD28 beads (α-CD3 α-CD28) to produce IL-2,
IFN-γ, MIP-1β, TNF-α, IL-17A, and up-regulation of the expression of
CD107a. Cells left unstimulated were used as negative control (Media,
C). Cumulative data comparing baseline activation levels of gastric
LPMC CD8⁺ (C) and CD4⁺ (D) T RM, white portion of the bar to PBMC
(black portion of the bar). In (C,D) significant differences between T RM
and T RM are indicated with asterisks on top of each bar; *p < 0.05;
**p < 0.005; ***p < 0.0005.

we also compared the percentage of cells producing cytokines
from adult volunteers stimulated with mitogens (SEB and anti-CD3/CD28)
produced most of the assessed cytokines at higher levels than media control
cells (Figures 5A,B; Tables 1–4). In general, higher percentages
were observed in gastric CD8⁺ T RM and CD4⁺ T RM cells producing
cytokines and expressing CD107a than in CD8⁺ T EM and
CD4⁺ T EM cells. In some instances, the cytokine production differences
were statistically significant (Tables 1 and 2). Overall, results were similar in all age groups (Tables 1 and 2).

We next compared cytokine production by adults, children,
and the elderly in both PBMC and LPMC populations (results
are summarized in Table 3). CD8⁺ T EM cells from children did
not show statistically significant differences when compared to
adults for any of the cytokines evaluated or CD107a expression. In contrast the elderly group demonstrated significantly higher number of CD8⁺ T RM cells (p < 0.05) producing MIP-1β at baseline
levels (compared to adults) and this cytokine was identified in a
higher percentage of cells following stimulation (Table 3). Other
cytokines observed in a higher percentage of cells following stimulation in the elderly were TNF-α (anti-CD3/CD28) and CD107a expression (SEB and anti-CD3/CD28) (Table 3). In children, a
significantly higher percentage of CD8⁺ T RM cells (LPMC) expressed
CD107a at baseline than in adults (Table 3). Neither children nor
the elderly showed differences in the percentage of CD4⁺ T EM
cells (PBMC) producing cytokines compared to adults. On the
other hand, in children, at baseline, the percentage of CD4⁺ T RM
cells (LPMC) producing IFN-γ and TNF-α was higher than in
adults. However, no differences were noted following stimulation
(Table 3). In the elderly, the percentage of CD4⁺ T RM cells
producing TNF-α, at basal levels were also significantly higher than
in adults. Moreover, the percentage of CD4⁺ T RM cells producing
significantly higher levels of IL-2 (anti-CD3/CD28), IFN-γ (anti-
CD3/CD28), and TNF-α (SEB and anti-CD3/CD28) following
stimulation was also enhanced (Table 3).

We also compared the percentage of cells producing cytokines
following SEB and anti-CD3/CD28 stimulation to the control cells
in all age groups. The results are summarized in Table 4. SEB and
anti-CD3/CD28 beads efficiently induced CD8⁺ T EM and CD4⁺
T EM cells from adults and the elderly to produce cytokines and
CD107a expression compared to media control (Table 4). Interestingly, even though both stimulants induced CD8\(^+\) TEM\(_{\text{EM}}\) cells to produce cytokines in children (Table 1), the results were not statistically significant as compared to media (Table 4), and only CD107a up-regulation was significantly induced by SEB in children. SEB and anti-CD3/CD28 beads were unable to stimulate CD8\(^+\) TEM\(_{\text{RM}}\) cells to produce IL-17A in any of the three age groups and IL-2 was significantly induced only in the elderly (Table 4). CD8\(^+\) TEM\(_{\text{RM}}\) cells were efficiently induced to produce IFN-\(\gamma\) and CD107a expression in all three age groups by both SEB and anti-CD3/CD28 beads (p < 0.05). Furthermore, MIP-1\(\beta\) was efficiently induced in adults and the elderly, but not in children by both stimulants. TNF-\(\alpha\) was also significantly induced in all three age groups but only by anti-CD3/CD28 beads. Neither SEB nor anti-CD3/CD28 beads were able to increase the percentage of CD4\(^+\) TEM\(_{\text{RM}}\) cells producing IL-2, MIP-1\(\beta\), and expressing CD107a in children and IL-17A in adults. However, at least one of these stimulants was able to stimulate CD4\(^+\) TEM\(_{\text{RM}}\) to produce IL-2, IFN-\(\gamma\), MIP-1\(\beta\), TNF-\(\alpha\), and CD107a in adults and the elderly (Table 4).

MULTIFUNCTIONAL GASTRIC CD8\(^+\) TEM\(_{\text{RM/EM}}\) CD4\(^+\) and CD8\(^+\) T cells that produce two or more cytokines simultaneously (multifunctional) have enhanced functionality and are more likely to correlate with protection from disease when compared to single cytokine-producing cells (25–28). The induction of multifunctional cells in the human gastric mucosa has not yet been reported and whether these cells play a role in the development or resolution of pathogenesis remains unknown. Thus, we investigated whether CD4\(^+\) and CD8\(^+\) TEM\(_{\text{RM}}\) (LPMC) obtained from the three age groups had multifunctional properties following SEB stimulation. All possible combinations (64 in total) for five cytokines (IFN-\(\gamma\), TNF-\(\alpha\), IL-2, IL-17A, MIP-1\(\beta\)) and expression of CD107a were analyzed in multidimensional space using the WinList FCOM function. Similar analyses were performed in CD4\(^+\) TEM\(_{\text{EM}}\) and CD8\(^+\) TEM\(_{\text{EM}}\) (PBMC) populations. The results demonstrated that stimulation elicits multifunctional responses in gastric CD4\(^+\) TEM\(_{\text{RM}}\) and CD8\(^+\) TEM\(_{\text{RM}}\) cells. Similarly, CD4\(^+\) TEM\(_{\text{EM}}\) and CD8\(^+\) TEM\(_{\text{EM}}\) cells demonstrated multifunctionality, which is consistent with previous results from our group as well as others (Figures 6 and 7) (6, 24, 25, 29). For simplicity, shown are only the six highest expressing multifunctional CD8\(^+\) TEM\(_{\text{RM}}\) and CD8\(^+\) TEM\(_{\text{EM}}\) cell groups (Figures 6A,B). Double, triple, quadruple, and quintuple cytokine secreting cells CD8\(^+\) TEM\(_{\text{RM}}\) and CD8\(^+\) TEM\(_{\text{EM}}\) were found in all age groups albeit at different percentages. Interestingly, of the six highest multifunctional groups in gastric CD8\(^+\) TEM\(_{\text{RM}}\) cells, four were also found in CD8\(^+\) TEM\(_{\text{EM}}\) cells (dotted boxes) (Figures 6A,B). We then compared the magnitude of multifunctional T cells between the age groups and identified some differences. A significantly

Table 1 | Comparison of PBMC CD8\(^+\) TEM\(_{\text{EM}}\) and gastric LPMC CD8\(^+\) TEM\(_{\text{RM}}\) cells activation responses in adult, children, and the elderly.

| Cytokine | Stimulant | Adult | Children | Elderly |
|----------|-----------|-------|----------|---------|
|          | CD8\(^+\) TEM\(_{\text{EM}}\) | CD8\(^+\) TEM\(_{\text{RM}}\) | CD8\(^+\) TEM\(_{\text{EM}}\) | CD8\(^+\) TEM\(_{\text{RM}}\) | CD8\(^+\) TEM\(_{\text{EM}}\) | CD8\(^+\) TEM\(_{\text{RM}}\) |
|          | Median | % (Range) | Median | % (Range) | Median | % (Range) |
| IL2 | Media | 0.1 (0–2) | 1.2 (0–2)** | 0.3 (0–1) | 1.0 (0–2) | 0.1 (0–1) | 1.7 (0–3)** |
|      | SEB | 1.7 (0–17) | 1.6 (0–42) | 0.3 (0–2) | 1.5 (1–2) | 1.8 (1–13) | 3.9 (1–22) |
|      | α-CD3/CD28 | 2.0 (0–12) | 1.7 (0–11) | 0.3 (0–1) | 1.0 (0–3) | 1.4 (1–9) | 4.0 (1–12) |
| INF\(\gamma\) | Media | 0.2 (0–6) | 1.3 (0–3)* | 0.4 (0–3) | 1.6 (1–3) | 0.6 (0–2) | 1.7 (0–5) |
|      | SEB | 8.6 (0–37) | 15.7 (8–27) | 4.3 (1–19) | 10.3 (6–17) | 6.3 (2–57) | 11.5 (2–28) |
|      | α-CD3/CD28 | 3.1 (0–19) | 13.8 (3–30)** | 2.7 (1–6) | 12.3 (6–23)* | 6.5 (1–15) | 20.9 (10–26)* |
| MIP-1\(\beta\) | Media | 0.2 (0–6) | 1.9 (0–8)** | 0.5 (0–4) | 0.6 (0–6) | 1.0 (0–4) | 4.8 (1–10)* |
|      | SEB | 5.0 (0–20) | 15.6 (5–31)** | 4.4 (0–7) | 8.6 (3–15) | 15.5 (1–55) | 17.8 (3–30) |
|      | α-CD3/CD28 | 2.3 (0–20) | 16.7 (3–49)** | 1.4 (0–14) | 8.9 (2–42) | 14.6 (1–39) | 33.2 (8–55) |
| TNF-α | Media | 0.2 (0–3) | 1.4 (0–4)* | 0.9 (0–3) | 1.9 (1–3) | 0.9 (0–2) | 0.7 (0–4) |
|      | SEB | 2.6 (0–22) | 9.1 (4–22) | 2.6 (2–21) | 3.4 (1–15) | 9.4 (1–49) | 7.6 (1–17) |
|      | α-CD3/CD28 | 2.9 (0–15) | 7.8 (2–18)* | 1.9 (1–5) | 8.5 (4–11) | 9.9 (1–21) | 9.7 (1–13) |
| IL17A | Media | 0.1 (0–2) | 0.7 (0–2)* | 0.5 (0–1) | 1.1 (0–2) | 0.1 (0–1) | 0.8 (0–2) |
|      | SEB | 1.1 (0–4) | 1.1 (0–7) | 0.3 (0–2) | 2.2 (0–9) | 0.2 (0–1) | 0.4 (0–10) |
|      | α-CD3/CD28 | 2.0 (0–6) | 1.0 (0–3) | 0.2 (0–1) | 1.9 (0–2)* | 0.2 (0–1) | 0.9 (0–5)* |
| CD107a | Media | 0.1 (0–4) | 1.4 (0–3)** | 0.8 (0–4) | 3.4 (2–4) | 1.1 (0–4) | 2.6 (1–5) |
|      | SEB | 5.2 (0–21) | 12.9 (7–40)* | 4.6 (3–21) | 9.8 (7–23) | 14.2 (5–45) | 12.9 (3–26) |
|      | α-CD3/CD28 | 1.3 (0–12) | 10.8 (2–18)** | 3.0 (2–7) | 16.6 (7–28)** | 12.7 (1–21) | 12.3 (10–19) |

Gastric LPMC CD8\(^+\) TEM\(_{\text{EM}}\) T cell responses (IL-2, IFN-\(\gamma\), MIP-1\(\beta\), TNF-\(\alpha\), IL-17A, and CD107a) to two stimulants (SEB and α-CD3/CD28 beads) were compared to PBMC CD8\(^+\) TEM\(_{\text{EM}}\) responses obtained from adults, children, and the elderly. Significant differences are shown in highlighted colors as determined by Mann–Whitney tests. Adults: n = 10; children: n = 10; elderly: n = 10.

*Light green color = significant increase in the frequency of CD8\(^+\) TEM\(_{\text{EM}}\) (LPMC) compared to CD8\(^+\) TEM\(_{\text{EM}}\) (PBMC); *p < 0.05; **p < 0.005.
Table 2 | Comparison of PBMC CD4⁺ T EM and gastric CD4⁺ T EM cells activation responses in adult, children, and the elderly.

| Cytokine | Stimulant | Adult CD4⁺ T EM | Children CD4⁺ T EM | Elderly CD4⁺ T EM |
|----------|-----------|----------------|-------------------|------------------|
|          | Median    | CD4⁺ T EM % (Range) | Median    | CD4⁺ T EM % (Range) | Median    | CD4⁺ T EM % (Range) |
| IL-2     | Media     | 0.3 (0–3) | 0.5 (0–2) | 0.5 (0–2) | 1.3 (0–3) | 0.3 (0–4) | 1.6 (0–9) |
|          | SEB       | 2.5 (0–60) | 3.4 (0–35) | 1.5 (1–5) | 0 (0–6) | 14.8 (1–31) | 8.7 (0–32) |
|         | α-CD3/CD28 | 2.2 (0–38) | 8.9 (0–21) | 1.2 (0–6) | 8.8 (20) | 7.9 (3–27) | 214 (5–32) |
| INFγ     | Media     | 0.2 (0–2) | 0.3 (0–2) | 0.7 (0–3) | 4.8 (0–9) | 0.6 (0–3) | 0.0 (0–5) |
|          | SEB       | 3.0 (0–15) | 7.0 (0-11) | 5.8 (2–9) | 3.7 (0–25) | 6.2 (3–18) | 3.6 (1–19) |
|         | α-CD3/CD28 | 0.6 (0–5) | 5.6 (5–22)*** | 4.1 (1–9) | 10.9 (2–25) | 3.8 (1–14) | 14.1 (2–27) |
| MIP-1β   | Media     | 0.2 (0–1) | 1.5 (0–8) | 0.3 (0–2) | 4.7 (0–6) | 0.7 (0–1) | 1.3 (0–8) |
|          | SEB       | 1.5 (0–13) | 4.8 (–33) | 0.8 (0–5) | 11.4 (0–35) | 1.5 (1–3) | 13.5 (1–21)*** |
|         | α-CD3/CD28 | 0.8 (0–9) | 9.2 (1–38)** | 0.3 (0–3) | 15.0 (0–31) | 1.2 (0–3) | 0.03 (7–34)*** |
| TNFα     | Media     | 0.1 (0–3) | 2.3 (0–6) | 1.2 (0–6) | 4.2 (1–6) | 2.8 (0–5) | 1.2 (0–6) |
|          | SEB       | 0.9 (0–55) | 10.8 (2-30) | 15.7 (5–21) | 6.3 (2–14) | 41.1 (1–53) | 9.9 (0–21)*** |
|         | α-CD3/CD28 | 0.6 (24) | 5.8 (14–42)*** | 12.2 (5–25) | 24.6 (8–60) | 23.7 (2–31) | 278 (4–33) |
| IL-17A   | Media     | 0.2 (0–2) | 0.9 (0–3) | 0.3 (0–3) | 1.4 (0–3) | 0.3 (0–1) | 0.0 (0–3) |
|          | SEB       | 2.1 (1–16) | 2.4 (0–12) | 3.1 (0–7) | 6.3 (2–25) | 1.5 (1–3) | 2.1 (1–9) |
|         | α-CD3/CD28 | 3.8 (0–14) | 4.8 (0–12) | 1.6 (1–5) | 14.1 (3–19)* | 0.9 (0–2) | 2.9 (0–14) |
| CD107a   | Media     | 0.2 (0–1) | 0.2 (0–3) | 0.3 (0–1) | 1.3 (0–5) | 0.2 (0–2) | 0.0 (0–1) |
|          | SEB       | 1.5 (1–7) | 4.0 (0–11) | 1.2 (0–3) | 5.7 (2–19)* | 1.6 (1–3) | 2.2 (0–11) |
|         | α-CD3/CD28 | 0.7 (0–5) | 5.9 (0–8)* | 0.7 (0–2) | 6.3 (12–12)* | 1.4 (0–3) | 3.5 (0–12) |

Gastric LPMC CD4⁺ T EM responses (IL-2, INFγ, MIP-1β, TNFα, IL-17A, and CD107a) to two stimulants (SEB and anti-CD3/CD28) were compared to PBMC CD4⁺ T EM responses obtained from adults, children, and the elderly. Significant differences are shown in highlighted colors as determined by Mann–Whitney tests. Adults: n = 10; children: n = 10; elderly: n = 10.

*Light green color = significant increase in the frequency of CD4⁺ T EM (LPMC) compared to CD4⁺ T EM (PBMC).

Light blue color = significant decrease in the frequency of CD4⁺ T EM (LPMC) compared to CD4⁺ T EM (PBMC). *p < 0.05; **p < 0.005; ***p < 0.0005.

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higher percentage of CD8⁺ T EM cells from the elderly contained double (CD107a⁺ MIP-1β⁺) cytokine-producing cells than adults and children (Figure 6A). Similarly, quintuple (CD107a⁺ INF-γ⁺ TNF-α⁺ IL-2⁺ MIP-1β⁺) cytokine-producing cells in the elderly were significantly more abundant than in adults (Figure 6A). In peripheral blood, differences between the age groups within the multi-cytokine-producing sets were also noted. The percentage of double (TNF-α⁺ IL-2⁺) and triple (INF-γ⁺ TNF-α⁺ IL-2⁺), as well as CD107a⁺ INF-γ⁺ MIP-1β⁺) CD8⁺ T EM cells in the elderly group was significantly higher than in children (Figure 6B). Furthermore, the percentage of triple (CD107a⁺ INF-γ⁺ MIP-1β⁺) CD8⁺ T EM cells was higher in the elderly than in adults (Figure 6B).

We also assessed multifunctionality in CD4⁺ T EM and CD4⁺ T EM cells (Figure 7). Both of these cell populations have the potential to become multifunctional and showed differences in the age groups within various multifunctional sets. Of the six highest multifunctional populations in gastric CD4⁺ T EM cells (LPMC), only two were also found in CD4⁺ T EM cells (PBMC) (dotted boxes) (Figures 7A,B). Double and quadruple cytokine-producing cells were observed in gastric CD4⁺ T EM cells. The percentage of double producer cells (TNF-α⁺ IL-2⁺ and IL-2⁺ MIP-1β⁺) in the elderly was significantly higher than in children (Figure 7A).

Moreover, the percentage of gastric CD4⁺ T EM cells producing IL-2 and MIP-1β in elderly volunteers was also higher than in adults (Figure 7A). Interestingly, the percentage of dual producer CD4⁺ T EM cells consisting of IL-17 (CD107a⁺ IL-17A⁺ and TNF-α⁺ IL-17A⁺) cells in children was significantly higher than adults and the elderly (Figure 7A). Similarly, PBMC CD4⁺ T EM stimulated with SEB display activated cells that contained double, triple, and quadruple cytokine-producing cells (Figure 7B). In the elderly, a significantly higher percentage of CD4⁺ T EM cells produced double (INF-γ⁺ TNF-α⁺ or TNF-α⁺ IL-2⁺), triple (CD107a⁺ IL-2⁺ TNF-α⁺ and IFN-γ⁺ TNF-α⁺ IL-2⁺), and quadruple (INF-γ⁺ TNF-α⁺ IL-2⁺ MIP-1β⁺) cytokines than children and adult volunteers (Figure 7B).

**DISCUSSION**

Recent reports have described the presence of T EM cells (CD8⁺) in mucosal surfaces (4–6, 9). These cells, originally described in the mouse model, were very recently identified in the lungs of humans (9), along with CD4⁺ T EM cells, which have been less well characterized. In the stomach mucosa, several immune cells have been described and although different methodologies for isolation of mononuclear cells from biopsies have been reported, the optimal conditions remain largely undefined. In this manuscript,
Table 3 | Summary of gastric LPMCTRM and PBMC TEM (CD4+ and CD8+) responses between children and the elderly to adults.

| Cytokine | Stim | PBMC CD8+ TEM | LPMC CD8+ TEM | PBMC CD4+ TEM | LPMC CD4+ TEM |
|----------|------|---------------|---------------|---------------|---------------|
|          |      | Ch a | El b | Ch | El | Ch | El | Ch | El |
| IL-2     | Med  |      |      |      |      |      |      |      |      |
| SEB      |      |      |      |      |      |      |      |      |      |
| α-CD3/CD28* |      |      |      |      |      |      |      |      |      |
| IFN-γ    | Med  |      |      |      |      |      |      |      |      |
| SEB      |      |      |      |      |      |      |      |      |      |
| α-CD3/CD28 |      |      |      |      |      |      |      |      |      |
| MIP-1β   | Med  |      |      |      |      |      |      |      |      |
| SEB      |      |      |      |      |      |      |      |      |      |
| α-CD3/CD28 |      |      |      |      |      |      |      |      |      |
| TNFα     | Med  |      |      |      |      |      |      |      |      |
| SEB      |      |      |      |      |      |      |      |      |      |
| α-CD3/CD28 |      |      |      |      |      |      |      |      |      |
| IL-17A   | Med  |      |      |      |      |      |      |      |      |
| SEB      |      |      |      |      |      |      |      |      |      |
| α-CD3/CD28 |      |      |      |      |      |      |      |      |      |
| CD107a   | Med  |      |      |      |      |      |      |      |      |
| SEB      |      |      |      |      |      |      |      |      |      |
| α-CD3/CD28 |      |      |      |      |      |      |      |      |      |

LPMC CD8+ and CD4+ TEM and PBMC TEM responses (IL-2, IFN-γ, MIP-1β, TNFα, IL-17A, and CD107a) to stimulation by two mitogens (SEB and anti-CD3/CD28) were determined and compared between children (Ch) or elderly (El) to adults (children vs. adult and elderly vs. adult). Significant differences are shown in highlighted colors as determined by Mann–Whitney test. Adults: n = 8; children: n = 5; elderly: n = 7. Empty (white) cells indicate non-significant differences between children or elderly vs. adults as determined by Mann–Whitney tests.

*Children; **Elderly; *Anti-CD3/CD28 beads; Light green color = significant in cytokine production compared to *p < 0.05; **p < 0.005.

we report an optimized method for the isolation of human gastric leukocytes from stomach biopsies and, using this method, the identification of CD8+ TEM and CD4+ TEM cells. Moreover, we explored the ability of these cells to produce cytokines following stimulation with various mitogens, as well as demonstrated the multifunctional nature of these responses. Finally, we investigated whether there are differences in the quality and magnitude of the responses in various age groups.

Our cell isolation protocol (LPMC) from gastric biopsies involved the removal of epithelial cells and a mild enzymatic digestion step (13) that was combined with a mild mechanical disruption step using stainless steel beads (BB). This additional step allowed for maximum dislodgement of cells with minimal damage and provided a more consistent and uniform homogenization, decreasing variation between samples and generating higher viable cell yields. Compared to previously published reports, our method, at a minimum doubled the number of viable LPMC isolated from gastric biopsies (12, 30). However, given that biopsies size and weight varies considerably during sampling and that in most studies the biopsy weights have not been reported, the real efficiency of the methods cannot be directly compared. Cell yields expressed as total number of viable cells per milligram of tissue in “dried” biopsies would be optimal to enable this assessment across studies. Interestingly, the number of cells per milligram of tissue in children was higher than in the elderly (Figure 2B). These results were similar to those reported by Bontems et al., who suggested that there was higher cellularity in children than in adults; however, no statistical differences were reported in that study (17). The data from elderly volunteers allowed us to extend the time frame of evaluation and confirmed that as the age of the volunteers increased, the number of mononuclear cells isolated in the stomach decreased.

Consistent with previous reports, the frequency of CD3+ cells was lower as a percentage of total LPMC cells in the gastric mucosa than in PBMC (19, 31). Additionally, CD4+ and CD8+ T cells from gastric LPMC were found at similar frequencies as reported by others (14, 16) and the vast majority of gastric CD8+ and CD4+ T cells showed a TEM phenotype (CD62L−, CD45RA−). These results provide evidence that the optimized cell isolation method described in the present manuscript did not result in cell subset selection bias. Therefore, these cells appeared to be of the newly defined tissue-TRM cells in human intestinal tissues (32). This presumption was confirmed by investigating the expression of hallmark receptors for these cells, including CD103 and CD69, which were expressed by T cells isolated from gastric tissues. Interestingly, differences were noted between CD8+ TRM and CD4+ TEM...
**Table 4 | Summary of T<sub>RM</sub> and T<sub>EM</sub> responses to stimulation in children, elderly, and adults.**

| Cytokine | Age     | PBMC | CD8<sup>+</sup> T<sub>RM</sub> | LPMC | CD8<sup>+</sup> T<sub>RM</sub> | PBMC | CD4<sup>+</sup> T<sub>EM</sub> | LPMC | CD4<sup>+</sup> T<sub>EM</sub> |
|----------|---------|------|-----------------|------|-----------------|------|-----------------|------|-----------------|
|          |         | SEB  | α3/28<sup>a</sup> | SEB  | α3/28<sup>a</sup> | SEB  | α3/28<sup>a</sup> | SEB  | α3/28<sup>a</sup> |
| IL-2     | Ad<sup>b</sup> | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
|          | Ch<sup>c</sup> | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
|          | El<sup>d</sup> | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
| IFN-γ    | Ad      | +++ |  | +++ |  | +++ |  | +++ |  | +++ |  |
|          | Ch      | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
|          | El      | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
| MIP-1β   | Ad      | ++  |  | ++  |  | +++ |  | ++  |  | ++  |  |
|          | Ch      | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
|          | El      | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
| TNFα     | Ad      | +++ |  | +++ |  | +++ |  | +++ |  | +++ |  |
|          | Ch      | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
|          | El      | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
| IL-17A   | Ad      | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
|          | Ch      | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
|          | El      | ++  |  | ++  |  | ++  |  | ++  |  | ++  |  |
| CD107a   | Ad      | +++ |  | +++ |  | +++ |  | +++ |  | +++ |  |
|          | Ch      | +++ |  | +++ |  | +++ |  | +++ |  | +++ |  |

Gastric LPMC CD8<sup>+</sup> and CD4<sup>+</sup> T<sub>RM</sub> and PBMC T<sub>RM</sub> responses (IL-2, IFN-γ, MIP-1β, TNFα, IL-17A, and CD107a) to stimulation by two mitogens (SEB and anti-CD3/CD28) were determined and compared to media stimulation (negative control) in adults (Ad), children (Ch), and the elderly (El). Significant differences are shown in highlighted colors as determined by Mann–Whitney tests. Adults: n = 6; children: n = 5; elderly: n = 7. Empty (white) cells indicate non-significant differences between stimulated and non-stimulated (media) cultures as determined by Mann–Whitney tests.

<sup>a</sup>Anti-CD3/CD28 beads; <sup>b</sup>Adults; <sup>c</sup>Children; <sup>d</sup>Elderly; <sup>e</sup>Light green color = significant increase in cytokine production compared to media stimulation, *p < 0.05; **p < 0.005; ***p < 0.0005.

T<sub>RM</sub> cells in gastric tissues. For example, the large majority of CD8<sup>+</sup> T<sub>RM</sub> cells co-expressed CD103 and CD69, whilst only a small proportion expressed CD103 alone. In contrast, only ~35% of CD4<sup>+</sup> T<sub>RM</sub> cells co-expressed CD103 and CD69. Therefore, CD8<sup>+</sup> T<sub>RM</sub> and CD4<sup>+</sup> T<sub>RM</sub> in the human gastric lamina propria exhibited a differential expression pattern of molecules reported to define T<sub>RM</sub> in other human mucosal tissues (7, 8). These observations suggest that CD4<sup>+</sup> T<sub>RM</sub> cells are a more heterogeneous and complex population than CD8<sup>+</sup> T<sub>RM</sub>, possibly composed by various subsets. Future studies are necessary to address this important question. Of note, CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>RM</sub> were present in children, adult, and the elderly at similar frequencies.

Migration of immune cells from peripheral blood to gut tissues is driven by the expression of tissue-specific homing receptors such as integrin a4β7 and CCR9 (33, 34). Since gastric T<sub>RM</sub> cells are expected to permanently reside in this tissue, we reasoned that up-regulation of CD103, which binds to E-cadherin and allows homing at the mucosal level, will result in down-regulation of integrin a4β7. Consistent with this, CD8<sup>+</sup> T<sub>RM</sub> and CD4<sup>+</sup> T<sub>RM</sub> cells showed a significant down-regulation of integrin a4β7 compared to its expression levels in CD8<sup>+</sup> T<sub>EM</sub> and CD4<sup>+</sup> T<sub>EM</sub> (PBMC). These results are consistent with data reported in the mouse model in tissues isolated from the small intestine (32, 35). Similar results were seen in adults, children, and the elderly. Whether CCR9 is also down-regulated in CD8<sup>+</sup> and CD4<sup>+</sup> T<sub>RM</sub> cells remains to be explored. The fact that we were able to identify CD8<sup>+</sup> T<sub>RM</sub> and CD4<sup>+</sup> T<sub>RM</sub> cells in LPMC, which contain cells from the lamina propria, suggests that T<sub>RM</sub> cells are either constantly mobilizing between the epithelial and lamina propria layers of the stomach or reside mainly in the lamina propria layer. In an attempt to address this question, we assessed the IEL fraction in some volunteers and identified mainly CD8<sup>+</sup> T cells, most of which co-expressed CD103 and CD69 (data not shown). CD4<sup>+</sup> T cells were also identified in the IEL fraction, but at such low frequencies that we were unable to ascertain their levels of expression of CD103 and CD69 (data not shown). It is reasonable to speculate that T<sub>RM</sub> cells mainly reside in the lamina propria and once they migrate to the epithelium (i.e., becoming part of the traditional IEL subset) are unable to re-enter the lamina propria. In the latter scenario, T<sub>RM</sub> cells in the lamina propria will constantly supply cells that migrate to the epithelium. This would require a change in expression of homing markers and would suggest that, in addition to CD103, other receptor(s) yet to be identified is(are) involved in the homing of these cells from the lamina propria to the epithelium. At this time our data are unable to determine which of these hypotheses is correct. However, it is likely that T<sub>RM</sub> cells shuttle between these...
two compartments working as sentinel cells and when a specific antigen is encountered, these cells are rapidly activated, producing cytokines and acquiring CTL activity.

CD8$^+$ T$_{RM}$ cells have been described in mice as well as humans, and reactivity of these cells to antigens derived from pathogens has been demonstrated (4–6, 9). We identified CD8$^+$ T$_{RM}$ and CD4$^+$ T$_{RM}$ cell in the stomach of volunteers confirmed to be *H. pylori* negative (as determined by CLO test) (36). While *H. pylori* is well recognized for its role in the development of gastritis, peptic ulcer, and adenocarcinoma, it does not affect the composition of the gastric microbiota (37). The gastric microbiota has been shown to contain a diverse community of 128 phylotypes (37), which could provide the underlying T cells with unidentified infectious agents or the gastric microbiota, through antigen(s) to regulate their development. It can be speculated that phylotypes (37), which could provide the underlying T cells with conserved epitopes that resemble those of pathogens, play a role in the development of T$_{RM}$ cells (38). Whichever the event(s) that triggers their development, it appears that they occur at a young age, since even the youngest children evaluated in our studies (i.e., 7-year-old) showed the presence of these unique cells. Future experiments designed to address these questions will include the investigation of the role of the microbiota in the development of T$_{RM}$ cells and a comparison of the cytokine production by CD4$^+$ T$_{RM}$ and CD8$^+$ T$_{RM}$ cells from *H. pylori* positive and healthy volunteers following stimulation with *H. pylori* antigens.

Gastric CD8$^+$ T$_{RM}$ and CD4$^+$ T$_{RM}$ cells obtained from biopsies of children, adults, and the elderly were responsive to SEB and anti-CD3/CD28 beads stimulations by secreting Th1 cytokines (IL-2, IFN-γ, TNF-α, IL-17A, MIP-1β) and up-regulating the cytotoxicity marker CD107a. These results confirm and extend studies in which gastric CD4$^+$ T cells obtained from healthy adults (*H. pylori* negative) secreted Th1 cytokines (IFN-γ and TNF-α) when stimulated with PMA/Ionomycin (16, 31). Interestingly, CD8$^+$ T$_{RM}$ cells appeared to produce cytokines constitutively; a higher percentage of T$_{RM}$ cells cultured in media only showed cytokine production compared to CD8$^+$ T$_{EM}$ cells. Similar results, albeit not statistically significant, were identified in CD4$^+$ T$_{RM}$ cells. These observations...
suggest that T<sub>RM</sub> cells are more prone to activation and possibly have a lower antigenic threshold for stimulation than their peripheral blood counterparts. However, it is important to consider that while the volunteers were <i>H. pylori</i> negative, they were referred for EGD due to the presence of clinical symptoms (e.g., dysphagia, heartburn, GERD, etc.). Therefore, to determine if gastric T<sub>RM</sub> cells were activated in response to an inflammatory environment resulting from the underlying clinical condition(s), we stratified the baseline cytokine levels based on the pathology findings from each volunteer (normal and “mild inflammation”) (Figure S2 in Supplementary Material). Neither CD8<sup>+</sup> T<sub>RM</sub> nor CD4<sup>+</sup> T<sub>RM</sub> cells showed statistically significant differences between the normal and mild inflammation groups. These results support the idea that T<sub>RM</sub> cells show a persistent activation state in “normal volunteers.” An alternative explanation for the persistent activation state of T<sub>RM</sub> cells could involve the role of the gastric microbiota. Thus, future studies should be directed to explore this and other alternative explanations. Whether the higher percentage of cells producing cytokines and up-regulating CD107a spontaneously have a deleterious effect at the gastric mucosal level or that this enhanced inflammatory environment benefits the host by limiting colonization with pathogens remains to be explored. Overall, CD8<sup>+</sup> T<sub>RM</sub> cells from adult, children, and the elderly responded to the stimuli and the cytokine production was higher compared to PBMC, but more evident in adults and elderly than in children.

There is little information on the induction of local immune responses in the gastric mucosa from children (39, 40). Few studies have evaluated the cytokine responses in the gastric mucosa of this age group and the results are contradictory (17, 41, 42). One study found that lower levels of IFN-γ were produced in culture supernatants of gastric mucosa tissues from children compared to adults, but no differences in TNF-α, IL-2, or IL-10 were detected regardless of their <i>H. pylori</i> status (17). On the other hand, a recent study showed that in <i>H. pylori</i> infected children, the gastric concentration of IL-1α and TNF-α were significantly higher than that in infected adults whereas IL-2, IL-12p70, and IFN-γ were lower in infected children than in infected adults (42). Of note, differences in cytokine profiles were observed between infected and uninfected individuals in both age groups (42). Epidemiological studies have also suggested that unlike adults, children rarely develop peptic ulcers or gastric atrophy (43–45). This suggests that children may display a unique immunological milieu that limits gastric mucosal damage. In our study, even though the phenotype and abundance of gastric CD8<sup>+</sup> T<sub>RM</sub> and CD4<sup>+</sup> T<sub>RM</sub> in children were similar to those of adults and the elderly, their responses were different. CD4<sup>+</sup> T<sub>RM</sub> and CD8<sup>+</sup> T<sub>RM</sub> cells from children responded only moderately to the mitogenic stimulations and secreted lower amounts of cytokines than their adult and the elderly counterparts. Our results are consistent, and markedly extend, previous studies demonstrated that gastric T cells from children are less responsive to stimulation than adults. Moreover, our results provide novel information on cells isolated from elderly subjects. Additionally, it has been shown that Th1 and Th17 responses in children are down-regulated, resulting in reduced gastritis due to <i>H. pylori</i> infections (46). This observation contrasts with that of adults, in whom <i>H. pylori</i> infections usually result in significant inflammation. Furthermore, in children who are positive for <i>H. pylori</i>, the levels of regulatory T cells (T<sub>regs</sub>) and IL-10 secreting cells in the gastric mucosa are higher than in <i>H. pylori</i> infected adults (46, 47). Therefore, these observations suggest that in children the regulatory mechanisms at the gastric level are more active than in adults and this could contribute to the limited reactivity identified in CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>RM</sub> cells. Future studies involving the investigation of the presence and functional properties of T<sub>regs</sub> in the gastric mucosa of children as compared to adults/elderly will shed light into this important question.

Multifunctionality analysis following SEB stimulation confirmed that T<sub>RM</sub> cells are multifunctional and also reinforced the idea that age is a significant factor. Interestingly, various multi-cytokine production patterns demonstrated that in the elderly a higher percentage of cells produced multiple cytokines than in children, suggesting that elderly cells are more reactive to stimulation. This data further confirmed and extend the observations in this manuscript that cells from children are less susceptible to activation. Of note, there were a few instances in which cells from children produced more cytokines than those isolated from the elderly (e.g., CD4<sup>+</sup> T<sub>RM</sub> dual producers CD107a and IL-17A, as well as TNF-α and IL-17A). This reinforces the indication that cytokine production is age-related.

In summary, we developed a consistent method for isolation of immune cells from the gastric biopsies that increased cell yields and allowed the identification of CD8<sup>+</sup> T<sub>RM</sub> and CD4<sup>+</sup> T<sub>RM</sub> cells in children, adults, and the elderly. We demonstrated that these cells were functional and responsive to various categories of stimulants. Finally, we show that gastric cells of children respond differently to stimuli than adults and the elderly in terms of cytokines and multi-cytokine production suggesting that unique regulatory mechanisms are operative in the children's gastric mucosa.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/Journal/10.3389/fimmu.2014.00294/abstract

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