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Research Article

Broadly reactive human CD4$^+$ T cells against Enterobacteriaceae are found in the naïve repertoire and are clonally expanded in the memory repertoire

Antonino Cassotta[*1,2], *Jérémie D. Goldstein*[*1], *Greta Durini*[1], *David Jarrossay*[1], *Franca Baggi Menozzi*[3], *Mario Venditti*[4], *Alessandro Russo*[5], *Marco Falcone*[5], *Antonio Lanzavecchia*[1], *Maria Cristina Gagliardi*[6], *Daniela Latorre*[*1,2] and *Federica Sallusto*[*1,2]

[1] Institute for Research in Biomedicine, Università della Svizzera italiana, Bellinzona, Switzerland
[2] Institute of Microbiology, ETH Zurich, Switzerland
[3] Servizio di Microbiologia EOLAB, Ente Ospedaliero Cantonale, Bellinzona, Switzerland
[4] Department of Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy
[5] Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
[6] Center for Gender Specific Medicine, Istituto Superiore di Sanità, Rome, Italy

Enterobacteriaceae are a large family of Gram-negative bacteria that includes both commensals and opportunistic pathogens. The latter can cause severe nosocomial infections, with outbreaks of multi-antibiotics resistant strains, thus being a major public health threat. In this study, we report that Enterobacteriaceae-reactive memory Th cells were highly enriched in a CCR6$^+$CXCR3$^+$ Th1*/17 cell subset and produced IFN-γ, IL-17A, and IL-22. This T cell subset was severely reduced in septic patients with *K. pneumoniae* bloodstream infection who also selectively lacked circulating *K. pneumonie*-reactive T cells. By combining heterologous antigenic stimulation, single cell cloning and TCR Vβ sequencing, we demonstrate that a large fraction of memory Th cell clones was broadly cross-reactive to several Enterobacteriaceae species. These cross-reactive Th cell clones were expanded in vivo and a large fraction of them recognized the conserved outer membrane protein A antigen. Interestingly, Enterobacteriaceae broadly cross-reactive T cells were also prominent among in vitro primed naïve T cells. Collectively, these data point to the existence of immunodominant T cell epitopes shared among different Enterobacteriaceae species and targeted by cross-reactive T cells that are readily found in the pre-immune repertoire and are clonally expanded in the memory repertoire.

Keywords: cross-reactivity · Enterobacteriaceae · human memory T cells · human naïve T cells · Th1*/17

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Correspondence: Dr. Federica Sallusto or Dr. Daniela Latorre
e-mail: sallustf@ethz.ch; latorred@biol.ethz.ch

*These authors contributed equally to this work.

Introduction

Enterobacteriaceae are a vast family of Gram-negative bacteria that includes harmless symbionts and potentially harmful pathogens [1, 2]. Among pathogenic Enterobacteriaceae, thyphoidal *Salmonella enterica* serovars, such as *S. typhi* and *S. paratyphi*, are responsible for life-threatening enteric fever with more than 20 million people affected worldwide annually [2–4], whereas non-typhoidal *S. enterica* serovars, such as *S. typhimurium* and *S. enteriditis*, are common causes of gastroenteritis due to food poisoning and can induce serious systemic infections in immunocompromised individuals [5, 6]. *Shigella* species, such as *S. flexneri*, *S. sonnei*, and *S. dysenteriae*, can cause a highly contagious and severe inflammatory diarrhea, which affects around 164 million people per year, predominantly children under the age of 5 years [7], while *Klebsiella pneumoniae*, which is part of the normal microbiome colonizing mucosal sites [8, 9], can become, under certain conditions (i.e., immunocompromised patients), an opportunistic pathogen and cause healthcare-associated infections [9, 10]. Given the lack of effective vaccines to prevent Enterobacteriaceae infections [11, 12] as well as the constant increase of multidrug resistant strains [10, 13-15], Enterobacteriaceae can cause severe deadly infections, thus representing a global health threat. Recently, carbapenem-resistant Enterobacteriaceae bacteria have been included in the list of antibiotic-resistant "priority pathogens" by the WHO [16].

Increasing evidence suggests that $CD4^+$ T cells can recognize commensals-derived antigens under homeostatic conditions and during gut dysbiosis or inflammation [17,18]. Although several studies have investigated the human T cell response to commensals and pathogens [4,19–22], a characterization of Enterobacteriaceae-reactive $CD4^+$ T cells in terms of phenotype, antigen specificity and TCR repertoire composition in physiological and pathological conditions remains elusive. Here, we combined several experimental approaches, including heterologous stimulation of bacteria-specific memory T cells, high-throughput TCR-Vβ sequencing, and antigen-specific priming of naïve T cells, to perform a systematic analysis of the $CD4^+$ Th cell response to multiple Enterobacteriaceae species in healthy individuals as well as in patients suffering from *K. pneumoniae* systemic infections. We report that the $CCR6^+CXCR3^+$ Th1*/17 subset contains most Enterobacteriaceae-reactive memory $CD4^+$ T cells. This subset is significantly reduced and *K. pneumoniae*-reactive T cells are absent in septic patients with *K. pneumoniae* bloodstream infection. Interestingly, most Enterobacteriaceae-reactive memory $CD4^+$ T cells cross-react to several Enterobacteriaceae species and a sizable fraction target outer membrane protein A (OmpA), which is a highly conserved antigen among Enterobacteriaceae species. Importantly, broadly cross-reactive $CD4^+$ T cells are already present in the naïve repertoire and become highly expanded in the memory repertoire. Collectively, these data provide a better understanding of the human immune responses against Enterobacteriaceae and provide insights for the development of new strategies for prevention of severe Enterobacteriaceae infections.

Results

Enterobacteriaceae-reactive memory $CD4^+$ T cells in healthy donors have a Th1*/17 phenotype

To investigate the human $CD4^+$ T cell response to Enterobacteriaceae, we isolated by FACS-sorting memory $CD4^+$ Th cells from PBMCs of a large number of healthy donors (Supporting Information Fig. S1A). Cells were labeled with CFSE and stimulated in vitro with autologous monocytes either untreated or pulsed with different bacteria (Fig. 1A and B). In most of the donors tested, a clear proliferative T cell response to the Enterobacteriaceae species *E. coli, K. pneumoniae, E. aerogenes, Shigella*, and *S. typhimurium* was detected, as shown by the CFSE profiles, although the magnitude of the response was significantly lower ($p < 0.0001$) compared to the response elicited by *S. aureus* or *M. tuberculosis* in control cultures (Fig. 1B). Enterobacteriaceae-induced T cell proliferation was inhibited by anti-MHC class II (HLA-DR, -DP, and -DQ) blocking antibodies, indicating that Th cells responded to bacterial antigens in the context of MHC class II molecules (Supporting Information Fig. S1B and C). The Enterobacteriaceae-reactive Th cells produced IFN-γ in combination with IL-17A and IL-22 (Fig. 1C and D) and, consistent with this phenotype, were significantly enriched in the $CCR6^+CXCR3^+$ Th1*/17 cell subset (Supporting Information Fig. S2). *S. aureus*-reactive $CD4^+$ T cells produced also IFN-γ in combination with IL-17A and IL-22, while *M. tuberculosis*-reactive cells produced mainly IFN-γ, as previously reported [23, 24].

Collectively, these results indicate that Enterobacteriaceae-reactive memory $CD4^+$ T cells are present in the blood of healthy individuals, are mainly confined in the $CCR6^+CXCR3^+$ Th1*/17 cell subset and produce IFN-γ, IL-17A, and IL-22.

Low rate of $CXCR3^+$ Th and lack of *K. pneumoniae*-reactive Th cells in systemic infection patients

Enterobacteriaceae are among the major causes of systemic infections [10,14,15,25–27]. Reduction in the overall frequency of blood-circulating $CD4^+$ T cells and impaired T cell functions have been described in animal models of sepsis and in patients, as reviewed recently [28, 29]. To evaluate the presence and function of Enterobacteriaceae-reactive T cells in pathological conditions, we analyzed memory $CD4^+$ Th cells in the blood of patients with severe sepsis caused by systemic carbapenem-resistant *K. pneumoniae* infections. In these patients, frequency of circulating $CXCR3^+$ cells ($CCR6^-$ Th1 and $CCR6^+$ Th1*/17) was significantly reduced compared to healthy controls, while frequency of $CCR4^+$ cells ($CCR6^-$ Th2 and $CCR6^+$ Th17) was comparable (Fig. 2A). In addition, $CCR6^+CXCR3^+$ memory T cells from septic patients did not proliferate in response to *K. pneumoniae*, while they proliferated in response to *M. tuberculosis*, although to a variable extent (Fig. 2B). Of note, none of the other Th

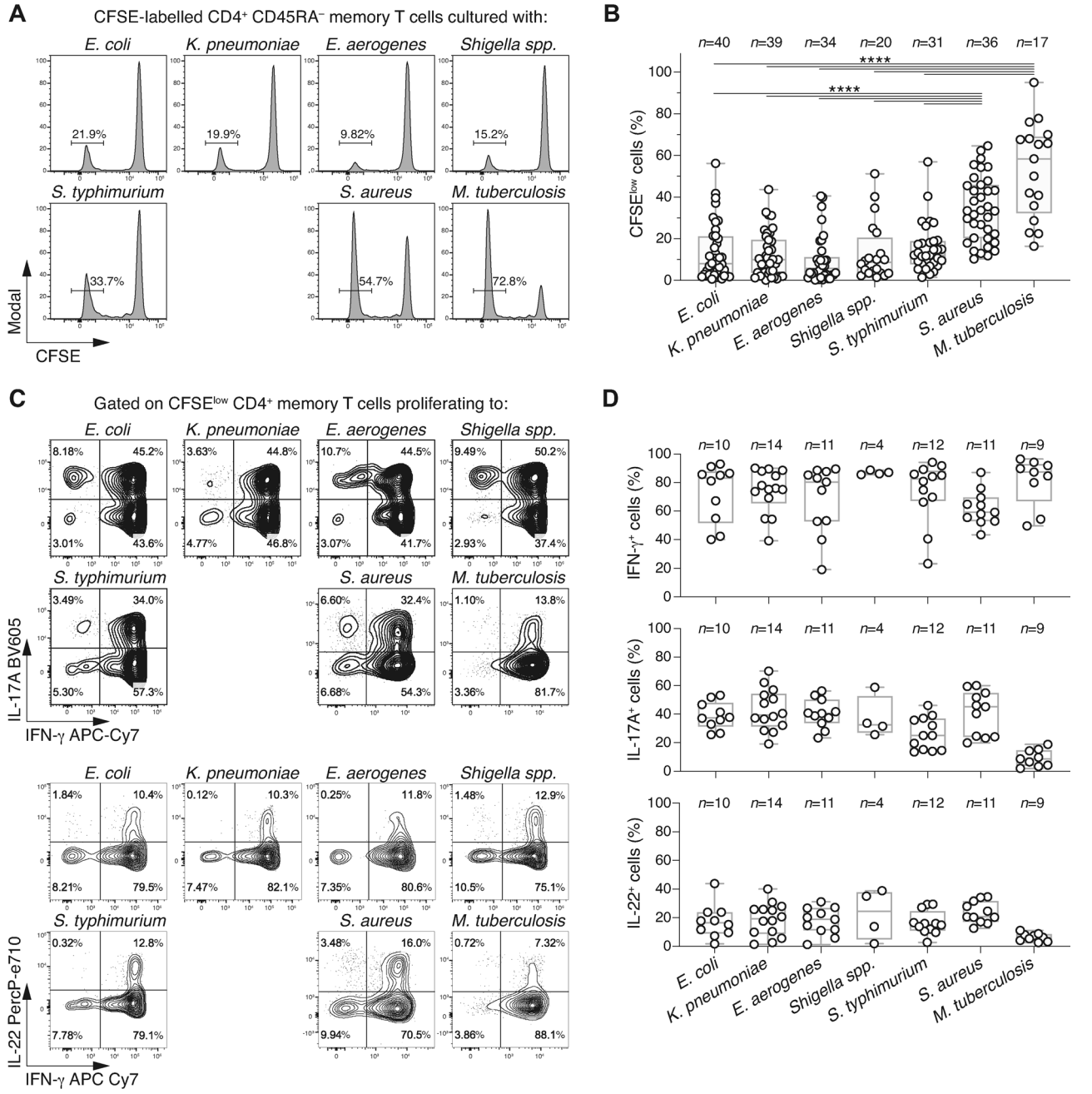


Figure 1. Enterobacteriaceae-reactive memory CD4$^+$ T cells are present in the blood of healthy donors and show a Th1*/17 phenotype. Human memory CD4$^+$ T cells were isolated from PBMCs, labeled with CFSE and stimulated with the indicated heat-inactivated bacteria in the presence of autologous monocytes and analysed by flow cytometry. (A) CFSE profiles on day 6 in a representative donor. Numbers indicate percentage of CFSE^{low} proliferating T cells. (B) Percentages of CFSE^{low} cells in n=17-40 different donors analyzed in 20 independent experiments (1 or 2 donors per experiment). Each dot represents a donor, boxes are quartile values, whiskers represent the highest and lowest values, and lines represent the median values. Numbers on top indicate the total number of donors analyzed with the indicated bacteria ****p-value < 0.0001, as determined by two-tailed unpaired t-test. (C and D) IFN-γ, IL-17A, and IL-22 production by CFSE^{low} cells was measured by intracellular staining after stimulation with PMA and ionomycin in the presence of Brefeldin A in a representative donor (C) and in n = 4–14 different donors in seven independent experiments (D). In (C), numbers in quadrants indicate percent cells in each throughout. In (D), each dot represents a donor, boxes are quartile values, whiskers represent the highest and lowest values, and lines represent the median values. Shown are total percentages of cells producing IFN-γ, IL-17, or IL-22, irrespective of the fact that the cells produced these cytokines alone or in combination. Numbers on top indicate the total number of different donors analyzed with the indicated bacteria.

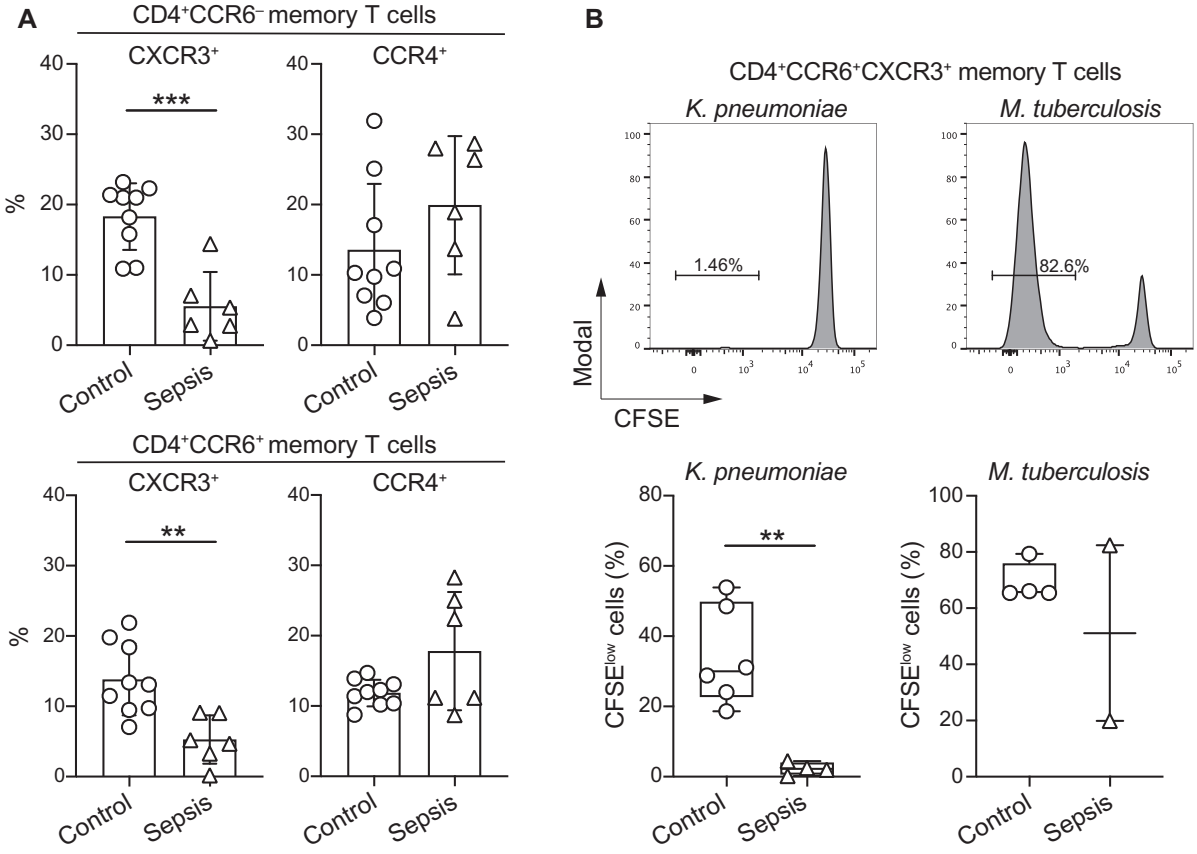


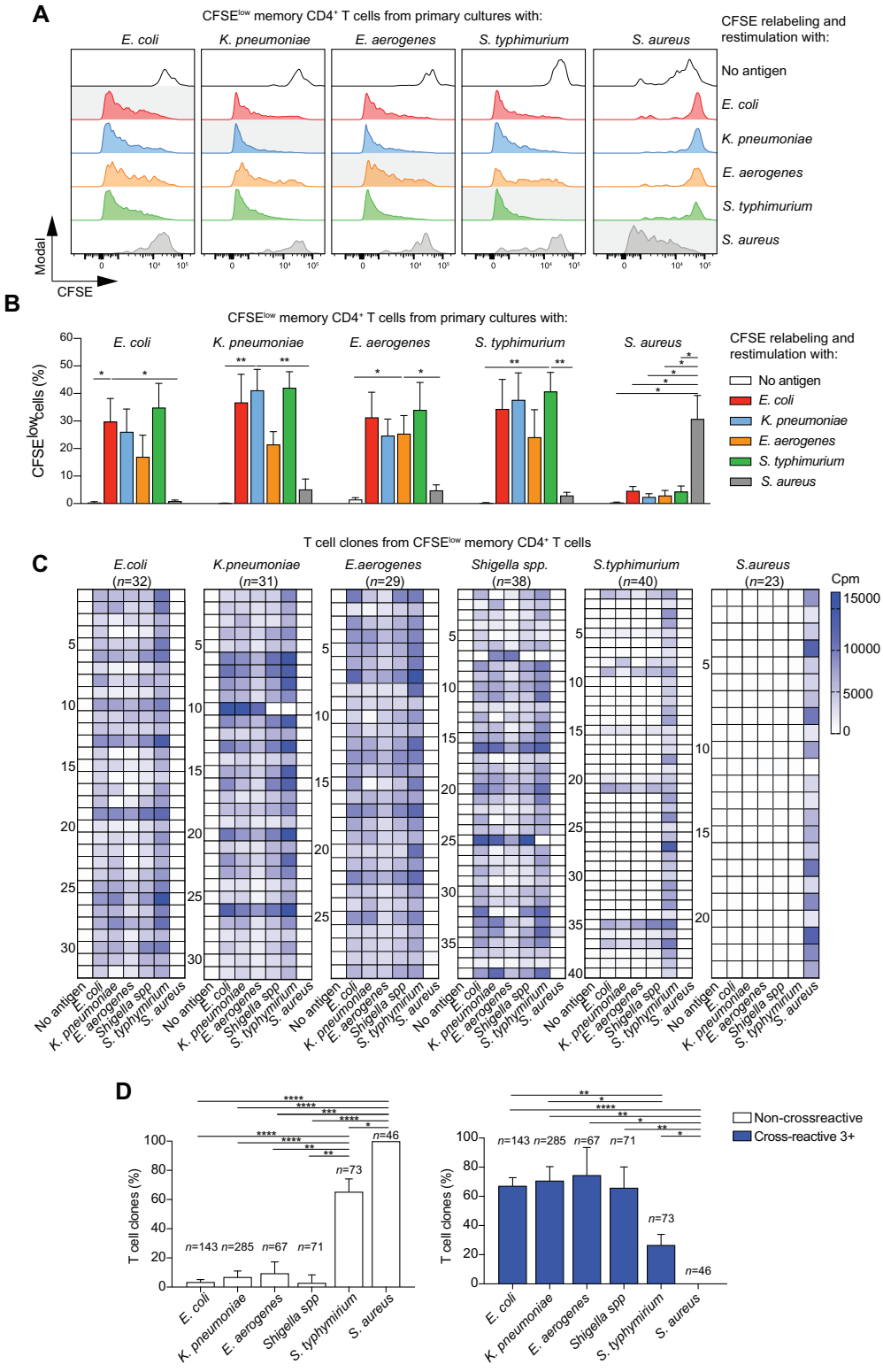
Figure 2. Septic patients with systemic *K. pneumoniae* infection show reduced frequency of circulating CXCR3$^+$ T cells and selectively lack *K. pneumoniae*-reactive T cells. (A) The surface expression of chemokine receptors by memory CD4$^+$ T cells from PBMCs of $n = 9$ healthy controls and $n = 6$ septic patients with systemic *K. pneumoniae* infection was analyzed by flow cytometry. Shown are pooled data (mean ± SD, five independent experiments with samples from one or two septic patients and one or two healthy controls per experiment) of percentage of memory CD4$^+$ T cells positive for combinations of chemokine receptors. (B) Human memory CCR6$^+$CXCR3$^+$CCR4$^-$ Th cells were sorted, labeled with CFSE, and stimulated with the indicated antigens in the presence of autologous monocytes. Shown are CFSE profiles on day 6 in a representative septic patient (upper panel), and pooled data of percentage of CFSE^{low} cells in healthy controls or septic patients (lower panel). Each dot represents a donor, boxes are quartile values, whiskers represent highest and lowest values, and lines represent the median values. $^{**}p$-value < 0.01, $^{***}p$-value < 0.001, as determined by two-tailed unpaired *t*-test. The data are from three independent experiments with samples from one or two septic patients and two healthy controls per experiment.

subsets isolated from septic patients proliferated in response to *K. pneumoniae* stimulation (data not shown), thus indicating a lack of a compensatory T cell response.

Memory CD4$^+$ T cells are broadly cross-reactive to Enterobacteriaceae species

Previous studies provided evidence of CD4$^+$ T cells able to cross-recognize multiple intestinal microbes in physiological and pathological conditions [19,21]. To thoroughly define the extent of T cell cross-reactivity in the response to Enterobacteriaceae, we used two different approaches. First, we isolated the CFSE^{low} memory CD4$^+$ T cells proliferating in response to Enterobacteriaceae, relabeled the cells with CFSE and performed homologous or heterologous restimulation. As expected, the Th cell lines obtained from the primary cultures with *E. coli, K. pneumoniae, E. aerogenes, S. typhimurium*, or *S. aureus* showed robust proliferation upon secondary restimulation with the same bacteria (Fig. 3A, shaded plots). Notably, the same Th cell lines proliferated also to heterologous stimulation with all the other Enterobacteriaceae species tested, but not to *S. aureus* (Fig. 3A, B). In contrast, Th cell lines obtained from the primary stimulation with *S. aureus* proliferated upon secondary restimulation with *S. aureus* but not with Enterobacteriaceae species.

In the second approach, we isolated from eight donors a large number of T cell clones from the CFSE^{low} polyclonal memory CD4$^+$ T cells proliferating in response to Enterobacteriaceae. The T cell clones ($n = 685$) were screened for their capacity to proliferate in response to autologous monocytes pulsed with different Enterobacteriaceae species or *S. aureus* as control (Fig. 3C and D; Supporting Information Tables S1 and S2). Specific Th cell clones (i.e., reactive only to the original bacteria used in the primary stimulation) were very rare among clones obtained from *E. coli-*, *K. pneumoniae-*, *E. aerogenes-*, and *Shigella*-cultures (range 3–9.5%), while, strikingly, around 60% of them cross-reacted with



three or more Enterobacteriaceae species (Fig. 3C and D). As an exception, *S. typhimurium*-specific Th cell clones were 65.4% while less than 30% cross-reacted with three other Enterobacteriaceae species (Fig. 3C and D), suggesting the possibility that memory T cells may have been primed by antigens encoded by *S. typhimurium* pathogenicity islands. As control, Enterobacteria-specific Th cell clones did not proliferate when stimulated by monocyte alone (no antigen) or by monocytes exposed to LPS (Fig. 3C; Supporting Information Fig. S3), while *S. aureus*-specific Th cell clones did not cross-react to any of the Enterobacteriaceae species tested (Fig. 3C and D).

Collectively, these results reveal an extensive cross-reactivity in the human memory $CD4^+$ T cell repertoire against Enterobacteriaceae antigens.

Extensive clonotype sharing among Enterobacteriaceae-reactive memory $CD4^+$ T cell repertoires

To define the TCR repertoire composition of Enterobacteriaceae-reactive $CD4^+$ T cells, we sequenced TCR Vβ genes in $CFSE^{low}$ memory $CD4^+$ T cells obtained after primary stimulation, as previously described [24]. In all donors analyzed, the TCR repertoire of Enterobacteriaceae-reactive Th cells was polyclonal and comprised a variable number of clonotypes (mean ± SD: *E.coli* 379 ± 370; *K. pneumoniae* 318 ± 182; *E. aerogenes* 525 ± 337; *Shigella ssp* 261 ± 99; *S. typhimurium* 907 ± 543), which were fewer compared to the number of clonotypes in control *S. aureus*- or *M. tuberculosis*-reactive Th cell cultures (mean ± SD: *S. aureus* 1512 ± 386; *M. tuberculosis* 1190 ± 658) (Fig. 4A). A nonmetric multidimensional scaling analysis showed that Enterobacteriaceae-reactive TCR Vβ repertoires co-clustered together and separately from *S. aureus*- or *M. tuberculosis*-reactive repertoires, suggesting a high degree of overlap (Fig. 4B). Indeed, as shown in Figure 4C for one representative donor, many *E. coli*-, *K. pneumoniae*-, *E. aerogenes*-, and *S. typhimurium*-reactive TCR Vβ clonotypes were found shared with 1 (green), 2 (yellow), and even 3 (red) additional Enterobacteriaceae-reactive TCR Vβ clonotypes. Of note, the most expanded TCR Vβ clonotypes in each Enterobacteriaceae-reactive repertoire (top 5%) were mainly broadly cross-reactive, being found in all four Enterobacteriaceae-reactive T cell populations (Fig. 4C). In addition, although the Enterobacteriaceae cross-reactive clonotypes comprised 26–49% of the unique TCR Vβ productive rearrangements of each Enterobacteriaceae-responding repertoire, the cumulative frequency accounted for most of the total T cell response (range 66–93% cumulative percentage of templates; Fig. 4D). Consistent with the lack of cross-reactivity observed in polyclonal cultures and T cell clones, very rare TCR Vβ clonotypes were shared between *S. aureus*- or *M. tuberculosis*-reactive and Enterobacteriaceae-reactive T cells.

To evaluate the level of clonal expansion of Enterobacteriaceae-reactive TCR Vβ clonotypes *in vivo*, we compared in 2 blood donors the Enterobacteriaceae-reactive TCR Vβ clonotypes of $CFSE^{low}$ memory $CD4^+$ T cells with that of total memory $CD4^+$ T cells sequenced directly *ex vivo* (Fig. 4E). Several Enterobacteriaceae-reactive clonotypes could be identified in circulating T cells. Interestingly, broadly cross-reactive TCR clonotypes (red circles) showed higher clonal expansion compared to specific TCR clonotypes, with 11 out of 19 and 21 out of 34 broadly cross-reactive TCR Vβ clonotypes being within the top 5% expanded clonotypes in circulating memory $CD4^+$ T cells of donor A and B, respectively.

Collectively, these findings demonstrate that the human memory $CD4^+$ T cell response to Enterobacteriaceae comprises specific and broadly cross-reactive T cell clones, with cross-reactive clones being the most expanded in vivo.

High rate of Enterobacteriaceae cross-reactive T cells are present in the naïve repertoire

The high extent of cross-reactivity of memory $CD4^+$ T cells against Enterobacteriaceae raised the question as whether cross-reactivity is already set in the naïve repertoire. To address this question, we performed in vitro priming experiments following a protocol previously adopted to study *S. aureus* and *C. albicans* naïve

Figure 3. Memory $CD4^+$ T cells are broadly cross-reactive to Enterobacteriaceae. Bacteria-reactive polyclonal $CD4^+$ T cell lines were obtained by flow cytometry-sorting of $CFSE^{low}$ cells from primary cultures of memory $CD4^+$ T cells. Following expansion, the cells were re-labeled with CFSE and restimulated with the same bacteria (homologous restimulation) or with different bacteria (heterologous restimulation) in the presence of autologous monocytes and measured by flow cytometry. (A) CFSE profiles of polyclonal T cell lines from a representative donor reactive for the bacteria indicated on top and restimulated for 5 days with the bacteria indicated on the right. Homologous restimulation for each culture is highlighted with a grey shadow. (B) Percentage of $CFSE^{low}$ cells after secondary homologous or heterologous restimulation in $n = 5$ different donors (mean values ± SEM) in five independent experiments. $CFSE^{low}$ cells are reported as percentage of total events after normalization on live lymphocytes. $^*p$-value < 0.05, $^{**}p$-value < 0.01, as determined by two tailed paired *t*-test compared to positive control (homologous antigen in the re-stimulation). (C and D) $CFSE^{low}$ cells from primary cultures of memory $CD4^+$ T cells were also used to isolate a large panel of Enterobacteriaceae-reactive T cell clones by limiting dilution. The T cell clones specific for the bacteria indicated on top of the heatmaps in C were then screened for cross-reactivity with a panel of bacteria as indicated at the bottom of the heatmaps in the presence of autologous monocytes. Proliferation was assessed on day 3 after a 16-h pulse with $[^3H]$-thymidine and expressed as counts per min (Cpm). The heatmaps report the Cpm of one of at leat two independent experiments with T cell clones isolated from a representative donor. The number of clones tested are reported on top of each heatmap. (D) Pooled data of the patterns of cross-reactivity of T cell clones isolated from the $CFSE^{low}$ fractions of memory $CD4^+$ T cells obtained from n=8 different donors in 8 independent experiments. Screening of T cell clones was performed in at least two independent experiments as described in panel (C). The barplots represent the frequency of non-cross-reactive T cell clones (left) or of T cell clones cross-reactive to other three or more Enterobacteriaceae species (right). The bar indicates mean ± SEM. Numbers on top indicate the total number of T cell clones analyzed with the indicated bacteria. $^*p$-value < 0.05, $^{**}p$-value < 0.01, $^{***}p$-value < 0.001, $^{****}p$-value < 0.0001 as determined by two-tailed unpaired *t*-test.

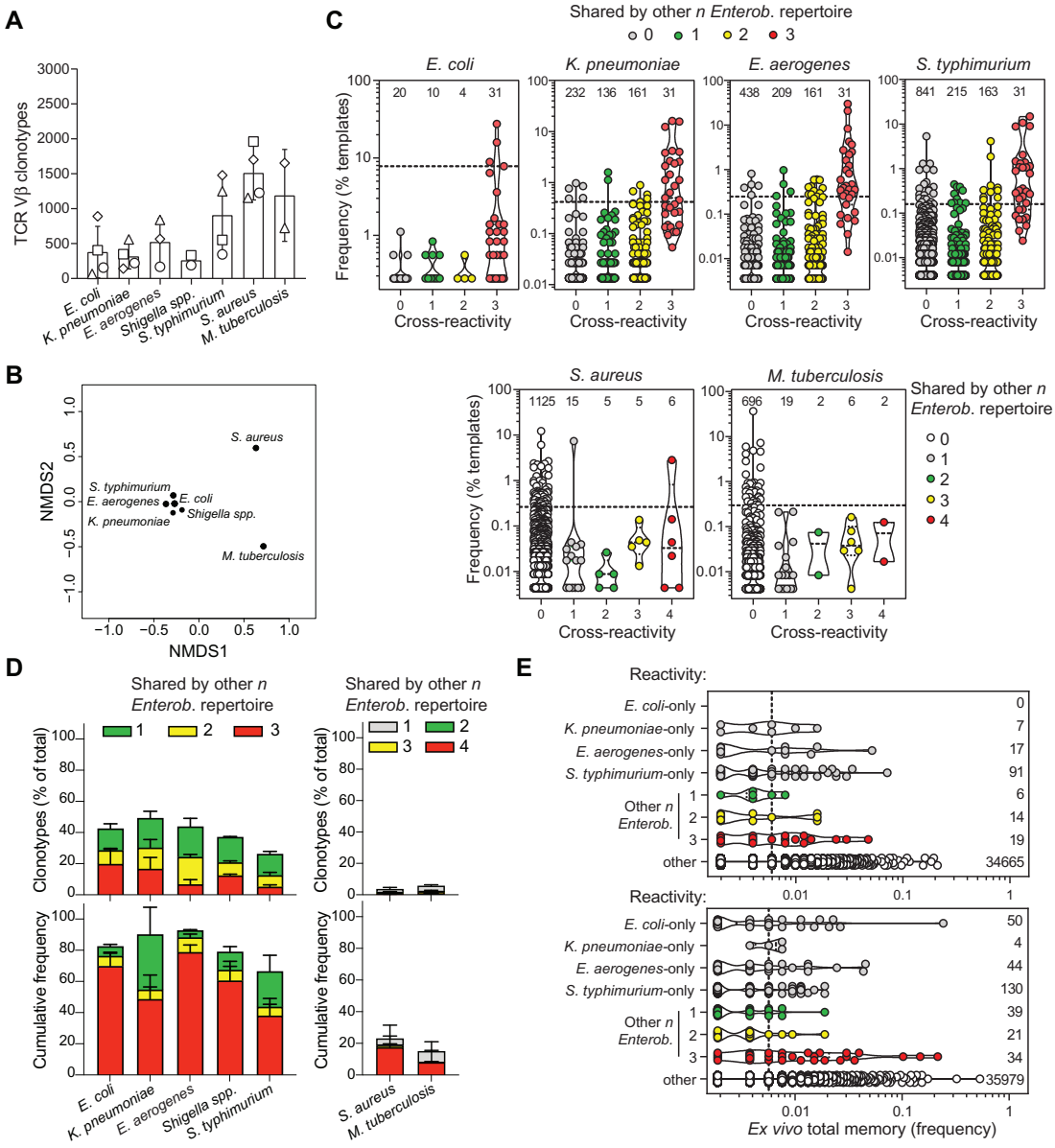


Figure 4. Extensive clonotype sharing among Enterobacteriaceae-reactive memory CD4$^+$ T cell repertoires. Bacteria-reactive CFSE^{low} cells were flow cytometry-sorted from stimulated memory CD4$^+$ T cells, and their TCR Vβ repertoire was determined by deep sequencing. (A) Number of unique productive TCR Vβ nucleotide rearrangements resolved from each bacteria-responsive memory repertoire in $n = 2$–4 different donors in two independent experiments. Each symbol represents a different donor. Bars indicate mean ± SD ($n = 2$–4). (B) Pairwise similarity of each antigen-reactive TCR Vβ repertoire was calculated by Chao-Jaccard overlap. The reciprocal distance of each TCR Vβ repertoire was plotted using Kruskal's nonmetric multidimensional scaling. Each point of the graph represents a distinct bacteria-reactive memory CD4$^+$ TCR Vβ repertoire (average from $n = 2$–4 donors): the closer the points, the more similar the repertoires in terms of sharing of TCR Vβ clonotypes. (C) Violin plots of the frequency distribution of TCR Vβ clonotypes reactive to Enterobacteriaceae or control bacteria from a representative donor. Each dot represents a unique TCR Vβ nucleotide rearrangement, and the color code indicates the number of distinct Enterobacteriaceae-reactive repertoires in which the clonotype was found. Median values are reported as dashed lines. The number of clonotypes for each class of reactivity is reported on top. Dotted lines in the graphs indicate the frequency of the top 5% expanded clonotypes. Because of limited cell number, *Shigella* was not tested in this donor. (D) Stacked bar plots of percentage of unique TCR Vβ rearrangements (upper panels) and corresponding cumulative frequency of templates (lower panels) of Enterobacteriaceae cross-reactive TCR Vβ clonotypes in $n = 2$–4 different donors analyzed in two independent experiments. Each sub-bar indicates the mean ± SD ($n = 2$–4). Color code indicates cross-reactive clonotypes found shared between the indicated Enterobacteriaceae-reactive repertoires or control repertoires, and other one, two, three, or four Enterobacteriaceae-reactive repertoires. (E) Violin plots of the frequency distribution of Enterobacteriaceae-reactive TCR Vβ clonotypes from $n = 2$ different donors measured by deep sequencing of total memory CD4$^+$ T cells directly *ex vivo* after flow cytometry-sorting (upper panel: donor A; lower panel: donor B). The reactivity of each clonotype was determined by comparison with the collection of TCR Vβ nucleotide sequences measured in CFSE^{low} cultures obtained by stimulation of memory CD4$^+$ T cells from the same donors. The color code indicates clonotypes specific (grey circles) or cross-reactive with one (green circles), two (yellow circles), or three (red circles) Enterobacteriaceae species. The number of clonotypes for each class of reactivity is reported on the right. The number and frequency distribution of all other clonotypes in the memory CD4$^+$ T cell repertoire is also reported (white circles). Dotted lines in the graphs indicate the frequency of the top 5% expanded clonotypes.

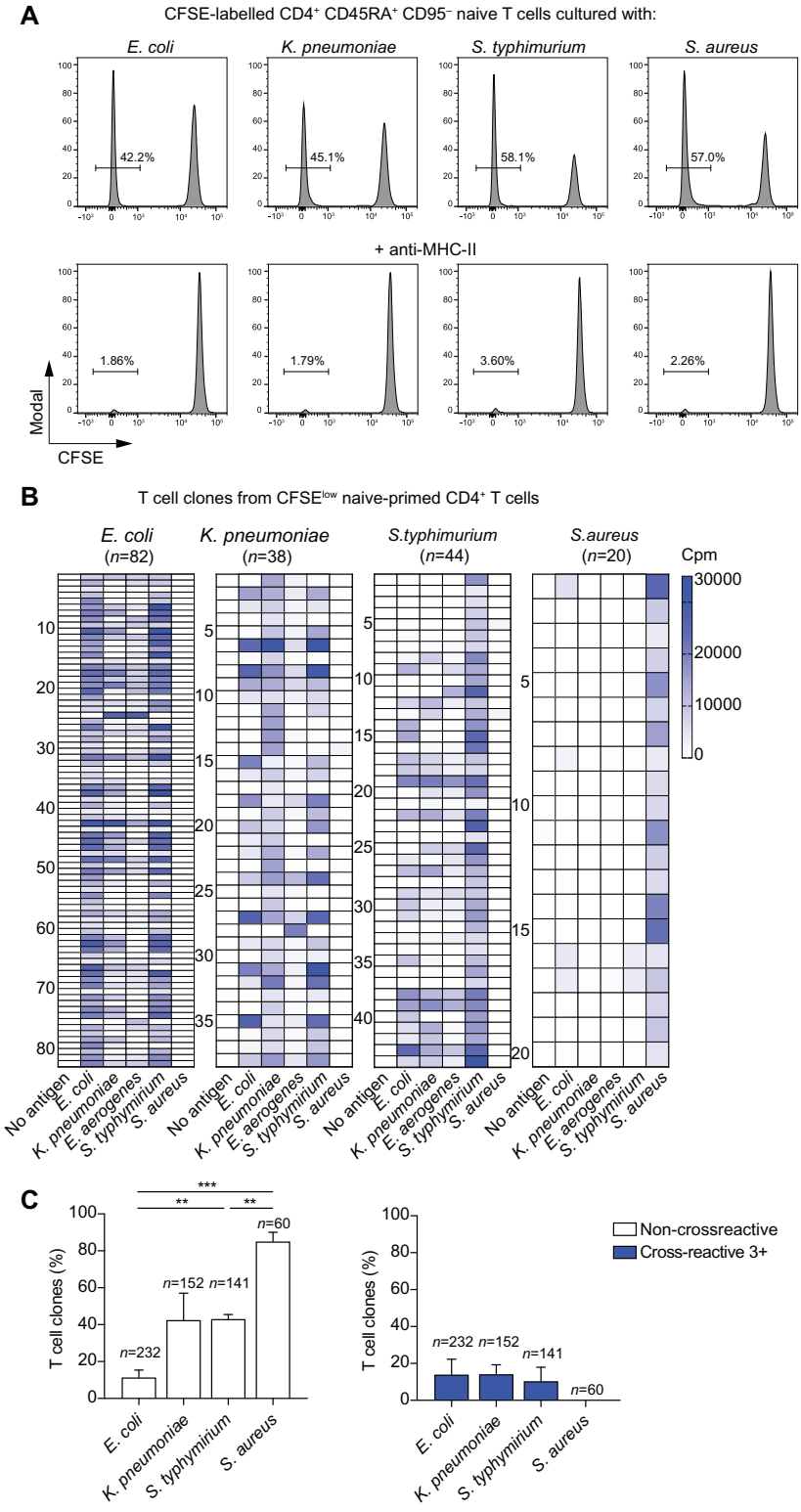


Figure 5. Cross-reactive T cells to Enterobacteriaceae are already detected in the naïve repertoire. (A) Human naïve $CD4^+CD45RA^+CD95^-$ T cells were flow cytometry-sorted at high purity, labeled with CFSE and primed in vitro for 10 days with the indicated heat-inactivated bacteria and autologous monocytes in absence (upper panel) or presence (lower panel) of anti-MHC-II blocking antibodies. Shown are the CFSE profiles by flow cytometry on day 10 after priming and the percentages of $CFSE^{low}$ proliferating cells from a representative donor out of a total of 6 different donors tested in 6 independent experiments. (B, C) Antigen-primed $CFSE^{low}$ T cells were cloned by limiting dilution. T cell clones isolated from each $CFSE^{low}$ fraction were screened with a panel of heat-inactivated bacteria in the presence of autologous monocytes. Proliferation was assessed on day 3 after a 16-h pulse with $[^3H]$-thymidine and expressed as counts per min (Cpm). The heatmaps report the Cpm of T cell clones isolated from $CFSE^{low}$ fractions of a representative donor. The data are representative of at least two independent experiments (B). The antigen used for the initial in vitro priming and the number of clones tested are reported on top of each heatmap. Each row of the heatmaps refers to an individual T cell clone, stimulated with the antigens reported at the bottom. (C) Pooled data of the patterns of reactivity of T cell clones isolated from each $CFSE^{low}$ fraction of in vitro primed naïve $CD4^+$ T cells from $n = 6$ different donors from six independent experiments. The reactivity of T cell clones was assessed as described in B in at least two independent experiments. The barplots represent the frequency of non-cross-reactive T cell clones (*left*) or of T cell clones cross-reactive to other three or more Enterobacteriaceae species (*right*). The bar indicates mean ± SEM. Numbers on top indicate the total number of T cell clones analyzed with the indicated bacteria. $^*p$-value < 0.05, $^{**}p$-value < 0.01, $^{***}p$-value < 0.001, as determined by two tailed unpaired *t*-test.

T cells [24, 30]. Briefly, highly pure naïve $CD4^+$ T cells were FACS-sorted from the blood of healthy donors, labelled with CFSE and cultured with autologous monocytes pulsed with the different Enterobacteriaceae species or *S. aureus*, as control. Proliferating $CFSE^{low}$ T cells were detected in all stimulated cultures but not in control cultures or in cultures performed in the presence of MHC-II blocking antibodies (Fig. 5A), consistent with a high frequency of naïve T cell precursors for these complex antigens. A large panel of Th cell clones ($n = 585$) were then isolated from primed $CFSE^{low}$ T cells isolated from six donors and tested for

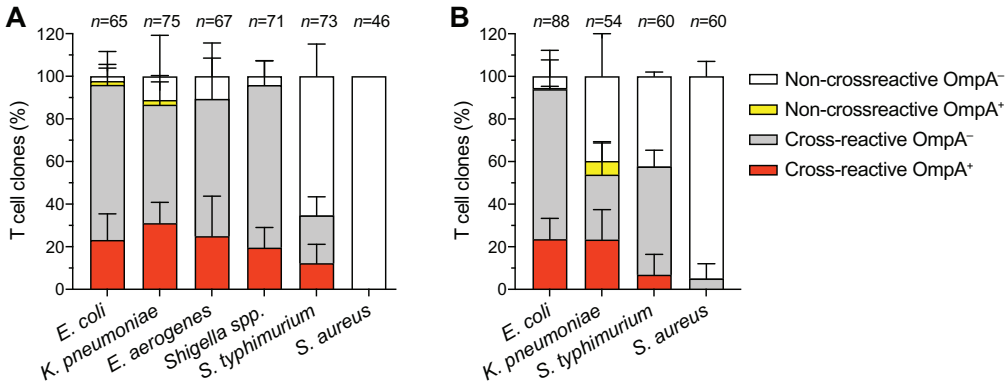


Figure 6. Outer membrane protein A is an important antigen determinant of cross-reactivity to Enterobacteriaceae. Enterobacteriaceae-specific T cell clones isolated from naïve and memory CD4$^+$ T cells of $n = 3$ different donors in three independent experiments were stimulated with autologous monocytes in the absence or presence of recombinant Outer membrane protein A (OmpA) from *K. pneumoniae*. Proliferation was assessed on day 3 after a 16-h pulse with [^{3}H]-thymidine. OmpA-reactive (OmpA$^+$) T cell clones were scored based on the proliferative response (stimulatory index, S.I. > 5, Δ Cpm >3000) in at least 2 independent experiments. The stacked barplots represent the percentage of cross-reactive (to 3 or more Enterobacteriaceae species) T cell clones (red and grey bars) and specific T cell clones (yellow and white bars) isolated from CFSE^{low} fractions obtained after stimulation with the indicated bacteria that reacted (red and yellow bars) or did not react (grey and white bars) to OmpA. Each sub-bar indicates the mean ± SEM. Numbers on top indicate the total number of T cell clones analyzed with the indicated bacteria.

their reactivity against different Enterobacteriaceae species. As shown in Figure 5B for a representative donor, a sizable fraction of the Enterobacteriaceae-reactive Th cell clones isolated from in vitro primed naïve T cells were cross-reactive to at least another Enterobacteriaceae species. Analysis of all donors revealed that up to 14% of the T cell clones cross-reacted to three additional Enterobacteriaceae species (Fig. 5C). Interestingly, a few Th cell clones derived from in vitro *S. aureus*-primed naïve T cells were able to cross-react with phylogenetically distant Enterobacteriaceae species (Fig. 5B).

Collectively, these findings show that Enterobacteriaceae cross-reactive T cells are already present in the naïve CD4$^+$ T cell compartment, which concurrently holds a sizable fraction of precursors specific for individual Enterobacteriaceae species.

Outer membrane protein A is a target of Enterobacteriaceae-cross-reactive T cells

The T cell cross-reactivity observed for Enterobacteriaceae may be associated with the presence of conserved antigenic epitopes in different bacteria species or due to an intrinsic TCR binding degeneracy [31–33]. To address this point, we interrogated the literature for potentially immunogenic proteins conserved within the Enterobacteriaceae family. Outer membrane protein A (OmpA) is a porin widely expressed by gram-negative bacteria, highly conserved among the Enterobacteriaceae family (Supporting Information Fig. S4) and throughout evolution [34, 35], and an important target of both humoral and cellular protective immune responses in vivo [36–38]. To evaluate the immunogenicity of OmpA for CD4$^+$ T cells, Enterobacteriaceae-reactive T cell clones isolated from memory and naïve compartments were stimulated with recombinant OmpA from *K. pneumoniae* in presence of autologous monocytes. We observed reactivity to OmpA in 12% to 33% (n=397) of clones isolated from memory CD4$^+$ T cells, and in 7–30% ($n = 262$) of clones from in vitro primed naïve CD4$^+$ T cells (Fig. 6A and B). Importantly, most OmpA-specific T cell clones were broadly cross-reactive to three or more Enterobacteriacaeae.

These results indicate OmpA as one of the immunogenic antigens targeted by a large percentage of Enterobacteriaceae broadly cross-reactive CD4$^+$ T cells, which likely recognize conserved epitopes with similar amino acid sequences encoded by different bacterial species.

Discussion

In this study, we performed a thoroughly analysis of the CD4$^+$ T cell repertoire to commensals and pathogens belonging to the Enterobacteriaceae family in humans. In line with recent studies [19,20,39], we show that Enterobacteriaceae-specific memory CD4$^+$ T cells have a Th1*/17 phenotype characterized by the expression of CCR6 and CXCR3 and production of IFN-γ, IL-17A, and IL-22. By combining multiple approaches that allow different levels of sensitivity and throughput, we report a high level of cross-reactivity of memory CD4$^+$ T cells at the monoclonal (T cell clones), oligoclonal (T cell lines), and polyclonal (TCR Vβ sequencing) level. Surprisingly and in contrast to previous reports [19], we found Enterobacteriaceae cross-reactive naïve T cells in all donors analyzed. Thus, while it is generally accepted that crossreactive T cells are the progeny of rare T cells that are selected by serial stimulations with different antigens, a phenomenon referred to as original antigenic sin [40], our results indicate that, in the cases of Enterobacteriaceae, cross-reactive T cells already represent a sizable fraction of the antigen-inexperienced naïve T cell repertoire.

We showed that Enterobacteriaceae broadly cross-reactive clonotypes are those most frequently found after *in vitro* stimulation of memory T cells and, importantly, are among the most expanded clonotypes of circulating memory T cells. The selection of a pool of clonally expanded cross-reactive memory T cells might be on the one hand advantageous for host protection, since it could potentially confer heterologous immunity to an extended range of pathogens prior to antigen exposure [41,42]. On the other hand, pre-existing cross-reactive immunity could also be detrimental if the pool of expanded T cells holds an effector phenotype not suitable to provide protection to the newly encountered pathogens. Development of antigen-specific Th responses with inappropriate phenotypes can result in failure of host protection, as shown in humans for *Mycobacterium leprae* [43,44], *M. tuberculosis* [45] and *Candida albicans* [46]. Cross-reactive T cells can be a double-edged sword, as shown recently in the case of *Candida*-induced Th17 cells that, while ensuring intestinal homeostasis, can contribute to lung inflammation and immunopathology upon cross-recognition of airborne *Aspergillus fumigatus* [47]. Finally, T cells against gut microbiota and other environmental microbes cross-reactive against neoantigens may affect immunogenicity of cancer and can account for the efficacy of immune checkpoint inhibitors [reviewed in 48].

We identified OmpA as one of the antigenic determinants of Th cells broadly cross-reactive with Enterobacteriaceae. Sequence analysis of OmpA homologs in different bacterial species revealed high sequence identity, thus suggesting that cross-reactivity to Enterobacteriaceae is mainly due to T cell recognition of conserved epitopes in different, although phylogenetically closely related bacteria. Given the evidence of OmpA as an important target of antibody response to Enterobacteriaceae [36–38], our findings are consistent with a tight connection existing between T- and B-cell immune responses, often converging to the same antigenic targets. T cell cross-reactivity to multiple antigens can be also due to promiscuous TCR engagement of many MHC-II molecules loaded with highly different peptides. The latter phenomenon is known as TCR degeneracy and relies to the intrinsic weak affinity of TCR-pMHC-II interaction [31–33] that can allow unexpected patterns of cross-recognition [41, 42]. TCR degeneracy might be at the basis of the observed cross-reactivity of rare naïve and memory Th cell clones that respond to phylogenetically distant bacteria, e.g., *S. aureus* and Enterobacteriaceae.

An interesting observation of this study was that septic patients suffering from *K. pneumoniae* systemic infection have a selective reduction of circulating CXCR3$^+$ Th cells (both Th1 and Th1*/17) and lack *K. pneumoniae*-reactive memory Th cells, while *M. tuberculosis*-reactive T cells are still detectable. The selective defect in *K. pneumoniae*-reactive Th cells in these patients suggests that sepsis-induced immunosuppression can be elicited in an antigen-specific, TCR-mediated fashion, for instance as the result of impaired T cell function or of activation-induced cell death (AICD) caused by massive antigen exposure [28, 29]. Interestingly, in the blood of two patients that survived *K. pneumoniae* sepsis, the Th1*/17 response to *K. pneumoniae* measured after recovery was unaffected and comparable to the one observed in healthy controls (data not shown). These data suggest that the presence of a circulating pool of *K. pneumoniae*-reactive T cells may correlate with protection from sepsis, although further studies are needed to precisely define this point.

In spite of extensive research and clinical trials, for many pathogens of the Enterobacteriaceae family such as *Shigella* spp, *K. pneumoniae* or *Yersinia pestis* there are no safe and effective vaccines [12, 49, 50], and currently available vaccines for other species, such as thyphoidal *Salmonella enterica* serovars, showed mild efficacy and conferred short-lasting immunity [11]. In light of our findings, we propose that the large level of Enterobacteriaceae cross-reactivity observed in the memory CD4$^+$ T cell repertoire can have a profound impact on immunization outcome and development of protective immunity to closely related pathogens, and therefore should be a parameter to take carefully in consideration to improve vaccine efficacy. The identification of antigenic determinants of cross-reactive and specific T cell responses to commensals and pathogens might be relevant for the rational design of subunit vaccines against newly emerging multidrug resistant Enterobacteriaceae. Directing the immune response to common or unique antigenic targets might allow to hijack, or alternatively overcome, the pre-existing immunity to ensure host protection.

Materials and methods

Cells and cell sorting

Blood from healthy donors was obtained from the Swiss Blood Donation Center of Basel and Lugano. Blood from septic patients was obtained from Policlinico Umberto I, Department of Public Health and Infectious Diseases (University of Rome "Sapienza", Italy). All blood donors provided written informed consent for participation in the study. The study was approved by the Ethical committees of Cantone Ticino, Switzerland (Ref. 2018-02166/CE 3428) and of the University of Rome "Sapienza", Italy (Ref. 3640). Human primary cell protocols were approved by the Federal Office of Public Health (no. A000197/2 to F.S.). PBMCs were isolated with Ficoll-Paque Plus (GE Healthcare). Monocytes and total CD4$^+$ T cells were isolated by positive selection using CD14 and CD4 magnetic microbeads, respectively (Miltenyi Biotech). Total CD4$^+$ cells obtained by positive selection were stained on ice for 15-20 min with the following fluorochrome-labeled mouse monoclonal antibodies: CD8-PE–Cy5 (clone B9.11; cat. no. A07758), CD14-PE–Cy5 (clone RMO52; cat. no. A07765), CD16-PE–Cy5 (clone 3G8; cat. no. A07767), CD56-PE/Cy5 (clone N901; cat. no. A07789), CD19-PE/Cy5 (clone J3-119; cat. no. A07771), CD25-PE–Cy5 (clone B1.49.9; cat. no. IM2646) from Beckman Coulter, CD4–PE–Texas Red (clone S3.5; cat. no. MHCD0417), CD45RA-Qdot 655 (clone MEM-56; cat. no. Q10069), CD95-PerCP-eFluor 710 (clone DX2; cat. no. 46-0959-42) from

ThermoFisher Scientific, CCR7–BV421 (clone G043H7; cat. no. 353208), CCR6-BV605 (clone G034E3; cat. no. 353420), CXCR3-Alexa Fluor 647 (clone G025H7; cat. no. 353712) from BioLegend, CCR4-PE-Cy7 (clone 1G1; cat. no. 557864) from BD Biosciences. Naïve and memory $CD4^+$ T cells were sorted to over 98% purity on a FACSAria III (BD) after exclusion of $CD8^+$, $CD14^+$, $CD16^+$, $CD56^+$, $CD19^+$, and $CD25^{bright}$ cells. Naïve T cells were sorted as $CD4^+CD45RA^+CCR7^+CD95^-$ cells; the remaining $CD4^+$ T cells were sorted as total memory. Memory Th cell subsets were sorted as follows: $CXCR3^+CCR4^-CCR6^-$ (enriched in Th1), $CCR4^+CXCR3^-CCR6^-$ (enriched in Th2), $CCR6^+CXCR3^+CCR4^-$ (enriched in Th1*/17), $CCR6^+CCR4^+CXCR3^-$ (enriched in Th17). Flow cytometry analysis was performed using FlowJo and adhering to published guidelines [51].

Microbes and antigens

The following microbial strains were produced at the Servizio di Microbiologia EOLAB, Ente Ospedaliero Cantonale (Bellinzona, Switzerland): *Escherichia coli* (ATCC DH10B), *Klebsiella pneumoniae* (ATCC 43816), *Enterobacter aerogenes* (ATCC 13048), *Shigella flexneri* (ATCC 12021), *Shigella sonnei* (ATCC 9290), *Salmonella typhimurium* (ATCC 14028), and *Staphylococcus aureus* (ATCC 29213). Bacteria were cultured in aerobic condition at 37°C in Luria-Bertani broth. After expansion, bacteria were extensively washed in PBS and heat inactivated at 60°C for 2 h. Bacteria count of each isolate was determined by staining with 4', 6-diamidino-2'-phenylindole, dihydrochloride (DAPI, Fisher Scientific) and counting at the fluorescence microscope. Ratio used for stimulation assays was two bacteria particles per monocyte. Whole cell lysate of *Mycobacterium tuberculosis* (strain CDC1551, cat. no. NR-14823) was obtained through BEI Resources (NIAID, NIH), and was used at a final concentration of 3 μg/ml. Recombinant OmpA from *Klebsiella pneumoniae* (ATCC 43816) was kindly provided by Humabs BioMed (Bellinzona, Switzerland), and used at 5 μg/ml for stimulation assays.

T cell stimulation

T cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 1% (v/v) nonessential amino acids, 1% (v/v) sodium pyruvate, penicillin (50 U/ml), streptomycin (50 μg/ml) (all from Invitrogen) and 5% human serum (Swiss Red Cross). Sorted memory $CD4^+$ T cells or naïve $CD4^+CD95^-$ T cells were labeled with CFSE and cultured at a ratio of 2:1 with untreated or antigen-pulsed irradiated autologous monocytes. Depending of the antigen, monocytes were pulsed 3-5 h with heat-inactivated bacteria (two particles per monocytes) or *M. tuberculosis* whole cell lysate (3 μg/ml). To determine MHC restriction, the assay was performed in the absence or presence of blocking anti-MHC-II monoclonal antibodies produced in house from hybridoma cell lines (anti-HLA-DR, clone L243 from ATCC, cat. no. HB-55; anti-HLA-DQ, clone SPVL3 [52]; anti-HLA-DP, clone B7/21 [53]). Stimulated memory and naïve T cell cultures were collected at day 6-7 and 8-10, respectively, and stained with antibodies to CD25–PE (clone M-A251; cat. no. 555432) from BD Biosciences and ICOS–APC (clone C398.4A; cat. no. 313510) from BioLegend. Proliferating activated T cells were FACS-sorted as $CFSE^{low}CD25^+ICOS^+$ and expanded in vitro in the presence of IL-2 (500 IU/ml). In some experiments, $CFSE^{low}$ cultures were labeled again with CFSE and challenged in secondary restimulation with antigen-pulsed irradiated autologous monocytes. Readout of T cell proliferation was determined at day 4-5 after secondary stimulation. Percentage of $CFSE^{low}$ cells were normalized on live lymphocytes as follows: (% lymphocytes) × (% live cells) × (% $CFSE^{low}$ cells).

Intracellular cytokine staining

$CFSE^{low}$ cultures from *ex vivo* stimulated memory T cells were stimulated with PMA and ionomycin for 5 h in the presence of Brefeldin A for the last 2 h (all reagents from Sigma–Aldrich). Cell viability was determined by staining with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFisher), according to the manufacturer's instructions. Subsequently, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and then stained with the following antibodies: IL-22-PerCP-eFluor710 (22URTI, cat. no. 46-7229-42) from eBioscience, IL-17A-BV605 (BL168, cat. no. 512326) and IFN-γ-APC-Cy7 (4S.B3, cat. no. 502530) from BioLegend. Stained cells were analyzed using a BD LSRFortessa (BD Biosciences), and flow cytometry data were analyzed with FlowJo software (Tree Star).

Isolation of T cell clones

$CFSE^{low}$ cultures FACS-sorted from ex vivo stimulated memory $CD4^+$ T cells or from in vitro primed naïve $CD4^+CD95^-$ T cells were cloned by limiting dilution, as previously described [54]. T cell clone reactivity was determined by stimulation with untreated or antigen-pulsed irradiated autologous monocytes. Depending of the antigen, monocytes were pulsed 3-5 h with heat-inactivated bacteria (two particles per monocytes), *M. tuberculosis* whole cell lysate (3 μg/ml) or recombinant OmpA (5 μg/ml). More than 50% of the T cell clones growing from the $CFSE^{low}$ fractions were bacteria-specific and were selected for further analyses. The remaining clones, which were not autoreactive, were excluded from the study. To determine MHC restriction, stimulation of T cell clones was performed in the absence or presence of the blocking anti-MHC-II monoclonal antibodies. In all experiments proliferation was assessed on day 3, after incubation for 16 h with 1 μCi/ml [methyl-^{3}H]thymidine (Perkin Elmer). Data were expressed as counts per min (Cpm). Positive T cell clones were scored based on a cut-off value of (i) $\Delta Cpm \geq 3\times10^3$ (Cpm with antigen and APCs – Cpm with APCs only) and (ii) stimulation index (SI) ≥ 5 (Cpm with antigen and APCs / Cpm with APCs only), according to well established criteria.

TCR Vβ deep sequencing

Ex vivo-sorted total memory $CD4^+$ and $CFSE^{low}$ fractions from antigen-stimulated memory $CD4^+$ T cell cultures ($2.5–5 \times 10^5$ cells) were analyzed by deep sequencing. In brief, cells were centrifuged and washed in PBS, and genomic DNA was extracted from the pellet using QIAamp DNA Micro Kit (Qiagen), according to manufacturer's instructions. Genomic DNA quantity and purity were assessed through spectrophotometric analysis. Sequencing of TCR Vβ CDR3 was performed by Adaptive Biotechnologies using the ImmunoSEQ assay (http://www.immunoseq.com). In brief, following multiplex PCR reaction designed to target any CDR3 Vβ fragments; amplicons were sequenced using the Illumina HiSeq platform. Raw data consisting of all retrieved sequences of 87 nucleotides or corresponding amino acid sequences and containing the CDR3 region were exported and further processed. The assay was performed at deep level for *ex vivo*-sorted total memory $CD4^+$ (detection sensitivity, 1 cell in 200 000) and at survey level for $CFSE^{low}$ antigen-reactive cultures (detection sensitivity, 1 cell in 40 000). Each clonotype was defined as a unique productively rearranged TCR Vβ nucleotide sequence; data processing was done using the productive frequency of templates provided by ImmunoSEQ Analyzer V.3.0 (http://www.immunoseq.com). For each repertoire, a frequency corresponding to the top 5^{th} percentile in the frequency-ranked list of unique clonotypes was chosen as threshold (top 5%). Chao-Jaccard overlap between pairs of TCR repertoires was calculated using R package "fossil" [55]; Kruskal's Non-metric Multidimensional Scaling (NMDS) of average Chao-Jaccard overlaps was performed using R package "MASS" [56].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software. Significance was assigned at p value < 0.05 unless stated otherwise. Specific tests are indicated in the figure legends for each comparison. Analysis of TCR Vβ repertoires was performed using R software version 3.5.1. Multiple sequence alignment was performed using CLC Genomics Workbench version 8 (QIAGEN).

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Author contributions: A.C. characterized the T cell response in healthy donors and septic patients, isolated T cell clones, performed bioinformatics analyses of TCR Vβ deep sequencing, analyzed the data, and wrote the manuscript; J.G. characterized the T cell response in healthy donors, isolated T cell clones, analyzed the data, and wrote the manuscript; G.D. provided assistance for the characterization of the T cell response in healthy donors; D.J. performed cell sorting; F.B.M. provided microbes and assistance for antigen preparation; M.V., A.R., and M.F. collected biological samples; A.L. provided supervision and wrote the manuscript; M.C.G. provided supervision and designed the experiments in septic patients; D.L. designed the experiments, characterized the T cell response in healthy donors and septic patients, isolated T cell clones, analyzed the data, and wrote the manuscript; F.S. provided overall supervision, designed the experiments, and wrote the manuscript.

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Abbreviations: **AICD**: activation-induced cell death · **Cpm**: counts per min · **OmpA**: outer membrane protein A · **SI**: stimulation index

Full correspondence: Federica Sallusto or Daniela Latorre, Institute of Microbiology, ETH Zurich, Vladimir-Prelog-Weg 1-5/10, 8093 Zurich, Switzerland, Tel +41 44 633 65 28; Fax +41 44 633 12 83; e-mail: sallustf@ethz.ch; latorred@biol.ethz.ch

Present address: Jérémie D Goldstein, Department of Pathology-Immunology, Faculty of Medicine, University of Geneva, Geneva, Switzerland

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