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Mixed mucosal-parenteral immunizations with the broadly conserved pathogenic *Escherichia coli* antigen SslE induce a robust mucosal and systemic immunity without affecting the murine intestinal microbiota

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ABSTRACT

Emergence and dissemination of multidrug resistance among pathogenic *Escherichia coli* have posed a serious threat to public health across developing and developed countries. In combination with a flexible repertoire of virulence mechanisms, *E. coli* can cause a vast range of intestinal (InPEC) and extraintestinal (ExPEC) diseases but only a very limited number of antibiotics still remains effective against this pathogen. Hence, a broad spectrum *E. coli* vaccine could be a promising alternative to prevent the burden of such diseases, while offering the potential for covering against several InPEC and ExPEC at once. SslE, the Secreted and Surface-associated Lipoprotein of *E. coli*, is a widely distributed protein among InPEC and ExPEC. SslE functions *ex vivo* as a mucinase capable of degrading mucins and reaching the surface of mucus-producing epithelial cells. SslE was identified by reverse vaccinology as a protective vaccine candidate against an ExPEC murine model of sepsis, and further shown to be cross-effective against other ExPEC and InPEC models of infection. In this study, we aimed to gain insight into the immune response to antigen SslE and identify an immunization strategy suited to generate robust mucosal and systemic immune responses. We showed, by analyzing T cell and antibody responses, that mice immunized with SslE via an intranasal prime followed by two intramuscular boosts developed an enhanced overall immune response compared to either intranasal-only or intramuscular-only protocols. Importantly, we also report that this regimen of immunization did not impact the richness of the murine gut microbiota, and mice had a comparable cecal microbial composition, whether immunized with SslE or PBS. Collectively, our findings further support the use of SslE in future vaccination strategies to effectively target both InPEC and ExPEC while not perturbing the resident gut microbiota.
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1. Introduction

Escherichia coli is a multifaceted Gram-negative bacterial organism. More than a laboratory workhorse, *E. coli* also colonizes and thrives within the healthy mammalian gut microbiota as a harmless facultative anaerobe. However, the remarkable ability of *E. coli* to allow for acquisition and loss of genetic material by horizontal gene transfer has driven the appearance of multiple pathogenic variants of *E. coli* via successful combinations of virulence factors. Today, at least nine different pathogenic variants, or pathotypes, account for the high versatility of *E. coli*. These pathotypes cause a wide range of human diseases, which bare the potential to be lethal, and carry a significant economic and public health burden.

The six commonly studied pathotypes within intestinal pathogenic *E. coli* (InPEC) are responsible for diarrheal diseases of various severity along the gastrointestinal (GI) tract, and differ by their mechanism of colonization and virulence, as well as the clinical symptoms they provoke [1–3]. A recently published comprehensive study by the World Health Organization (WHO) on the global burden of foodborne diseases estimated that in 2010, InPEC were responsible for over 324 million cases of diarrheal diseases, with more than one third of it affecting children under 5 years of age

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[4]. Among these cases, enteropathogenic *E. coli* (EPEC) was the second leading cause of deaths from diarrheal diseases in the world. Extraintestinal pathogenic *E. coli* (ExPEC) can invade distant sites like the urinary tract, the bloodstream or the central nervous system [2]. Among ExPEC, uropathogenic *E. coli* (UPEC) is the main etiological agent of urinary tract infections (UTIs), accounting for 75% of all uncomplicated UTIs [5]. Recurrent UTIs are a common problem for young women, causing significant morbidity and care-associated cost [6,7]. The neonatal meningitis-associated *E. coli* (NMEC) is one of the leading causes of early- and late-onset neonatal meningitis and sepsis [8,9].

Resistance to commonly used antibiotics such as fluoroquinolones, tetracycline and cephalosporins is now widespread in both InPEC and ExPEC [1,5,7,10,11]. The extent of multidrug resistance (MDR), including to last-line antibiotics such as carbapenems, tigecyclin and colistin [12–14], is becoming a growing concern, especially in the developing world, where treatment options are limited. For many in the scientific and clinical community, vaccines represent one of the most promising approaches to address the ever-increasing antibiotic resistance in pathogenic *E. coli*. The usual suspects such as toxins, adhesins, and siderophores, all important for colonization and virulence of the pathogen, have been targeted as potential vaccine antigens and explored *in vivo* for their immunological and protective properties [7,15–18]. Despite some positive leads, few have gone through clinical trials, and results have had limited success. Among the many difficulties, the high antigenic diversity and virulence factor redundancy of *E. coli* have undoubtedly hampered the identification of a vaccine protective against various members of a specific pathotype, and support the idea that broad protection against pathogenic *E. coli* will most likely be reached through a vaccine containing multiple antigens. In this regard, reverse vaccinology [19] has offered the opportunity to search and rapidly identify, using bioinformatics, potential vaccine candidates that would be secreted or present on the cell surface of multiple *E. coli* pathogenic strains.

Using this strategy on several ExPEC strains, our group has identified protein SslE (for Secreted and Surface-associated Lipoprotein of *E. coli*), also known as ECOK1_3385 or YghJ, as a protective vaccine candidate in a murine sepsis model with NMEC [20]. Additional immunization studies showed SslE to also be protective against other ExPEC strains in different animal models [20,21], as well as an InPEC strain. Furthermore, analysis of human sera of convalescent patients from urosepsis [22] or ETEC infections [23,24] revealed the presence of SslE-specific antibodies, confirming the immunogenicity of SslE. Functionally, SslE was characterized as a mucin-degrading metallopeptidase [25], and shown *in vitro* to degrade several mucins including MUC2, the most common intestinal mucin [21,25]. A series of assays has demonstrated that SslE mucinase activity helps *E. coli* penetration through the mucus layer, a step that could favor colonization by allowing bacteria to better reach the epithelial layer [25,26]. SslE has also been associated with biofilm formation in EPEC [27] and, more recently, with significant tissue damage and hemorrhage in mouse ilea [28], further supporting the role of SslE as an important virulence factor. The SslE-encoding gene is widely distributed in the *E. coli* phylogeny, with a higher presence in intestinal and extraintestinal pathogenic isolates (between 70% and 83%) compared to commensal isolates (59%) [20]. In a recent study, SslE was found to be present in 70% of all strains of an *E. coli* data set containing 1700 complete or draft genome sequences spanning commensals and pathotypes from human and animal origins [22].

Overall, SslE represents a very promising component for a broad-protective vaccine, as it is immunogenic, protective, conserved among different pathotypes, and could play a key role in pathogenic *E. coli* virulence and disease. Yet, very little is known about the mechanism behind SslE protective efficacy. In this study, we selected SslE as model antigen to further characterize the immune response against *E. coli* in mice, with the aim of determining whether a regimen exists that would give robust mucosal and systemic responses. Because the response generated mucosally and systemically can be heavily influenced by the route of immunization chosen, we wanted to take a comparative approach by looking at mucosal-only (intranasal, or i.n), parenteral-only (intramuscular, or i.m), as well as a mixed immunization regimen. It is now well-established that mixing mucosal and parenteral immunizations can have significant benefits in the immune response generated versus mucosal-only or parenteral-only [29–33]; we therefore hypothesized that using a mixed immunization approach would induce a greater immune response, both at the mucosa and systemically. Here, we indeed show that immunizations with SslE using as priming an i.n dose, followed by two i.m boosts (referred as i.n / i.m / i.m), provided the most robust cellular and humoral responses in both the small intestine lamina propria (LP) and the systemic environment, compared to either i.n-only or i.m-only immunizations. Additionally, considering the known presence of antigen SslE in several *E. coli* commensals, we were particularly interested at investigating the effect of an intestinal immune response to SslE on the resident gut microbiota. The influence of the gut microbiota on human health is now well-acknowledged; its disruption has a critical impact on the development and maintenance of the intestinal immune system, and on the onset of severe diseases [34]. Thus, ensuring that an immunization effect on the gut immune response does not perturb the microbiota is paramount. Analysis of the microbial population from both fecal and cecal contents of mice immunized i.n / i.m / i.m reveals no changes to a significant level of the overall composition at the phylum level, and the composition profiles at both family and genus levels appear undisturbed in the cecum before and after immunizations. Together, these results provide the first in-depth immunological profile associated with antigen SslE, and set important groundwork for the use of SslE in future vaccine and clinical studies.

2. Materials and Methods

2.1. Formulation with antigen SslE

Cloning, expression and purification of the NMEC IHE3034 strain SslE recombinant protein were performed as previously described [21]. The batch of recombinant SslE used to perform our experiments was determined to have a purity of 94%, and an endotoxin level below 0.07 EU/μg. Before each immunization, antigen SslE was freshly dialyzed using a Slide-A-Lyser dialysis cassette 10 K MWCO (ThermoFisher Scientific) overnight in PBS 1X in order to remove the glycerol used (40%) to store the antigen at - 20 °C. The antigen was then concentrated using a 15 ml centrifugal filter unit 50 K MWCO (Millipore); the final antigen concentration was determined by using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) according to the manufacturer's recommendations.

For i.n immunizations, we used cholera toxin (CT, Sigma) as adjuvant; we combined 10 μg of recombinant SslE with 3 μg of CT in formulations for a final volume of 10 μl per mouse. For i.m immunizations, we used an oil-in-water emulsion adjuvant, named SEA for Self-Emulsifying-Adjuvant, which was manufactured as previously described [35]; we combined 10 μg of antigen SslE in a 1:1 ratio with SEA in formulations for a final volume of 20 μl per mouse. Each formulation was freshly prepared the day of immunization, using PBS 10X (Ambion) diluted with Water For Injection (WFI). Each formulation batch was inspected for pH

(range of 7.4 ± 0.5) and osmolality (range of 300 ± 60 mOsm/kg). Antigen and adjuvant characterizations were also performed by running each formulation by SDS-PAGE to verify the presence and integrity of each component upon formulation. In particular, for intramuscular formulations, the soluble part of the formulation (the subnatant) was isolated by ultracentrifugation at 60,000 rpm and used in the SDS-PAGE to confirm the antigen stability in the formulation.

2.2. Mice and immunizations

Protocols were approved by the Italian Ministry of Health (authorization number 689/2015-PR). All mice were housed under specific pathogen-free (SPF) conditions, in accordance with institutional guidelines, at the GSK Vaccines Animal Resource Center, which is an AAALAC (Association of Assessment and Accreditation of Laboratory Animal Care) accredited facility. Four groups of ten eight-week old BALB/c mice were immunized with SslE or PBS three times, four weeks apart, at days 1, 29 and 57 (Fig. 1): an i.n-only group i.n / i.n / i.n, an i.m-only group i.m / i.m / i.m, a mixed immunization group i.n / i.m / i.m, and a naive group receiving PBS pH = 7.4 i.n / i.m / i.m. Immunizations were given as 5 μl per nostril on anesthetized animals when done intranasally, and in the quadriceps when done intramuscularly.

2.3. In vitro restimulation of antigen-specific CD4$^+$ T cells and intracellular cytokines staining

To measure CD4$^+$ T cell responses, spleens were harvested and single-cell suspensions were prepared. Splenocytes were plated at 2×10^6 cells/well in 96-well U-bottom plates in RPMI-1640 medium (Gibco-Life Technologies) supplemented with 25 mM HEPES (Gibco-Life Technologies), 10% heat-inactivated FBS (low endotoxin; HyClone, Logan, UT), 1X Pen/Strep/Glut (100X; Gibco-Life Technologies) and 50 μM B-mercaptoethanol (Sigma), and stimulated with 10 μg/ml SslE for 2 h before the addition of 5 μg/ml of Brefeldin A (Sigma) for 4 h at 37 °C + 5% CO_2. Cells were then washed and stained with Live/Dead Fixable Near-IR viability marker (Molecular Probes-Life Technologies). Cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), washed with Perm/Wash buffer (BD Biosciences) and stained with the following antibodies: BV605-labelled anti-CD3 (BD Biosciences), PE-CF594-labelled anti-CD8, BV510-labelled anti-CD4 (Biolegend), V450-labelled CD44, A-488-labelled anti-TNFα (BD Biosciences), BV785-labelled anti-IFNγ (Biolegend), PE-Cy5-labelled anti-IL-2 (Biolegend), PerCP eFluor710-labelled IL-4 and IL-14 (eBioscience), PE-Cy7-labelled anti-IL-17 (eBioscience), all resuspended in Perm/Wash 1X solution. Cells were acquired on an LSR II flow cytometer (BD Bioscences) and analyzed with FLOWJO software (TreeStar). Double-positive cells for CD44 and CD4 were gated on IL-2, IFN-γ, IL-17, IL-4 and IL-13.

2.4. ELISA assays

For all ELISA assays, we used 96 well Nunc-Immuno MicroWell MaxiSorp flat bottom plates (Thermofisher Scientific). Plates were coated with 100 μl of antigen SslE at 1 μg/ml in PBS pH 7.4 overnight at 4 °C. Plates were blocked for 1 h at 37 °C + 5% CO_2 with PBS + 0.05% Tween 20 + 2% BSA heat shock fraction (Sigma-Aldrich). Plates were then incubated with diluted serum, intestinal washes, or fecal pellet supernatants (in series of 2X dilutions) in PBS + 2% BSA for 2 h at 37 °C + 5% CO_2. For each assay, 100 μl of goat anti-mouse secondary antibody coupled to alkaline phosphatase was added at the appropriate dilution in PBS + 2% BSA and incubated for 2 h at 37 °C + 5% CO_2: anti IgG (H + L) at 1:2000, anti-IgA at a dilution of 1:1000, anti-IgG1, anti-IgG2a and anti-IgG2b at a dilution of 1:2000 (all antibodies from SouthernBiotech). Between each of these steps, plates were washed three times with PBS-0.05% Tween 20. Plates were then incubated with 100 μl of p-nitrophenyl-phosphate liquid substrate (Sigma-Aldrich) for 30 min at room temperature then immediately read on a SpectraMax microplate reader (Molecular Devices) at 405 nm. The linear part of the curve was used for calculating titers at a cutoff value of 0.1 (for sera) or 0.5 (for intestinal washes and fecal pellets).

2.5. Lamina propria cells (LPC) cytokine quantification

Purification of LPC from mouse small intestine (SI) was adapted from a previously described protocol [36]. At the end of the immunization schedule, whole SI tissue was cut and incubated twice in PBS 1X (Gibco-Life Technologies) supplemented with 10% heat-inactivated FBS (low endotoxin; HyClone, Logan, UT), 5 mM EDTA (Sigma) and 1 M HEPES (Gibco-Life Technologies) for 20 min at 37 °C, shaking. The precipitate, containing the LPC, was further cut before being digested in RPMI supplemented with 10% FBS, 100 U/ml collagenase type VIII (Sigma) for 20 min. Cells were then washed, passed through a 70 μm nylon cell strainer and resuspended in 100% Percoll (GE Healthcare Life Sciences), which was overlayered with 40% percoll, and centrifuged for 20 min at

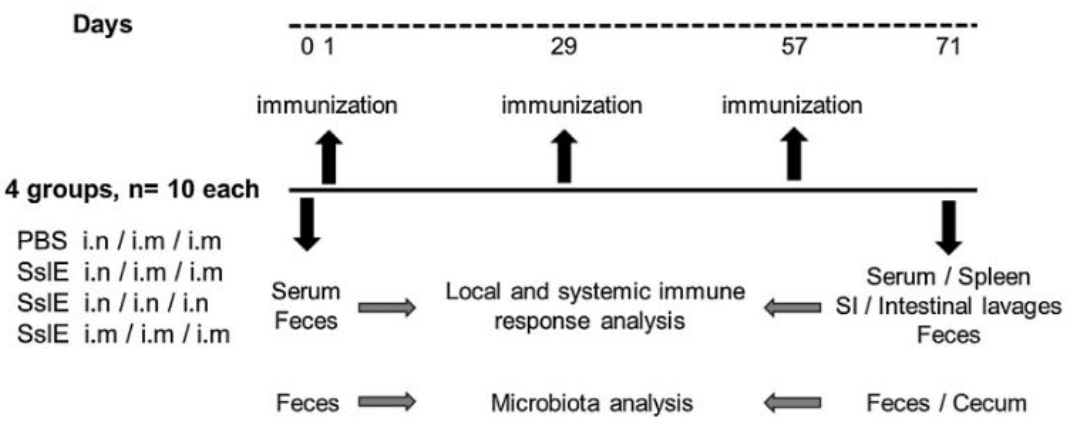

Fig. 1. Study design for the animal experiments. For each experiment (two independent experiments), three immunization groups with SslE and one control (PBS) group were used (n = 10 mice per group). i.n / i.m / i.m: intranasal immunization at day 1, intramuscular immunizations at days 29 and 57. i.n / i.n / i.n: intranasal immunizations at days 1, 29 and 57. i.m / i.m / i.m: intramuscular immunizations at days 1, 29, 57. For each group, samples were taken for analysis at day 0 (preimmune stage), or day 71. For microbiota analysis, only PBS i.n / i.m / i.m and SslE i.n / i.m / i.m were used, with n = 6.

2100 rpm with no break. LPC were recovered from the interface layer. Viability was verified to be > 80% using the Nucleocounter NC-250™ (Chemometec) and staining cells with a solution of acridine orange and DAPI (Solution 18; Chemometec). LPC were plated at 2 x 10^6 cells/well in 96-well U-bottom plates in RPMI-1640 medium (Gibco-Life Technologies) supplemented with 25 mM HEPES (Gibco-Life Technologies), 10% heat-inactivated FBS (low endotoxin; HyClone), 1X Pen/Strep/Glut (100X; Gibco-Life Technologies) and 50 μM B-mercaptoethanol (Sigma), and stimulated with 10 μg/ml SslE for 24 h at 37 °C + 5% CO_2. Plates were then centrifuged for 10 min at 2000 rpm and supernatants were collected and placed at - 80 °C until ready to process.

Each supernatant was quantified for IL-1β, IL-2, IL-4, IL-5, IL-13, TNFα, IFN-γ, IL-17A, IL-17F, IL-21 and IL-22 using a custom made Meso Scale Discovery U-PLEX mouse Biomarker Group 1 Assays (Meso Scale Discovery) according to the manufacturer's instructions. Plates were read using the QuickPlex SQ 120 imager (Meso Scale Discovery).

2.6. Genomic DNA extraction and microbiota composition analysis

Fecal pellets were collected at day 0 and day 71, along with cecal contents at day 71 (Fig. 1) for six mice of the PBS-immunized group and six mice of the i.n / i.m / i.m group (three mice per cage for each group). All samples were immediately stored at -80 °C until ready to process. Genomic DNA from all samples was isolated based on a previously published protocol [37]. Samples were sent for Illumina sequencing and analysis of bacterial population at Life Sequencing (Valencia, Spain), using the capture of the hyper-variable regions V3-V4 of the ribosomal gene 16 s according to previous literature [38]. On average, 75,000 sequences (62,794–97,213 sequences) were recovered from our samples.

2.7. Statistics

All statistics were made using GraphPad Prism (San Diego, California). Statistical significance (for all assays but the microbiota analysis) was calculated using analysis of variance (ANOVA) with Dunnett's post test. A Mann-Whitney two-tailed test was used to compare the microbiota relative abundance between PBS-immunized and SslE-immunized mice. Significance is calculated as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant ($P \geq 0.05$).

3. Results

3.1. Effect of various SslE immunization regimens on the gut immune response

We first investigated the effect of i.n-only, i.m-only, and i.n / i.m / i.m immunizations on the mucosal immune response. Lamina propria cells (LPC) were isolated from small intestines at day 71, and the levels of secreted cytokines released in the supernatant after 24 h of *in vitro* re-stimulation with antigen SslE were measured using MSD technology (Fig. 2). When comparing the i.n / i.m / i.m and i.n / i.n / i.n routes, our results showed that LPC produced cytokines associated with Th1 (IFN-γ, TNF-α, IL-2), Th2 (IL-4, IL-5, IL-13) and Th17 (IL-17A, IL-17F, IL-21 and IL-22) responses in both these groups. Interestingly, although the levels of Th1 cytokines produced were nearly identical in both protocols, we observed a statistically significant increase in Th2 cytokines when mice were immunized through the i.n / i.m / i.m routes, notably with a > 5-fold increase of IL-13. The i.n-only immunizations led, on the other hand, to a 2.5-fold increase of IL-17A compared to the i.n / i.m / i.m routes. The levels of recovered cytokine were similar between i.n-only and mixed immunization groups for IL-17F, as well as for IL-22, for which we roughly obtained a 12-fold increase compared to naive mice. As for i.m / i.m / i.m immunizations, this regimen only gave a very limited cytokine response in the small intestine (SI), at least for the panel of cytokines that we investigated.

These results showed that, within the protocols tested, an i.n prime immunization is important to induce a Th1, Th2 and Th17 responses in the SI. The i.m boosts seem to result in a greater induction of Th2 cytokines, while the i.n boosts increase production of IL-17A after antigen recall. Overall, both i.n / i.n / i.n and i.n / i.m / i.m immunization protocols appear to be efficient at inducing a robust intestinal T cell response to antigen SslE.

The gut humoral immune response often plays a critical role in protective immunity against enteric pathogens [39], so we looked at the effects of each of the various immunization protocols on the release of antigen-specific secreted immunoglobulin A (SIgA) antibodies. To do so, we quantified by ELISA SslE-specific SIgA titers recovered in both intestinal washes and fecal pellets at day 71 (Fig. 3). Similarly to the cellular response in the small intestine LP, results obtained with i.m / i.m / i.m immunizations were limited, with virtually no detectable levels of SIgAs. On the other hand, i.n / i.n / i.n or i.n / i.m / i.m regimens were able to induce detectable SIgA titers that were of the same magnitude when measured in intestinal washes; however, the median SIgA titer recovered from fecal pellets was 5 times higher with i.n / i.m / i.m than with i.n / i.n / i.n immunizations. Thus, the mixed i.n / i.m / i.m protocol generates just as high, if not more, levels of SslE-specific SIgAs versus a mucosal-only, i.n / i.n / i.n protocol.

3.2. Effect of various SslE immunization regimens on the systemic immune response

Since we consider SslE to be a strong candidate as part of a universal vaccine against both InPEC and ExPEC strains, we wanted to assess not only the intestinal immune response, but also the systemic immune response to antigen SslE. To look at and compare the cellular immune response to antigen SslE after our different immunization protocols, splenocytes were isolated at day 71 and re-stimulated *in vitro* with antigen SslE for 6 h (with the last 4 h with Brefeldin A). We then performed an intracellular staining in order to quantify by flow cytometry the cytokine response of effector memory T cells after antigen recall, which is presented as frequency of the total amount of $CD4^+/CD44^+$ T cells recovered (Fig. 4). For all of the cytokines tested, the i.n / i.m / i.m immunization protocol led to a greater percentage of cytokine-positive cells compared to either i.n / i.n / i.n or i.m / i.m / i.m regimens. Indeed, when compared to the i.n-only immunizations, i.n / i.m / i.m immunizations led to at least 4 times more TNF-α and IL-2- positive $CD4^+/CD44^+$ T cells, as well as nearly 3 times more IL-17-positive $CD4^+/CD44^+$ T cells. Noteworthy, we recovered IFN-γ and IL-4/IL-13- positive cells only in splenocytes from mice immunized i.n / i.m / i.m. On the other hand, very few cells recovered from mice immunized with the i.m-only protocol were positive for Th1 cytokines (IFN-γ, TNF-α and IL-2), and none were apparently positive for the Th2 or Th17 cytokines tested.

To look into the serum antibody response, we performed ELISA assays using sera from mice at day 71, which we compared to the pre-immune sera. The total IgG titers (Fig. 5A) showed that mice immunized via i.n / i.m / i.m routes produced higher amounts of IgGs in the sera compared to either i.n-only or i.m-only routes, for which mice produced similar levels of IgGs, approximately 3 times lower than the mixed immunization protocol. We also sought to look into various IgG subtypes to determine the IgG subclass dominance for each immunization protocol (Fig. 5B). All three immunization regimens led to a predominant production of IgG1

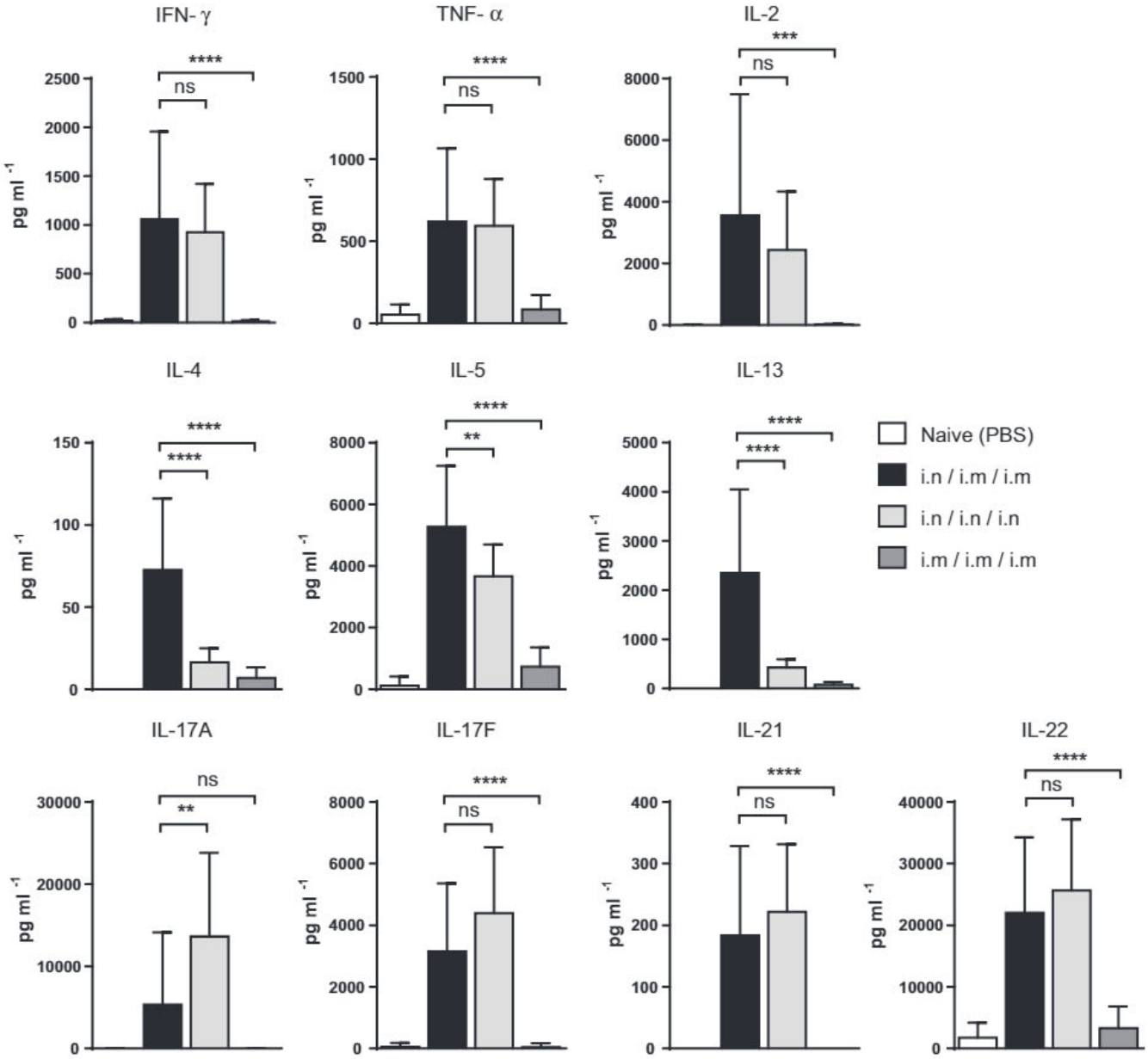

Fig. 2. Cytokine quantification of murine lamina propria cells (LPC) after immunizations. Balb/C mice were immunized intranasally (i.n) or intramuscularly (i.m) with SslE + adjuvant (CT for i.n, SEA for i.m) or PBS (Naive), and LPC were harvested and purified at day 71. Purified LPC were seeded at 10^6 cells/well and cultured with recall antigen SslE (10 μg/ml final concentration) for 24 h at 37 °C. Supernatants were collected and analyzed for cytokine quantification using the MesoScale Discovery U-PLEX mouse cytokine assay kits. Data are from two independent experiments with ten mice per group, and shown as mean ± standard deviation. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; IL, interleukin.

antibodies, while IgG2a levels were weaker, and IgG2b even lower. For all IgG subclasses tested, mice immunized with SslE via the i.n / i.m / i.m routes showed higher titers versus i.n-only or i.m-only routes. The ratio IgG2/IgG1 calculated for each of these regimens was lower than 0.5 in all cases, confirming a strong Th2 response for all three regimens (Supplemental Fig. 1). However, we did note that both i.n / i.m / i.m and i.n / i.n / i.n regimens had a statistically significant higher ratio value compared to the i.m / i.m / i.m regimen, indicating a bias, although small, towards a Th1 response with both regimens using i.n immunizations.

Overall, mice immunized with SslE via the i.n / i.m / i.m routes showed a stronger systemic immune response than those immunized via i.n-only or i.m-only routes, both at the cellular level with higher Th1, Th2 and Th17 cytokines, and at the humoral level with greater amounts of IgG antibodies, in any of the IgG subclasses tested.

3.3. Effect of a mixed i.n / i.m / i.m immunization regimen with antigen SslE on the gut microbiota of conventional mice.

With the gut being a prime example, it is now well established that mucosal immunity and microbiota constantly and dramatically shape the outcome of one another [40–42]. We focused on the i.n / i.m / i.m regimen, which gave us the strongest overall immune response locally and systemically, to investigate whether immunizations with antigen SslE would lead to an alteration of the mouse gut microbiota. The question was twofold: to determine whether these immunizations would provoke any sort of dysbiosis in the gut microbiota, and to specifically find out whether immunizations with antigen SslE, which is present in pathogenic as well as commensal *E. coli* [20], would affect the commensal population to significant, perhaps detrimental levels.

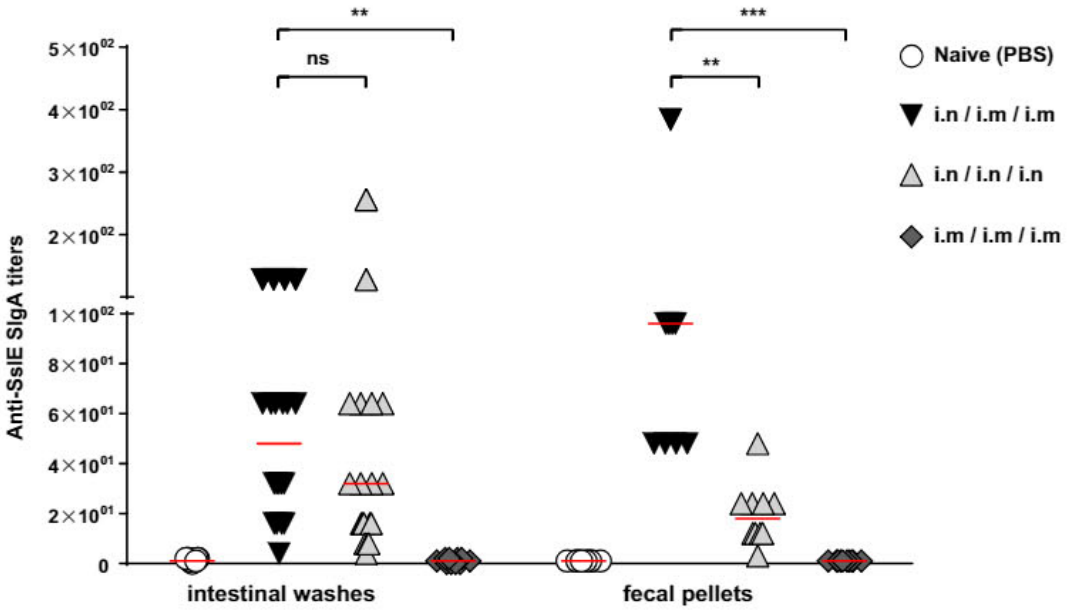

Fig. 3. Quantification of secreted IgA (SIgA) titers after immunizations. Intestinal washes and fecal pellets were harvested at day 71 from Balb/C mice immunized intranasally (i.n) or intramuscularly (i.m) with SslE + adjuvant (CT for i.n, SEA for i.m) or PBS (Naive). Specific SIgA antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). The bar represents the median of antibody titers for each immunization group. Data are from one (fecal pellets) or two (intestinal washes) independent experiment(s), where each dot represents one mouse.

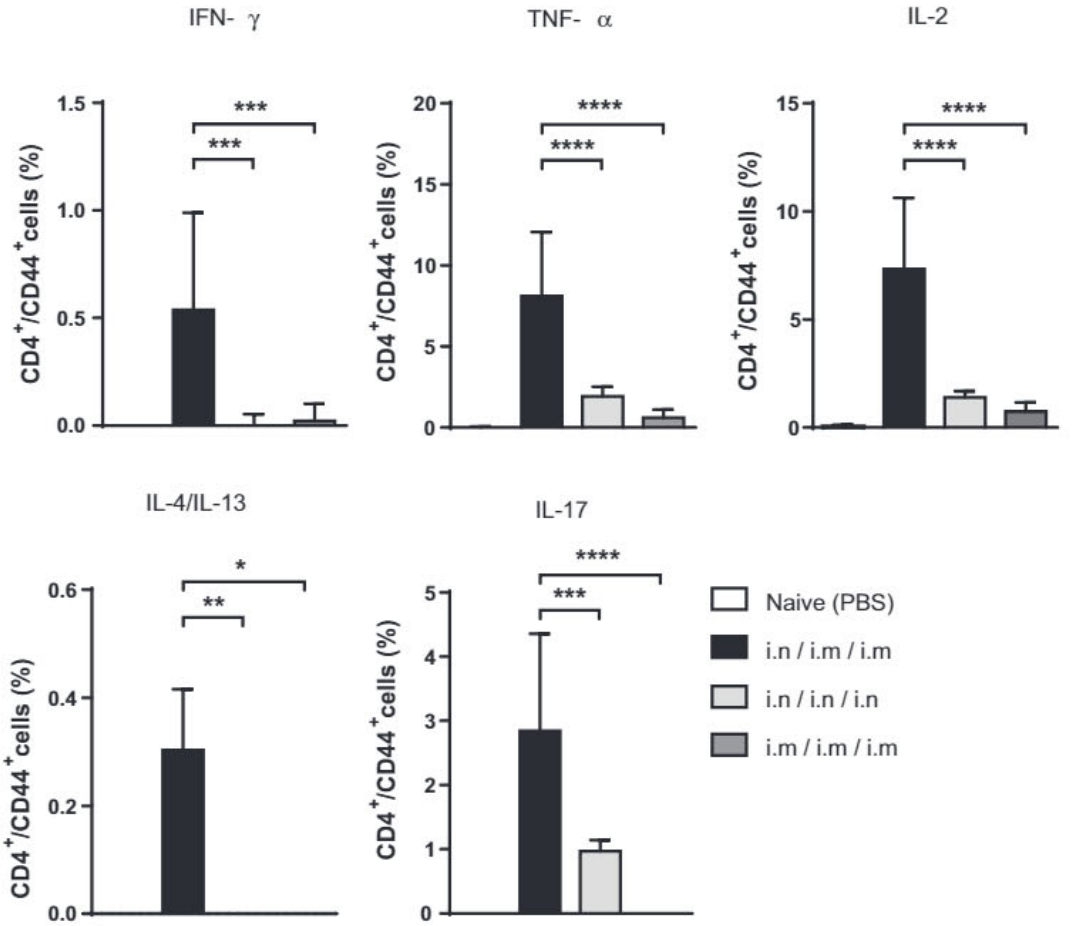

Fig. 4. Cytokine quantification of murine splenocytes after immunizations. Balb/C mice were immunized i.n / i.m / i.m, i.m / i.m / i.m or i.n / i.n / i.n with SslE + adjuvant (CT for i.n, SEA for i.m) or PBS (Naive), and splenocytes were harvested and purified at day 71. Splenocytes were seeded at 10^6 cells/well, cultured with recall antigen SslE (10 μg/ml final concentration) and were incubated for 6 h at 37 °C, including 4 h with Brefeldin A. Cells were stained with Live/Dead, fixed and permeabilized, then stained with fluorescent antibodies for flow cytometry acquisition. Data are from two independent experiments with ten mice per group, and shown as mean ± standard deviation. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; IL, interleukin.

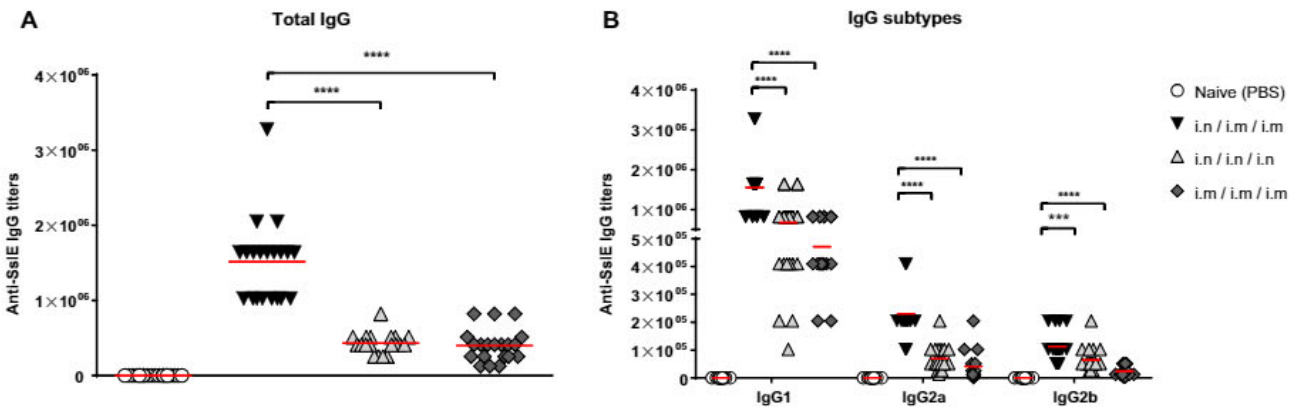

Fig. 5. Quantification of IgGs after immunizations. Sera from Balb/C mice immunized i.n / i.m / i.m, i.m / i.m / i.m or i.n / i.n / i.n with SslE + adjuvant (CT for i.n, SEA for i.m) or PBS (Naive) were taken at day 71. Specific (A) total IgG antibody titers, or (B) IgG1, IgG2a and IgG2b titers were determined by enzyme-linked immunosorbent assay (ELISA). Data are from two independent experiments, where each dot represents one mouse.

We collected fecal pellets from mice immunized with antigen SslE versus PBS using the i.n / i.m / i.m regimen at days 0 and 71, analyzed their genomic DNA content and compared the resulting data. After immunizations with PBS or SslE, we recovered the same phyla, and in similar proportions, than prior to immunizations (Fig. 6A): the Firmicutes and Bacteroidetes represented the vast majority of classified sequences, while the Actinobacteria and Proteobacteria were present in much lower amounts. We did notice a small increase of the Proteobacteria phylum in SslE-immunized mice at day 71 compared to PBS-immunized mice.

The Shannon diversity index, which accounts for abundance and evenness, remained high at roughly 1.5 at the family level (Fig. 6B), and 2.1 at the genus level (data not shown) in both PBS and SslE-immunized mice compared to pre-immunization. We looked more closely at the microbiota composition at the family level in the fecal pellets pre- and post-immunizations (Fig. 6C, upper panels). We recovered the same families before and after immunizations, but observed some fluctuations of the relative abundance over time. Notably, the relative abundance of the Firmicutes Lactobacillaceae and Lachnospiraceae changed substantially from pre- to post-immunizations. All pre-immunized mice showed a relative abundance of Lactobacillaceae of roughly 35% and a lower abundance of Lachnospiraceae at roughly 17%. In PBS-immunized mice, we recovered 24% of Lactobacillaceae and 30% of Lachnospiraceae in fecal pellets; in SslE-immunized mice, we obtained 10% of Lactobacillaceae and 41% of Lachnospiraceae. Interestingly, when we looked at the microbiota composition from cecal contents post-immunizations (Fig. 6C, lower panels), we did not observe such fluctuations between control and immunized mice at the family level; instead, we obtained a similar distribution for both PBS-treated and SslE-immunized mice which was comparable to the one recovered from fecal pellets of SslE-immunized mice from the fecal pellets at day 71, with about 7% of Lactobacillaceae and 44% of Lachnospiraceae. Additionally, the small raise in the relative abundance of Proteobacteria appeared to be the result of an increase in the family Desulfovibrionaceae (Fig. 6C), and more specifically the genus *Desulfovibrio* (data not shown), which was noticeable in both fecal and cecal contents post-immunizations. The relative abundance of *Desulfovibrio* in the cecum, where the difference was the highest, increased from 0.06 ± 0.01 in naive mice to 1.97 ± 0.8 in immunized mice; however, statistical analysis with a Mann-Whitney two-tailed test revealed no significance in this difference. To the extent of our analysis, we recovered neither the genus *Escherichia* nor the Enterobacteriacea family within the Proteobacteria phylum from our classified sequences prior or post-immunizations, whether in the fecal pellets or the cecum. Thus, we could not assess whether immunizations with antigen SslE would affect the commensal *E. coli* in the gut.

Overall, mice immunized with antigen SslE using the i.n / i.m / i.m regimen maintained the same richness of the microbiota. SslE-immunized and PBS-immunized mice showed some disparities in the fecal relative abundance of Lachnospiraceae and Lactobacillaceae, but maintained a strikingly similar distribution in the cecal relative abundance of families.

4. Discussion

In this study, we described the immune response to the ExPEC antigen SslE and the influence of immunizations with SslE on the gut microbiota. SslE is a mucin-degrading metalloprotease widely distributed among the many *E. coli* pathotypes, with a highly conserved zinc-metalloprotease core motif [20,21,43]. Functional studies have demonstrated that SslE is capable of degrading major intestinal mucins such as MUC2 and MUC3 [25]. When *E. coli* is in contact with adherent enterocytes, secretion of SslE is increased, which is thought to facilitate colonization by degrading mucus and allowing bacteria to reach the surface of enterocytes [26]. SslE immunizations confer protection, although to various extents, in murine models of ExPEC and InPEC infections, including sepsis, UTI, and intestinal colonization with enterotoxigenic *E. coli* (ETEC) [20,21]. Sera from patients recovering from UTIs or an ETEC infection contain anti-SslE antibodies [22–24]. Thus, SslE is conserved in sequence, widely distributed, secreted during infection, and immunogenic, all of which are important characteristics with respect to a potential vaccine candidate.

We sought to better explore this opportunity and investigate the immune response generated against SslE following various methods of immunization. Our goal was to define the immunization regimen that would deliver the highest mucosal and systemic immune responses, hence bare the potential to be cross-effective against multiple *E. coli* pathotypes. Based on previous studies demonstrating the benefits of combining mucosal with parenteral immunizations [29–33], we set up immunization regimens with either i.n immunizations only, i.m immunizations only, or an i.n prime followed by two i.m boosts. We conclusively showed that mice immunized i.n / i.m / i.m with SslE mounted a greater overall immune response than those immunized i.n / i.n / i.n or i.m / i.m / i.m.

In the SI, both mixed immunizations and i.n-only immunizations led to a significant cytokine response after *in vitro* antigen restimulation compared to i.m-only immunizations. It is very likely that these responses are antigen-specific $CD4^+$ T cell responses,

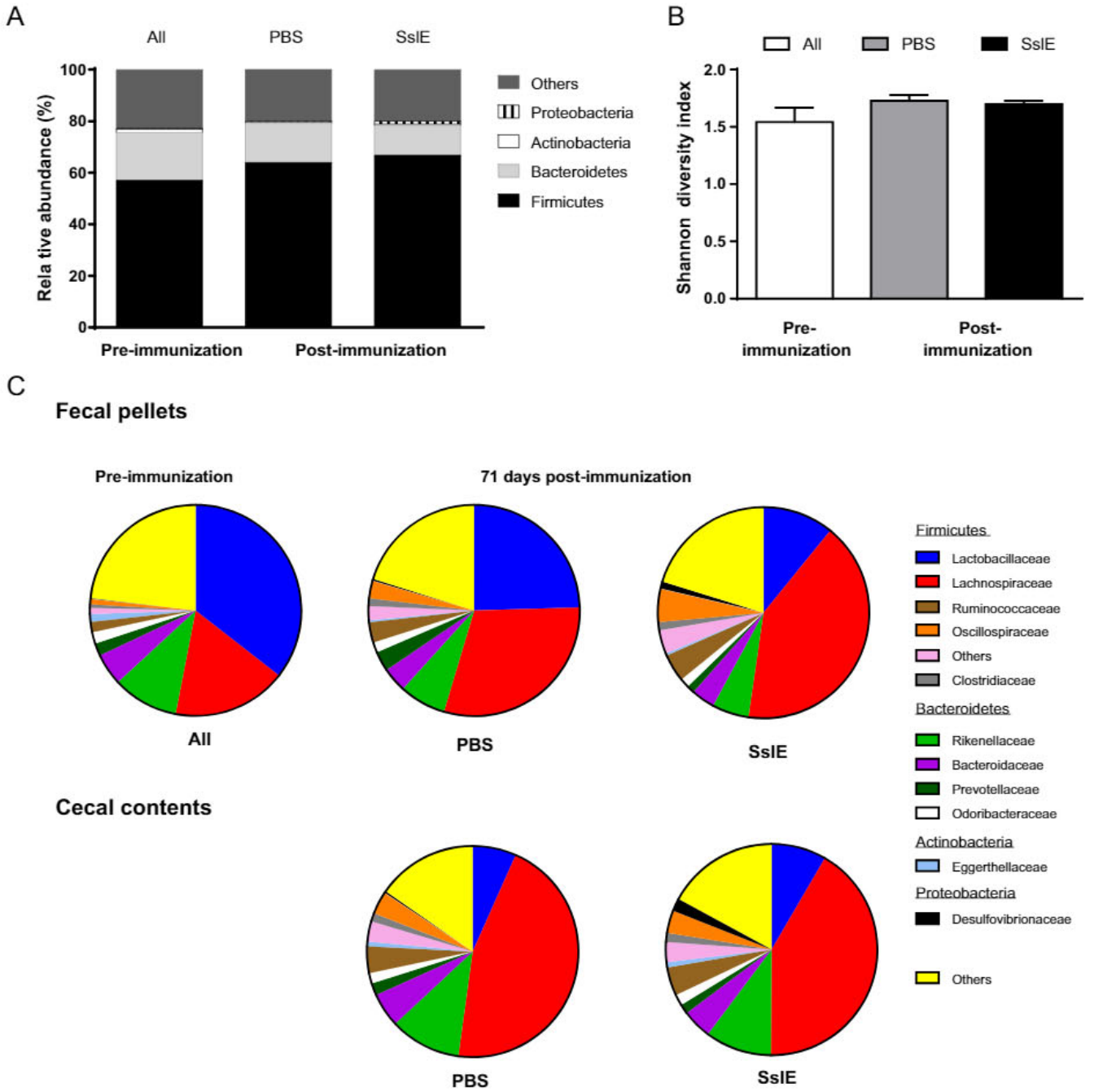

Fig. 6. Microbiota composition before and after immunizations with SslE. (A) Normalized phyla relative abundance obtained from fecal pellets of PBS-immunized or SslE-immunized mice (i.n / i.m / i.m) at days 0 (pre-immunization) and 71 (post-immunization); n = 6 for each group. (B) Shannon diversity index obtained at the family level for each group. (C) Microbiota composition (normalized to 100%) at the family level obtained from fecal pellets (upper panels) pre and post-immunizations or from cecal contents (lower panels) obtained post-immunizations. "Others" represents sequences in clusters where no association has been found.

though we cannot rule out, due to our experimental set up, a non-specific response of other LP lymphoid cells. Notably, for both regimens, we observed a strong induction of the IL-17A, IL-17F and IL-22 cytokines. IL-17A can be secreted by several cells of the LP, including Th17 cells and group 3 innate lymphoid cells (ILC3) [44]. IL-17A and IL-17F, which are thought to share similar biological activities, are important in the initial control of pathogen proliferation through the release of antimicrobial peptides and induction of neutrophil recruitment [45,46]. Deficiency in either one of these cytokines leads to full susceptibility of mice to *C. rodentium* [47], suggesting that these two cytokines are not absolutely redundant and may have some unique biological activities in early gut pathogen control. IL-22, also in part secreted by Th17 cells and ILC3, can act synergistically or additively with IL-17A and IL-17F by increasing antimicrobial peptide secretion and intestinal protection. Previous studies showed that Th17 cells developed in mice after infection with EHEC O157:H7 [48], or immunization with ETEC F4^{+} fimbriae [49]. Th17 cells are also known to be important for the induction of an antigen-specific IgA response by B cells in the germinal center of Peyer's patches in the small intestine [50]. Thus, the presence of these cytokines in the LP after recall with SslE suggests that IL-17 and IL-22-

secreting cells are specifically induced *in vivo* after SslE immunizations, mimicking what has been previously observed in other infection and vaccination models.

While we observed a bias towards a higher IL-17A secretion with i.n / i.n / i.n, the i.n / i.m / i.m regimen led to a significantly greater release of Th2-like cytokines. These differences may be attributed to an adjuvant effect. CT, the adjuvant used in i.n immunizations, has been recently shown to induce a balanced Th1/Th2/Th17 response, whether injected mucosally or parenterally [51]; the i.n-only regimen could thus potentially lead to more efficient priming by CT of Th17 cells at each immunization. Likewise, the enhanced Th2-like cytokine levels seen with i.n / i.m / i.m may possibly be attributed to the use of SEA, which has been suggested to behave like most delivery system type of adjuvants by priming Th2 $CD4^+$ T cells and enhancing the antibody response.

Besides these important mechanistic questions, our results made evident that the mucosal route of immunization, more than the adjuvant itself, was an essential element in mounting the immune response to SslE in the SI. Indeed, i.m-only immunizations, regardless of the use of SEA, performed very poorly in the mucosa, with little to no cytokine response recovered in the SI. Similarly, both mixed immunizations and i.n-only immunizations promoted the secretion of anti-SslE IgAs in the gut, while we could not observe any detectable levels of SIgAs after i.m-only immunizations. Interestingly, the SIgA titers recovered from intestinal lavages were fairly similar between i.n-only and mixed immunizations, yet titers in fecal pellets seemed to be significantly higher in mixed immunizations. Secretions of IgA antibodies in the intestinal lumen represent a crucial element in the immune response against gut bacterial pathogens. In experimental ETEC challenges, infected volunteers all produced mucosal IgAs upon challenge, [52], suggesting that IgA antibodies are indeed an effective arm in the control and/or clearance of ETEC infections.

Thus, with antigen SslE, i.n-priming seemed to be an essential element in the priming of B and T cells in the gut, and sufficient to induce an immune response of the same magnitude regardless of whether boost immunizations were performed i.n or i.m. It would be interesting to investigate whether the timing of the mucosal immunization is also a critical element for an effective mucosal immune response, by comparing i.n / i.m / i.m immunizations to i.m / i.m / i.n immunizations for example; however, previous studies on immunizations against influenza showed that priming with a mucosal immunization was essential to generate a mucosal immune response [32]. Additionally, the choice of i.n versus another mucosal route could be an important factor in the gut immune response obtained: preliminary results from an equivalent study with SslE using the sublingual route for mucosal immunizations showed no particular benefit in using the mixed immunization regimen, which instead led to a smaller cellular and humoral immune response in the SI compared to sublingual-only immunizations (unpublished data). Notably, the comparison between intranasal and sublingual immunizations with SslE revealed a much more reproducible and efficient immune response to the former versus the latter. That said, translating intranasal immunizations with SslE in potential clinical trials would ultimately warrant the use of another type of mucosal adjuvant; indeed, CT and the structurally similar ETEC heat-labile (LT) enterotoxin present safety risks when used intranasally, even in their detoxified mutant forms [53]. Alternatively, testing other mucosal routes of administration, such as the intradermal route, should be considered in future studies. The differences between i.n / i.m / i.m and i.n-only immunizations were more evident when we quantified the systemic immune response to antigen SslE. The $CD4^+$ T cell response obtained from splenocytes showed a consistently higher percentage of Th1/Th2/Th17 cytokine-positive $CD4^+$ T cells when mice were immunized i.n / i.m / i.m versus i.n-only. These results were paralleled by the serum IgG titers recovered post-immunizations, where i.n / i.m / i.m led to titers of all IgG1, IgG2a and IgG2b far higher than i.n-only immunizations. The i.m-only regimen showed very little systemic T cell response and a much lower IgG antibody titer compared to the mixed immunizations, regardless of the antibody subtype assessed. This finding suggests that an i.n prime immunization with SslE is critical not only for a mucosal immune response, but also for a systemic immune response. Further, the important difference in both B and T cell responses observed between i.n / i.m / i.m and i.n / i.n / i.n indicates that boosting with parenteral immunizations ensured an efficient mounting of the systemic immune response, at a magnitude not attained with i.n-only immunizations.

In the consideration of a broad-spectrum vaccine against both InPEC and ExPEC, the robust systemic immune response obtained with i.n / i.m / i.m is of particular interest. Indeed, studies have showed that a UTI infection with UPEC resulted in the proliferation of antigen-specific splenic T cells and increase of serum IgG titers, and transfer of either T cells or serum led to protection of naive mice from infection [54]. There may therefore be great benefits associated with a mixed immunization by effectively targeting multiple pathotypes of *E. coli*.

Mucosal immunizations, particularly with an antigen present on the surface of commensals [20,55], must raise the question of the impact of immunizations on the gut microbiota. Choosing i.n / i.m / i.m immunizations to address this possibility, we did not observe any significant change in the overall richness of the microbiota between PBS and SslE-immunized mice all the way down to the genus level, as indicated by the Shannon diversity index. Accordingly, we recovered the same families and genera between the two groups pre- and post-immunizations, which was an important indicator that immunizations with SslE did not significantly perturb the overall composition of the gut microbiota.

Within Firmicutes, the relative abundance of the families Lachnospiraceae and Lactospiraceae was markedly different in the fecal pellets of pre- versus post-immunized mice. The high abundance of Lactobacillaceae and lower abundance of Lachnospiraceae in pre-immunized mice was rather surprising, as Lactobacillaceae tend to be in high abundance up only until the ileum, while dropping below 10% in the cecum, colon and feces; as for the Lachnospiraceae, they normally constitute the largest part of the Firmicutes, with a relative abundance at 30% at least [56,57]. However, at day 71, we observed a shift in these two families, whose relative abundance became quite similar to previously reported results [56]; this was the case in fecal and cecal contents of SslE-immunized mice, and in the cecal content of PBS-immunized mice. This shift could have been due to the transfer of mice from the vendor to our facility. The length of our experimental study could also be at play: immunizations were performed and followed between weeks eight and eighteen; considering the significant age-dependent changes, even in an SPF environment, in the gut microbiota of laboratory mice [58,59], this factor could explain at least part of the changes observed between pre- and post-immunized mice. Additionally, we noticed a different profile of these two families between the fecal content of mock-immunized and SslE-immunized mice. While we cannot rule out that this difference is influenced by immunizations with SslE, we believe it is unlikely, as this difference was not perceived in the comparison of the cecal content between these two groups.

The Proteobacteria phylum represents a small component of the microbiota composition, and *E. coli* is generally a poor colonizer of the mouse GI tract [60]. To the extent and depth of our study, we did not find the Enterobacteriaceae family and thus the *Escherichia* genus in our sequence analysis of pre- and post-immunized mice, whether in the feces or the cecum. Although this result was to be expected, assessing whether immunization with SslE would affect

resident *E. coli* commensals still remains an important question to be addressed in the context of the human gut microbiota. The use of germ-free rats transplanted with a human fecal microbiota [61,62] could be, in this regard, an interesting model to assess this issue. Nevertheless, our results show that a mucosal immunization with SslE does not perturb the gut microbiota, which we believe is of importance.

In summary, we have described an efficient, mucosal-parenteral immunization schedule with antigen SslE to induce a robust intestinal and systemic immune response against SslE, while not significantly disturbing the resident gut microbiota. SslE thus appears as a potential key component of a broad spectrum vaccine against pathogenic *E. coli*. Although widespread and protective against different *E. coli* pathotypes, SslE does not cover all known strains [20]. Using the same reverse vaccinology approach against InPEC, our group is currently characterizing some promising vaccine candidates that have showed protective efficacy against an intestinal enterohemorrhagic *E. coli* model of infection. Another approach is to use reverse vaccinology to identify antigens present in the *E. coli* core genome from all pathogenic and non-pathogenic strains. A recent study has identified antigen YncE, present in > 99% of all *E. coli* genomes available, which shows protection against a bacteremia model of infection, and is recognized by antibodies present in the sera of convalescent urosepsis patients [22]. We and others have showed that immunizations with conserved *E. coli* antigens do not perturb to significant extent the murine gut microbiota [63]. Although *E. coli* is a prevalent member of our microflora, it only accounts for about 0.1% of its total composition. The idea of a multi-component broad-spectrum vaccine including candidates such as SslE and YncE thus deserves to be considered in future vaccine research strategies.

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Author contributions

IN, UD and CB conceived the study; IN, CB and BCB designed the experiments; IN and JV performed the experiments; IN, CB, AB-D and GJ analyzed the data; IN wrote the manuscript; all authors reviewed and approved the manuscript.

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Conflict of interest

CB, UD, BCB and MP are current permanent employees at GSK. JV is a former GSK employee and was supported by the People Programme (Marie Sklodowska-Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013 under the REA Grant agreement 317057 HOMIN-ITN. Ilham Naili is a former GSK employer and a Marie Curie PhD Research Fellow granted by ITN EID DISCo, whose work was performed at both GSK Vaccines and INRA. All other authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.10.008.

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