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Characterization of antigen-presenting cells induced by intragastric immunization with recombinant chimeric immunogens constructed from *Streptococcus mutans* AgI/II and type I or type II heat-labile enterotoxins

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SUMMARY

Intragastric (i.g.) immunization with recombinant chimeric proteins constructed from the saliva-binding region (SBR) of *Streptococcus mutans* surface antigen AgI/II and the A2/B subunits of enterobacterial heat-labile enterotoxins has been successfully used to induce salivary and circulating antibodies against *S. mutans* that have protective potential against dental caries. To investigate the mode of action of these vaccine constructs, mice were immunized i.g. with chimeric proteins constructed from SBR and cholera toxin (CT) or the type II enterotoxins of *Escherichia coli*, LT-IIa and LT-IIb. Antigen-presenting cells (APC) in Peyer's patches (PP) and mesenteric lymph nodes (MLN) were characterized by flow cytometry. Compared with immunization with SBR alone, chimeric proteins SBR-LTIIaA2/B and SBR-LTIIbA2/B increased the number of B cells and macrophages in PP and diminished B cell numbers in MLN, whereas SBR-CTA2/B diminished the numbers of B cells and macrophages in PP and MLN. Immunization with all three chimeric proteins led to upregulation of MHC class II molecules and co-stimulatory receptors CD40, CD80, and CD86 especially on dendritic cells in PP and also on APC in MLN. The results provide a molecular basis for the enhanced immune responses induced by chimeric proteins compared with uncoupled antigen, and for differential responses to chimeric proteins based on CT or type II enterotoxins.

INTRODUCTION

Given that the great majority of human infectious diseases are directly acquired through the mucosal surfaces, future generations of vaccines will most likely be administered by mucosal routes to elicit protective secretory immunoglobulin A (IgA) antibody responses at these portals of pathogen entry. However, one of

the limiting factors in developing mucosal vaccines has been the availability of suitable adjuvants and delivery systems that will effectively stimulate the mucosa-associated lymphoid tissues, which are known to be the principal inductive sites of the mucosal immune system (Brandtzaeg *et al.*, 2008). Among the most effective mucosal adjuvants demonstrated in animal models are cholera toxin (CT) and the related heat-labile enterotoxins of *Escherichia coli* (LT), but their inherent toxicity precludes their use in humans. Several approaches to overcoming this problem have been described, including the generation of detoxified mutants of CT and LT, and the use of the non-toxic B subunit pentamers of these toxins, CTB and LTB, respectively (Freytag & Clements, 2005; Holmgren & Czerkinsky, 2005). A huge and to some extent controversial literature exists on the different technologies and their modes of action (Hajishengallis *et al.*, 2005a; Liang & Hajishengallis, 2010).

Our approach, which was undertaken in an effort to develop a vaccine against dental caries (Russell *et al.*, 2004) has been to explore the use of CTB coupled to protein antigens initially by chemical conjugation (Czerkinsky *et al.*, 1989; Russell & Wu, 1991; Wu & Russell, 1993). The *Streptococcus mutans* surface antigen AgI/II was originally identified as a potential antigen for developing a vaccine against caries (reviewed in Russell *et al.*, 1999). We subsequently demonstrated that high levels of salivary IgA antibodies to AgI/II could be induced in rats or monkeys by mucosal immunization with AgI/II coupled to CTB, and that immunized rats were protected against caries (Katz *et al.*, 1993; Russell *et al.*, 1996). The N-terminal segment including the A-repeat region of AgI/II was found to be important in the adherence of *S. mutans* to saliva-coated hydroxyapatite (Hajishengallis *et al.*, 1994), although others found that the central P-repeat region was also involved in adherence (Crowley *et al.*, 1993). This apparent anomaly was resolved by the recent determination of the crystallographic structure of most of AgI/II (Larson *et al.*, 2010), which shows how the A- and P-repeat regions interact. The chimeric mucosal immunogen, SBR-CTA2/B, was constructed by fusing the 'saliva-binding region' (SBR; residues 186–577) of AgI/II to the A2 subunit of CT followed by assembly with the CTB subunit (Hajishengallis *et al.*, 1995), and was shown to induce protective antibodies against caries in the rat model by intranasal (i.n.) immunization (Hajishengallis *et al.*, 1998). In further elaboration of this principle, the type II heat-labile enterotoxins of *E. coli*, LT-IIa and LT-IIb (Connell, 2007), have also been used to create similar chimeric proteins, SBR-LTIIaA2/B and SBR-LTIIbA2/B (Martin *et al.*, 2001a; M. Martin, T. Conell & M. W. Russell, unpublished results). Superficially, all of these constructs in which AgI/II (or its SBR) are coupled to enterotoxin B subunits whether by chemical conjugation or recombinant DNA technology, are effective mucosal immunogens that induce mucosal and systemic antibody responses when administered intragastrically (i.g.) or i.n. However, careful analysis of the conditions and mechanisms involved reveals important differences in the outcomes obtained and modes of action of the different constructs, which might be exploitable for different immunological objectives. Specifically, i.g. immunization with chemically conjugated AgI/II-CTB requires the co-administration of an adjuvant dose of intact CT to induce antibody responses (Russell & Wu, 1991), whereas AgI/II-CTB conjugate (prepared from recombinant CTB) is effective by the i.n. route without additional adjuvant (Wu & Russell, 1998; Gockel & Russell, 2005). Others have reported that protein antigens chemically conjugated to recombinant CTB or LTB are tolerogenic by the i.g. route (Sun *et al.*, 1994; Lycke, 2005) although we could find no evidence for tolerance induction by i.g. administration of AgI/II-CTB (Gockel & Russell, 2005). In contrast, the recombinant chimeric immunogens delivered i.g. without adjuvant are effective immunogens (Hajishengallis *et al.*, 1995; Gockel & Russell, 2005). Moreover, the type II enterotoxins, LT-IIa and LT-IIb have been shown to have different immunostimulatory properties from the type I enterotoxins, CT and conventional LT (or LT-I) (Martin *et al.*, 2000; Hajishengallis *et al.*, 2005a; Nawar *et al.*, 2005). These different properties have been attributed to the ability of the different enterotoxins (or their B subunits) to recognize different ganglioside receptors, and in the case of LT-IIa and LT-IIb B subunits to engage Toll-like receptor 2 (Connell, 2007; Liang & Hajishengallis, 2010).

It is therefore clear that numerous factors impact upon the outcome of mucosal immunization with antigens coupled to enterotoxins, including the route of administration, the precise composition and nature of the construct, and the properties of the coupled anti-

gen (Hajishengallis *et al.*, 2005a). However, it is not known precisely which tissues and cells are targeted by these immunogens when given by the i.g. route, and how and where they are processed and presented to T cells to initiate the immune response. It is likely that these constructs increase immune responses to the coupled protein antigen not only by the immunostimulatory activity of the enterotoxin B subunits, but also by facilitating antigen uptake and processing. Therefore it is important to determine the tissue sites and cellular pathways of uptake of SBR-CTA2/B, SBR-LTIIaA2/B and SBR-LTIIbA2/B chimeric proteins, and to identify the type and location of the antigen-presenting cells (APC) involved. We hypothesize that the chimeric proteins are taken up more readily and perhaps by different cells than uncoupled SBR and that differences between chimeric proteins depend upon their different ganglioside-binding properties. In particular we propose that different immunization outcomes of immunization with type I or type II enterotoxins as carriers are determined by the initial interaction of the immunogens with APC in the mucosal immune inductive sites. To begin investigating these effects and to test this hypothesis, we immunized mice i.g. with SBR-CTA2/B, SBR-LTIIaA2/B, or SBR-LTIIbA2/B, in comparison with SBR alone as a control, and analysed by flow cytometry the APC populations in Peyer's patches (PP) and the mesenteric lymph nodes (MLN) that drain the intestinal tract.

METHODS

Antigens and adjuvants

The construction of *E. coli* clones that express the recombinant chimeric proteins, SBR-CTA2/B and SBR-LT-IIaA2/B, has been described previously (Hajishengallis *et al.*, 1995; Martin *et al.*, 2001a). The chimeric proteins were purified from whole cell lysates of IPTG-induced cultures by a combination of ammonium sulfate precipitation and fast protein liquid chromatography (Pharmacia, Uppsala, Sweden) on molecular size-exclusion (Sepharose S-100) and anion-exchange (MonoQ) columns (Hajishengallis *et al.*, 1995; Martin *et al.*, 2001a). The chimeric protein SBR-LT-IIbA2/B was similarly constructed with the inclusion of a His-tag sequence in the LT-IIb B subunit (Hajishengallis *et al.*, 2004), allowing for the protein to be purified from cell lysates by nickel-affinity chromatography. Likewise, DNA encoding the SBR fragment was cloned from SBR-CTA2/B with the inclusion of a His-tag sequence, and SBR was purified from cell lysates by nickel-affinity chromatography.

Purity of each protein preparation was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and identity of the protein was established by enzyme-linked immunosorbent assay and Western blotting using antiserum to *S. mutans* AgI/II raised in this laboratory. Chimeric proteins were also tested in enzyme-linked immunosorbent assay using plates coated with gangliosides GM1 (for SBR-CTA2/B), GD1b (for SBR-LT-IIaA2/B), or GD1a (for SBR-LT-IIbA2/B), and developed with monoclonal antibody to SBR (developed in this laboratory) or antiserum to CTB (List Biological Laboratories, Campbell, CA), LTIIaB, or LTIIbB (both provided by Dr T.D. Connell, University at Buffalo) to confirm the presence of coupled SBR and respective enterotoxin B subunits, as well as the preservation of ganglioside-binding. Protein concentration was assayed by means of the Micro BCATM Protein Assay Reagent kit (Thermo-Scientific, Rockford, IL). The CT was purchased from List Biological Laboratories (Campbell, CA).

Animals and immunizations

Female BALB/c mice, 6–8 weeks old, were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed at the University at Buffalo Laboratory Animal Facility in compliance with National Institutes of Health guidelines for animal care. The Institutional Animal Care and Use Committee approved all procedures used in this study. Mice were immunized i.g. with 100 μg of each chimeric protein, or an equimolar amount of SBR (40 μg), or 5 μg CT in 200 μl of 0.7 M $NaHCO_3$, as determined in previous studies (Russell & Wu, 1991; Toida *et al.*, 1997).

Preparation of cells

Mice were sacrificed 24 h after immunization, and single-cell suspensions were obtained by teasing PP and MLN apart with needles; tissue debris was removed by filtering through a cell strainer (Becton-Dickinson, San Jose, CA). The cells were washed

twice in phosphate-buffered saline (GIBCO BRL, Gaithersburg, MD) supplemented with 2% fetal calf serum and were finally resuspended in phosphate-buffered saline with 2% fetal calf serum. The number of viable cells, determined by use of trypan blue dye exclusion, was routinely ≥90% of total cell counts.

Flow cytometry

The following fluorescent antibody conjugates and control antibodies were obtained from BD Biosciences (Mountain View, CA): phycoerythrin (PE)-Cy7 hamster anti-mouse CD11c, allophycocyaninrat anti-mouse CD11b, allophycocyanin rat anti-mouse CD45R/B220, allophycocyanin rat anti-mouse CD8α, R-phycoerythrin (R-PE) hamster anti-mouse CD80 (B7-1), PE rat anti-mouse CD86 (B7-2), R-PE rat anti-mouse CD40, R-PE-conjugated rat anti-mouse MHC class II (MHC-II; I-A/I-E), allophycocyanin rat IgG2b-κ isotype control, allophycocyanin rat IgG2α-κ isotype control, PE hamster IgG2-κ isotype control, R-PE rat IgG2α-κ isotype control, PE rat IgG2b-κ isotype control, and rat anti-mouse CD16/CD32 (FcγRIII/II). LIVE/DEAD® Fixable Green Dead Cell Stain kit was obtained from Invitrogen (Carlsbad, CA).

Cells were blocked against non-specific staining with purified rat anti-mouse CD16/CD32, and first stained for phenotypic markers: $B220^+$ (B cells), CD11b (macrophages), $CD11c^+$ (dendritic cells; DC), or CD8α. Cells were then co-stained for co-stimulatory molecules CD40, CD80 or CD86, or for MHC-II. Each assay involved up to four contrasting fluorochromes, e.g. Live/Dead, PE-CD40/CD80/CD86/MHC-II, allophycocyanin-CD11b/B220/CD8α and PE-Cy7-CD11c. After fixing in 1% paraformaldehyde overnight, cells were analysed on a FACScalibur flow cytometer using CellQuest software (Becton-Dickinson). LIVE/DEAD® Fixable Green Dead Cell Stain was used in all instances to exclude non-viable cells from the analyses. Cell suspensions were gated on live cell populations and no fewer than 10^4 events were acquired for each experiment.

Statistical methods

Data were analysed for statistical significance using paired and unpaired analyses of variance and non-parametric tests with SPSS 15 (IBM, Somers, NY). Differences between groups were considered significant at the level of $P < 0.05$. The results are representative of duplicate experiments.

RESULTS

Phenotypes of APC in gut mucosal tissues after i.g. immunization with different constructs

Mice were immunized with SBR, SBR-CTA2/B, SBR-LTIIaA2/B, SBR-LTIIbA2/B or CT, and 24 h later PP and MLN were excised and processed for flow cytometry of the mononuclear cells. The PP and MLN from unimmunized control mice were similarly processed. The major types of APC were identified phenotypically according to the expression of CD11c (DC), CD11b alone (macrophages), or B220 alone (B cells). The DC were further characterized by their co-expression of CD11b, CD8α or B220.

In PP, the predominant type of APC was B cells, which accounted for ~75% of mononuclear cells in unimmunized control animals (Table 1). Numbers of $B220^+$ B cells were significantly increased to >80% in mice immunized with SBR-LTIIaA2/B or SBR-LTIIbA2/B, but were decreased in mice immunized with SBR-CTA2/B or CT alone (Table 1). A modest increase was also seen in mice immunized with SBR. Overall, $CD11c^+$ DC were few in number. However, compared with SBR alone, immunization with the chimeric proteins tended to increase the numbers of DC in PP, including the $CD11b^+$ subset and to some extent the $CD8\alpha^+$ subset (Table 1), although not all apparent increases reached statistical significance. Numbers of $CD11b^+$ macrophages were increased relative to controls in PP from animals immunized with SBR, SBR-LTIIaA2/B, or SBR-LTIIbA2/B, but decreased in those immunized with CT (Table 1). SBR-LTIIaA2/B and SBR-LTIIbA2/B also significantly increased the number of $B220^+$ B cells whereas SBR-CTA2/B decreased the numbers of B cells and macrophages, as did CT itself (Table 1).

Increased numbers of $B220^+$ B cells in PP after immunization with SBR-LTIIaA2/B or SBR-LTIIbA2/B were matched by decreased numbers of these cells in MLN (Table 2), and also by increased numbers of $CD11b^+$ macrophages in MLN (Table 2). Other alterations in APC numbers did not easily fit into a pattern: B cells were decreased in the MLN of mice immunized with SBR-CTA2/B; macrophages were elevated in mice immunized with SBR alone; and

Table 1 Antigen-presenting cell phenotypes in Peyer's patches 24 h after intragastric immunization with SBR, SBR-CTA2/B, SBR-LTIIaA2/B, or SBR-LTIIbA2/B

Immunization group	CD11c$^+$ DC	CD11c$^+$ DC subset			CD11b$^+$ macrophages	B220$^+$ B cells
		CD11b$^+$	CD8α^+	B220$^+$		
Control (n = 5)	3.34 ± 0.78	0.68 ± 0.07	0.53 ± 0.03	1.79 ± 0.13	5.12 ± 1.01	73.56 ± 0.86
SBR (n = 7)	2.37 ± 1.00	0.54 ± 0.11	0.38 ± 0.06**	1.08 ± 0.27**	7.35 ± 0.43*	76.31 ± 1.15*
SBR-CTA2/B (n = 5)	3.88 ± 0.79†	0.81 ± 0.21†	0.57 ± 0.26	1.72 ± 1.01	5.85 ± 0.47†	71.80 ± 1.72*††
SBR-LTIIaA2/B (n = 5)	2.70 ± 0.66	0.84 ± 0.04†	0.61 ± 0.02†	2.33 ± 0.55	7.38 ± 0.98**	80.57 ± 1.77**††
SBR-LTIIbA2/B (n = 5)	4.24 ± 0.69†	0.66 ± 0.07†	0.65 ± 0.39	1.18 ± 0.13**	7.85 ± 0.17**	81.77 ± 1.05**††
CT (n = 6)	3.35 ± 1.56	0.86 ± 0.17	1.00 ± 0.12*	2.42 ± 0.36	2.51 ± 0.46**	64.37 ± 1.43**

Data shown as % of total gated cells, mean ± SD.

*P < 0.05, **P < 0.01, relative to corresponding unimmunized controls (analysis of variance).

$^\dagger P$ < 0.05, $^{\dagger\dagger} P$ < 0.01, relative to immunization with SBR alone (analysis of variance).

Table 2 Antigen-presenting cell phenotypes in mesenteric lymph nodes 24 h after intragastric immunization with SBR, SBR-CTA2/B, SBR-LTIIaA2/B, or SBR-LTIIbA2/B

Immunization group	CD11c$^+$ DC	CD11c$^+$ DC subset			CD11b$^+$ macrophages	B220$^+$ B cells
		CD11b$^+$	CD8α^+	B220$^+$		
Control (n = 5)	3.95 ± 1.35	0.93 ± 0.09	1.56 ± 0.21	1.18 ± 0.14	15.06 ± 1.57	31.50 ± 1.50
SBR (n = 7)	3.04 ± 0.31	0.80 ± 0.05	1.04 ± 0.15	0.86 ± 0.07	28.82 ± 0.53**	28.96 ± 3.12
SBR-CTA2/B (n = 5)	3.53 ± 0.59	0.80 ± 0.06	1.46 ± 0.07†	0.88 ± 0.17	18.70 ± 0.27††	22.25 ± 0.77**
SBR-LTIIaA2/B (n = 5)	2.30 ± 0.57**†	1.01 ± 0.03†	1.38 ± 0.09††	1.17 ± 0.14	24.28 ± 0.18**††	25.15 ± 0.25*
SBR-LTIIbA2/B (n = 5)	5.35 ± 0.66**††	0.88 ± 0.18	1.10 ± 0.09	0.79 ± 0.16	24.68 ± 0.35**††	24.89 ± 1.31**
CT (n = 6)	5.42 ± 1.35*	2.14 ± 0.24**	5.65 ± 0.55**	4.93 ± 0.67**	8.66 ± 0.65*	28.80 ± 0.36

Data shown as percentage of total gated cells, mean ± SD.

*P < 0.05, **P < 0.01, relative to corresponding unimmunized controls (analysis of variance).

$^\dagger P$ < 0.05, $^{\dagger\dagger} P$ < 0.01, relative to immunization with SBR alone (analysis of variance).

CD11c$^+$ DC were decreased in mice immunized with SBR-LTIIaA2/B but increased in those immunized with SBR-LTIIbA2/B (Table 2). However, compared with SBR alone, immunization with the chimeric proteins led to decreased numbers of macrophages, but effects on DC numbers were variable overall (Table 2). Immunization with CT appeared to increase the numbers of DC in MLN, including all subsets, and decreased the number of macrophages (Table 2).

Activation status of APC in gut mucosal tissues after i.g. immunization with different constructs

To determine whether the different constructs affected the activation (in contrast to the numbers) of APC in PP or MLN, we analysed the expression of the co-stimulatory markers CD40, CD80 and CD86, and of the MHC-II. Upon activation by antigens or other stimulants, APC are known to upregulate the expression of MHC-II as they present processed antigenic peptides to T cells, and of the co-stimulatory molecules which are necessary to provide the 'second signals' to naive T cells. Hence these molecules serve as functional indicators of the activation status of APC.

Few changes in activation status of APC were apparent relative to the unimmunized control in PP (Fig. 1A), particularly for DC and macrophages (Fig. 1B,C). Expression of MHC-II was increased in the total APC population (DC, macrophages and B cells combined) after immunization with SBR-CTA2/B or SBR-LTIIbA2/B, whereas small decreases in co-stimulatory molecule expression were noted in isolated instances, most notably that CD40 and CD86 expression were diminished after immunization with CT alone (Fig. 1A). However, compared with SBR alone, immunization with the chimeric proteins resulted in upregulation of CD40 in the whole APC population, and in activation of CD11c$^+$ DC by almost all criteria (upregulation of CD40, CD80, CD86 and

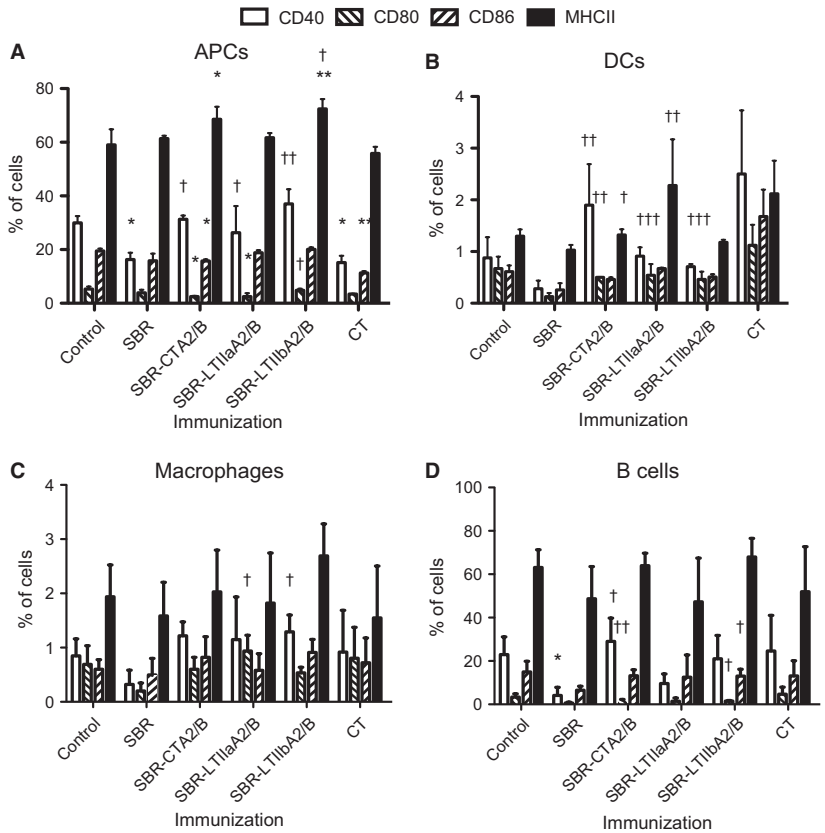

Figure 1 Surface expression of activation markers CD40, CD80, CD86, and MHC-II by antigen-presenting cells (APC) from Peyer's patches 24 h after intragastric immunization with SBR, SBR-CTA2/B, SBR-LTIIaA2/B, or SBR-LTIIbA2/B. (A) Whole APC; (B) $CD11c^+$ dendritic cells; (C) $CD11b^+$ macrophages; (D) $B220^+$ B cells. Bars show means ± SD ($n = 5$ to $n = 7$). $^*P < 0.05$, $^{**}P < 0.01$, relative to unimmunized controls; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, relative to immunization with SBR alone (analysis of variance).

MHC-II), as did immunization with CT (Fig. 1B). Fewer sporadic changes were seen in activation markers for macrophages and B cells (Fig. 1C,D).

Changes in whole APC activation were more evident in the MLN (Fig. 2). Immunization with the chimeric proteins appeared to enhance CD40, CD86 and MHC-II expression among APC, especially relative to SBR alone (Fig. 2A), and to upregulate CD40 and CD80 expression among DC (Fig. 2B). Immunization with CT increased DC activation markers, although compared with unimmunized controls this was not so evident in the whole APC population (Fig. 2A). It was notable that MHC-II expression on DC in both PP and MLN was low although immunization with CT increased this, especially in MLN (Fig. 2 B). Macrophages showed some evidence of activation in response to immunization with the chimeric proteins, with elevation of CD86 and MHC-II, whereas CT elevated mainly CD40 and CD80 (Fig. 2 C). B-cell activation was diminished after immunization with SBR, but the chimeric proteins and CT showed little effect (Fig. 2D).

DISCUSSION

Flow cytometric analysis of APC populations in PP and MLN after i.g. immunization has revealed that antigen (SBR) coupled to enterotoxin B subunits in the form of recombinant chimeric proteins engages APC in a manner that free SBR alone does not. Furthermore, differential effects were seen between different chimeric proteins that may be attributable to their different receptor-binding properties, because it is known that the principal receptor recognized by CT is GM1 ganglioside, whereas LT-IIa recognizes GD1b>GM1>GT1b>GQ1b>GM2>GD1a>GM3, and LT-IIb recognizes predominantly GD1a (Fukuta *et al.*, 1988). Moreover the B subunits of LT-IIa and LT-IIb, but not CT, interact with Toll-like receptor 2 (Hajishengallis *et al.*, 2005b).

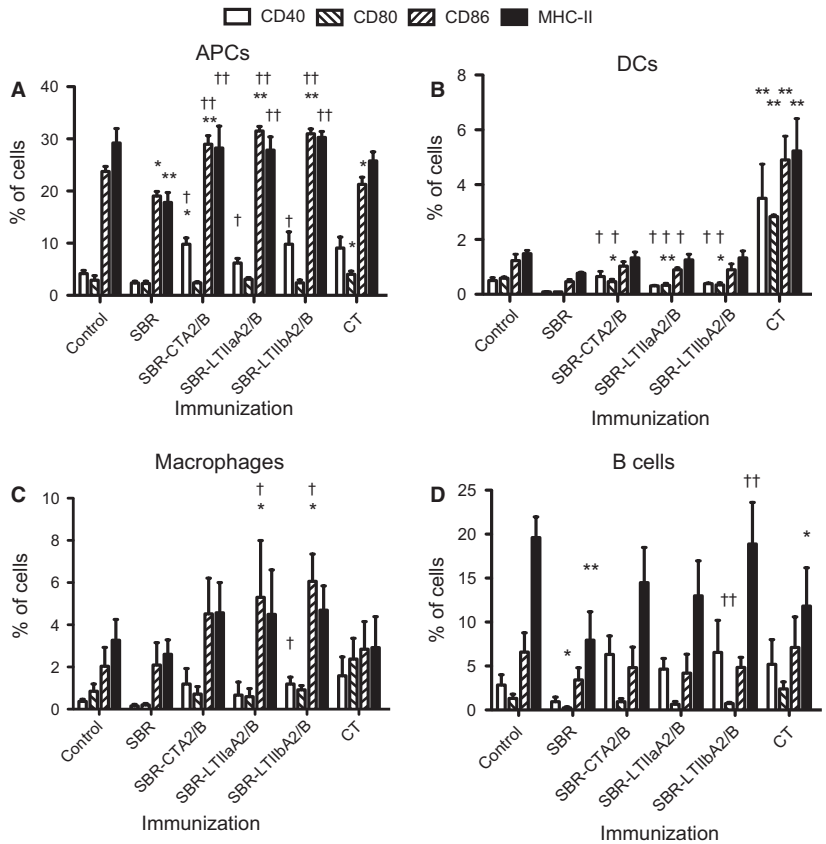

Figure 2 Surface expression of activation markers CD40, CD80, CD86, and MHC-II by antigen-presenting cells (APC) from mesenteric lymph nodes 24 h after intragastric immunization with SBR, SBR-CTA2/B, SBR-LTIIaA2/B, or SBR-LTIIbA2/B. (A) Whole APC; (B) $CD11c^+$ dendritic cells; (C) $CD11b^+$ macrophages; (D) $B220^+$ B cells. Bars show means ± SD ($n = 5$ to $n = 7$). $^*P < 0.05$, $^{**}P < 0.01$, relative to unimmunized controls; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, relative to immunization with SBR alone (analysis of variance).

The predominant APC detected in PP were B cells, which are known to occur within M-cell pockets (Brandtzaeg, 2010). It is therefore noteworthy that i.g. immunization with chimeras based on LT-IIa and LT-IIb resulted in elevated numbers of B cells in PP, and correspondingly diminished numbers of B cells in MLN. Conversely, immunization with the CT-based chimera or with CT alone decreased the numbers of B cells found in both PP and MLN. Differential effects of type II and type I enterotoxins on B cells have been observed previously: CT, but not LT-IIa or LT-IIb, enhanced expression levels of CD86, and only CT induced B-cell differentiation into plasma cells (Arce *et al.*, 2005, 2007). Only minor shifts in numbers of DC or their subsets were observed in PP and MLN after i.g. immunization with the chimeric proteins, although CT alone caused an elevation of all subsets of DC in MLN, with a concomitant decrease in numbers of macrophages. Notably, whereas SBR-LT-IIaA2/B and SBR-LT-IIbA2/B elevated the numbers of macrophages and B cells in the PP, SBR-CTA2/B did not, and CT itself markedly suppressed these cell numbers. Confocal microscopic studies have revealed that DC migrate into the follicular dome of PP within a few hours after intra-intestinal application of CT, but they decline after 12 h (Anosova *et al.*, 2008). However, our flow cytometric analysis of cell numbers could not detect such movements within tissues, and our observations were made 24 h after immunization. Initially, we also analysed cells harvested at 12 h, but found that the results were essentially similar, though at a lower level, to those reported here for 24 h.

To investigate further the effects of the chimeric proteins on APC, we determined their activation status by flow cytometric analysis of MHC-II, which is upregulated when APC take up and present exogenous antigens, and of the co-stimulatory molecules

CD40, CD80 and CD86, which are necessary to stimulate naive T cells. Expression of MHC-II was low on DC and macrophages in PP, even after i.g. immunization with CT or the chimeric proteins, suggesting that DC are in an immature state and not primarily involved in the response to these immunogens in PP. Whereas the combined APC population showed elevation of MHC-II in response to immunization with CT or with the SBR-LT-IIbA2/B chimera, and elevation of CD40 and CD86 in response to all three chimeric proteins, this was not clearly reflected in separate types of APC (DC, macrophages and B cells), although $B220^+$ B cells were the most activated regardless of immunization status. This probably reflects ongoing responses to luminal antigens (food and commensals), and was not much influenced by the experimental immunization. On the other hand, any specific effects of the immunogens administered would probably be confined to those few cells that take up these immunogens, which would be outnumbered and masked by the predominating responses to background antigens. We previously noted that CD86 expression was enhanced on human APC exposed to SBR-CTA2/B or SBR-LT-IIaA2/B chimeric proteins (Martin *et al.*, 2001b).

Recently there has been growing interest in the DC located in the intestinal lamina propria, because these cells have been shown to protrude their dendrites through the epithelium and sample antigenic material from the lumen (Rescigno *et al.*, 2001). It has been reported that these cells express CD103 and are largely suppressive in their response characteristics, i.e. that they induce T-regulatory cells rather than T-helper cells (Iliev *et al.*, 2009). The present investigation did not address these cells, but our findings have provided the basis for further studies using advanced imaging approaches to investigate lamina propria DC as well as those present in PP and MLN. These approaches are also allowing us to focus on the APC that take up the administered antigen or chimeric protein and present it to T cells whose characteristics and response patterns can then be determined. Because of controversies over the nature of the responses induced by antigens coupled to enterotoxin B subunits when these are given by mucosal routes, it becomes important to determine the precise nature and functional characteristics of APC engaged by these immunogens. We have repeatedly found that the recombinant chimeric proteins, SBR-CTA2/B, SBR-LT-IIaA2/B and SBR-LT-IIbA2/B are immunogenic in the absence of co-administered holotoxin adjuvants, when administered by i.g. or i.n. routes (Hajishengallis *et al.*, 1995, 1998, 2005a; Martin *et al.*, 2001a; Gockel & Russell, 2005). In contrast, *S. mutans* AgI/II (from which SBR is derived) chemically conjugated to CTB depends upon the co-administration of intact CT to induce responses by the i.g. route, though not by the i.n. route (Czerkinsky *et al.*, 1989; Russell & Wu, 1991; Wu & Russell, 1993, 1998). The precise molecular configuration of the coupled immunogen appears to be a factor in determining the outcome, and we suggest that this is determined at the level of the APC that are engaged.

Intranasal immunization with SBR-CTA2/B in rats induces protective immunity to dental caries similar to that induced by immunization with AgI/II-CTB conjugate (Hajishengallis *et al.*, 1998). The chimeric proteins based on type II enterotoxins have not been tested in this regard, but studies in mice have shown that i.n. immunization with SBR-LT-IIaA2/B, or with AgI/II adjuvanted with LT-IIa or LT-IIb induce similarly high levels of salivary IgA and circulating IgG antibodies (Martin *et al.*, 2001a; Nawar *et al.*, 2005). However, the immunomodulatory mechanisms responsible for the final outcome appear to be different because type I and type II enterotoxins engage different receptors and cells (Arce *et al.*, 2005). A particular advantage of the type II enterotoxins as adjuvants or coupled carriers is that they may pose less of a potential problem with respect to i.n. immunization. Both CT and LT-I have been found to traffic back to the olfactory bulb and brain via the olfactory nerve (Van Ginkel *et al.*, 2005), which may account in part for the observed adverse effects of using LT-I as an adjuvant in a human i.n. influenza vaccine (Mutsch *et al.*, 2004). However, the i.n. route is advantageous for inducing antibody responses in the saliva as well as upper respiratory and genital tracts (Wu & Russell, 1993), provided that safe adjuvants or delivery systems can be identified. The type II enterotoxins or their derivatives, which bind to different receptors than the type I enterotoxins (CT and LT-I), may be useful in this regard.

Streptococcus mutans AgI/II and its functionally important segment, SBR, have been identified as potential candidate antigens for the development of a mucosally delivered vaccine against dental caries (Russell *et al.*, 1999, 2004). As with many mucosal vaccines, a key issue is the development of a deliv-

ery or adjuvant system that will effectively elicit the desired responses in secretions, in this case, in the saliva. Coupling to the non-toxic B subunits of enterotoxins such as CT or type II LTs represents one approach that has been demonstrated to induce salivary IgA antibodies and protective immunity against caries in animal models (Katz *et al.*, 1993; Russell *et al.*, 1996; Hajishengallis *et al.*, 1998; Martin *et al.*, 2001a). Comprehending the mode of action of these constructs is necessary to optimize their application and further development. The impact extends beyond a caries vaccine, because the development of mucosal vaccines in general is limited by the requirement for effective adjuvant systems. One particular issue has been whether such vaccines will induce tolerance rather than active immunity (Mestecky *et al.*, 2007). Conversely, the development of vaccines to suppress undesirable immune responses would have a major impact on autoimmune and allergic diseases. Elucidating the mechanisms whereby enterotoxin-based adjuvant and delivery systems can be designed to induce either desired outcome is an important goal.

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