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Inhibition of interleukin-2 by a Gram-positive bacterium, *Streptococcus mutans*

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SUMMARY

Generation of an effective cellular immune response is key to the successful development of both humoral and cellular immune defences against most pathogens. However, while the type of cellular immune response elicited by any given pathogen is dictated by the entire array of antigens and molecules which comprise that pathogen, most studies of human immune responses to bacterial pathogens tend to focus on selected antigens. This is a result, in part, of a desire to find those antigens that will generate a desired immune response, as well as limited technology for monitoring the complex array of responses generated by an intact organism. Utilizing *Streptococcus mutans* as a model Gram-positive organism, a novel flow cytometric assay that permits the identification of individual cells within a responding population, and highly sensitive cytokine assays, we show for the first time that CD8 T cells and natural killer (NK) cells comprise a significant component of the response to this organism in humans. This is despite the fact that CD8 T cells are traditionally thought to respond to endogenously derived antigens only. In addition, we provide the first evidence that a Gram-positive organism can actively inhibit interleukin-2 (IL-2), an important autocrine growth factor for T cells. The latter observation could represent an additional mechanism by which Gram-positive organisms evade host defences.

INTRODUCTION

Generation of an effective and efficient cellular immune response to foreign antigen is key to the elimination of most pathogens.[1] In addition, the cellular immune response is not only critical for eliminating intracellular pathogens, but also dictates the amount and diversity of antibodies produced.[2,3] Studies examining the complex series of events following exposure of human peripheral blood immune cells to intact microorganisms are generally limited to examining responses to individual bacterial components. However, because the response to an intact organism may not necessarily be equal to the sum of the responses to its individual molecules, additional studies examining responses to whole organisms are still needed. Furthermore, in light of recent technological advances[4] and an increased understanding of T-cell subsets,[1] there are now more effective tools for examining such responses using human mixed lymphocyte populations such as peripheral blood mononuclear cells (PBMC). The goal of the studies presented in this paper was to examine the complex cellular immune response of human PBMC to a Gram-positive bacterium. Such studies could provide valuable information regarding the control of infectious organisms. We chose to use human PBMC because they are frequently the first cells to encounter foreign pathogens in humans.[1] *Streptococcus mutans* was selected as the Gram-positive organism to study because most individuals have been exposed to it by age 3,[5,6] and thus, *S. mutans*-specific responses are readily detectable in human peripheral blood.[7]

Utilizing assays to examine cytokine profiles generated in response to this organism, we show selective inhibition of interleukin-2 (IL-2), IL-2 being an important autocrine growth factor for T cells.[1] Furthermore, employing a novel assay which allows one to identify specific cell subsets proliferating in response to antigen,[4] we show that this Gram-positive bacterium not only stimulates CD4 T cells and natural killer (NK) cells, but also CD8 T cells, traditionally recognized for their ability to respond primarily to endogenously derived antigens.[8] Thus, these studies have important implications for regulation of immunity to *S. mutans* in particular, and possibly to Gram-positive organisms in general.

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Abbreviations: IL-2, Interleukin 2; LTA, lipoteichoic acid; PBMC, peripheral blood mononuclear cells; *S. mutans*, *Streptococcus mutans*; Tc, cytotoxic T cells; Th, helper T cells; TT, tetanus toxoid.

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MATERIALS AND METHODS

Antigens

Tetanus toxoid (TT) was obtained from Accurate Chemical and Scientific Corporation (Westbury, NY). *Streptococcus mutans*, strain 3209, was cultured in Todd Hewitt broth

overnight at 37°. The bacteria were then pelleted in 300 ml volumes using a Sorvall GSA rotor at 10 K for 10 min at 4°. Following centrifugation, bacteria were resuspended in HEPES-buffered RPMI (HRPMI) containing 0·1 μg/ml human albumin (Sigma, St. Louis, MO). To break up any chains of bacteria, organisms were sonicated for 5 s. at a setting of 7 using the Ultrasonic Model W220 Sonicator (Heat Systems-Ultrasonics Inc., Plainview, NY). This sonication procedure does not disrupt the cell wall, but rather leaves the organisms intact and viable,[9,10,11] as confirmed by light microscopy. Bacteria were resuspended in 10 ml 1 × phosphate-buffered saline (PBS) and aliquoted into 0·8 ml volumes ($2–7 \times 10^9$ bacteria/ml) to which 1 ml of 60% glycerol was added to maintain viability, and then stored at −70°. When thawed, bacteria were washed three times in 1 × PBS. If fixed organisms were used, the bacteria were fixed overnight with 0·08% methanol-free formalin (Sigma) in 1 × PBS and then washed three times with 1 ml of the same. Additionally, live organisms were used immediately after growth, eliminating the freeze in glycerol. All antigen concentrations were chosen based on preliminary experiments specifically designed for assay optimization.

Cells

PBMC were isolated using Ficoll–Hypaque as previously described,[12] and were the source of human immune cells. PBMC were obtained from normal, healthy donors ranging from 25 to 42 years of age. Purified monocytes were isolated by cold aggregation as previously described.[13] Briefly, PBMC were resuspended in HRPMI + 10% fetal bovine serum (FBS) at 50×10^6 cells/ml, rocked gently at 4° for 30–45 min and incubated on ice for 20 min, allowing the aggregates to precipitate. Once precipitated, the supernatant was removed and the pellet gently resuspended in 1·5 ml HRPMI + 10% FBS. The aggregates were then layered over 2 ml of ice-cold 100% FBS and allowed to precipitate through the FBS on ice for 30–45 min. The top layer was then removed leaving a population which was >85% pure monocytes as determined by fluorescence-activated cell sorting (FACS) analysis. The cells were then frozen in liquid nitrogen until use. All cell concentrations were chosen based on preliminary experiments specifically designed for assay optimization.

Cell proliferation measured by [^{3}H] thymidine incorporation

To initially analyse the cellular response to *S. mutans*, [^{3}H]thymidine incorporation was used as previously described,[12] with some modifications. Briefly, 100 μl of PBMC at $2·5 \times 10^5$ were added to individual wells of a 96-well plate (Costar). This was followed by the addition of 100 μl of *S. mutans* in Aim V medium at concentrations in 1:3 serial dilutions ranging from 0 to $8·2 \times 10^4$ fixed bacteria/ml and incubation at 37°, 5% CO_2 for 72 hr. The proliferative response to the antigen was then monitored by addition of [^{3}H]thymidine (ICN, Biomedicals, Los Angeles, CA), for a period of 24 hr at 37° and 5% CO_2. Cells were then harvested using a Skatron (Skatron Instruments, Ltd., Suffolk, UK) cell harvester, and [^{3}H] thymidine incorporation measured using a RackBeta (LKB Wallac, Turku 10, Finland) scintillation counter.

Analysis of cytokine profiles

The production of IL-2, interferon-γ (IFN-γ) and tumour-necrosis factor-β (TNF-β) (T helper 1; Th1), and IL-4, IL-5 and IL-10 (Th2) in the supernatants of 15×10^6 PBMC responding to $0·24 \times 10^6$ *S. mutans*/ml or 33·3 μg/ml TT was monitored and compared to that of PBMC alone. Culture supernatant was collected on day 3 following addition of antigen, and then frozen at −20° for future analysis. Cytokine enzyme-linked immunosorbent assay (ELISA) kits were obtained from commercial sources (Immunotech, Inc., Westbrook, ME and R&D Systems, Minneapolis, MN) and were carried out according to manufacturer's instructions. Sensitivities of the assays ranged from 1 (IL-5), 4 (IFN-γ), 5 (IL-4 and -10) and 7 (TNF-β) pg/ml to 500 (IL-5), 1000 (IL-4), 1250 (IFN-γ), 2000 (IL-10) and 10 000 (TNF-β) pg/ml.

Transwell experiments

To determine whether *S. mutans* was producing a soluble factor which was suppressing IL-2 in cells stimulated with TT, PBMC at a concentration of $1·2 \times 10^6$ cells/ml with or without each antigen were placed in the wells of a 12-well, 12-mm diameter, 0·4 μm pore size transwell plate (Costar) in the following manner: *S. mutans* at a concentration of $0·24 \times 10^6$ bacteria/ml in the top well and TT at a concentration of 33·3 μg/ml in the bottom well, each antigen alone and a no antigen control. Plates were then incubated for 3 days at 37° in 5% CO_2. Supernatants were harvested on the third day and assayed for IL-2 production using the cytokine assays outlined above.

Identification of proliferating cells using the 'cell census plus' assay

To analyse in more detail the specific cell populations proliferating in response to antigen, the 'cell census plus' system, which monitors cellular proliferation using flow cytometry, was utilized.[4] 'Cell census plus' kits were a generous gift of Sigma Chemical Co. (St Louis, MO). This is a novel assay which detects proliferation by monitoring the reduction in fluorescence of a membrane dye (PKH26) as a result of cell division.[4] Data can be expressed in terms of a proliferative index which takes into account the number of cells dividing, and the number of divisions undergone. Alternatively, results may also be expressed as the percentage of total cells proliferating. The assay was performed as indicated by the manufacturer and as previously described,[4] with some modifications. Briefly, PBMC were isolated from whole blood over Ficoll–Hypaque and washed three times with serum- and protein-free media (HRPMI only) at 400 ***g*** for 5 min. Cells were then resuspended at 5×10^6 cells/ml in Diluent C and added to an equal volume of dye diluted 1:250 in Diluent C. The cells were then incubated for 3 min at 25°, while inverting the tube periodically. An equal volume of FBS was then added followed by an equal volume of RPMI + 2% FBS and cells were then spun for 10 min at 400 ***g***. Cells were then washed twice in a clean tube, counted and resuspended at $1·25 \times 10^6$ cells/ml in Aim V. For every 1 ml of cells, 25 μl of autologous serum and either 6 μl *S. mutans* at 40×10^6 bacteria/ml and 5·1 μl Aim V or 11·1 μl Aim V were added. Then, 6·4 ml of each sample were added to the wells of a 6-well plate and incubated for 7 days at 37°, 5% CO_2. On day 7, the cells were harvested and stained with monoclonal antibodies (mAb) to CD3 (pan T),

CD4 (T helper cells (Th)), CD8 (cytotoxic T cells (Tc)), CD56 (NK cells) and CD19 (B cells) (Beckton Dickinson, Mountain View, CA) with the appropriate fluorescent labels as described in the section on flow cytometry. Following acquisition on a Beckman FACScan, data analysis was completed using the ModFit software package (Verity Software House, Inc., ME). In these studies, data are expressed either as the percentage of cells within the total cell population proliferating, or the percentage of cells that are of a specific type within the proliferating population.

Flow cytometry

Flow cytometry was used to identify specific cell populations based on surface marker expression. Staining procedures were adapted from those previously described, to accommodate multiple fluorochromes.[12] Briefly, to individual wells of a 96-well plate at 4°, 20 µl of PBMC at $12{\cdot}5 \times 10^6$ cells/ml were added, followed by 20 µl of 12 mg/ml human blocking immunoglobulin G (IgG) (Sigma) and 20 µl of mAb to CD3 which was directly labelled with PerCP, and either CD4, CD8, CD56 or CD19 which were all directly labelled with fluorescein isothio cyanate (FITC) (Beckton Dickinson). After a 1 hr incubation at 4°, plates were centrifuged and the cells washed three times with PBS bovine serum albumin (BSA) at 4° and resuspended in PBS/BSA containing 2% methanol-free formalin (Kodak, Rochester, NY). Cells were examined and mean fluorescence intensity (MFI) measured.

Analysis for suppressive factors

To determine whether the factor suppressing IL-2 was derived from lymphocytes, monocytes, or *S. mutans* itself, we devised the following assay. Live or fixed *S. mutans*, at a concentration of $0{\cdot}24 \times 10^6$ cells/ml, was incubated either alone or with 15×10^6 PBMC or 5×10^6 monocytes in Aim V. After 3 days at 37°, 5% CO_2, the resultant supernatants were passed through a 0·2 µm filter and then incubated in a 12-well plate (Costar) with $1{\cdot}9 \times 10^6$ PBMC and 33·3 µg/ml TT for 3 days at 37° and 5% CO_2. The supernatants generated were then assayed for IL-2 using the cytokine ELISA kits described above.

Statistical analysis

The data in Fig. 1 and Table 2 were analysed by the paired *t*-test and were considered significantly different when $P < 0{\cdot}03$.

RESULTS

S. mutans induces a potent proliferative response in human PBMC

We initially examined proliferation in response to intact *S. mutans* using [^{3}H] thymidine in order to confirm the presence of a detectable proliferative response to this organism. Potent proliferation in response to *S. mutans* was in fact observed for all donors tested, although the level of the response was donor dependent (Fig. 1).

Unique cytokine profiles generated in response to *S. mutans*

Previous studies using purified components of *S. mutans* had suggested that much of the proliferating cell population would consist of CD4 T cells.[7] We therefore attempted to determine if all the major cytokines common to the known T-cell subsets would be present following exposure of human PBMC to *S. mutans*. We found that *S. mutans* generated cytokine profiles that could not be clearly defined as belonging to either the Th1 or Th2 subsets. More significantly, with the majority of donors, despite the presence of IFN-γ, TNF-β and IL-10, the Th1 (IL-2) and Th2 (IL-4) cytokines important for T-cell proliferation were undetectable following the exposure of PBMC to *S. mutans* (Table 1). Yet, IL-2 was detectable when using TT, which has been shown to stimulate cytokine production characteristic of both Th1 and Th2 T cell subsets[14] (Table 1).

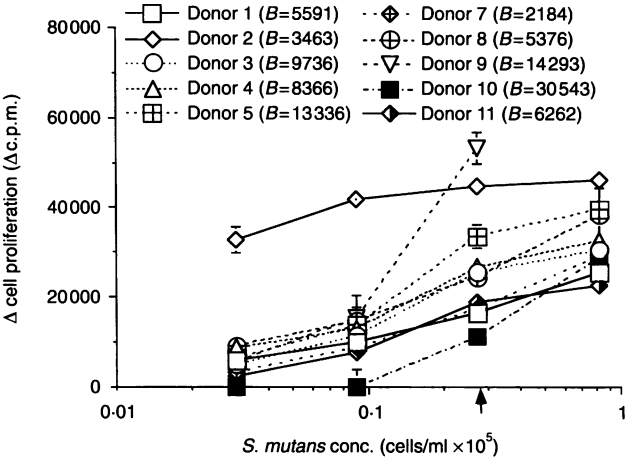

Figure 1. Detection of *S. mutans*-specific responses as measured by [^{3}H] thymidine incorporation. Varying doses of *S. mutans* were added to PBMC and [^{3}H] thymidine was added 3 days later. [^{3}H] thymidine incorporation was measured on day 4. Data represents the average of three replicates with backgrounds subtracted to facilitate comparison of *S. mutans*-specific responses between individual donors. Data is expressed as counts/minute (c.p.m.) ± SD. B = background values for each respective donor. Statistical analysis was performed using a paired *t*-test which compared responses at an antigen concentration of $2{\cdot}7 \times 10^4$ with the background values for each donor (arrowhead on the *x*-axis indicates concentration chosen). The values for nine of 10 donors were statistically different with $P < 0{\cdot}03$.

Inhibition of IL-2 by soluble factors induced by *S. mutans*

To determine whether the absence of IL-2 in PBMC exposed to *S. mutans* could be explained by active inhibition of IL-2, additional experiments were conducted. To ensure that *S. mutans* did not secrete or induce secretion of a substance that blocks detection of IL-2 in our ELISA, a 1:1 mixture of supernatants generated from TT-specific and *S. mutans*-specific T cells were assayed for IL-2. Detection of IL-2 in response to TT was not blocked by the presence of supernatants from PBMC stimulated with *S. mutans* (data not shown). In contrast, we found our detection of IL-2 was significantly reduced when *S. mutans* and TT were combined during T-cell stimulation (Table 2). Using a transwell system, we observed that when *S. mutans*- and TT-stimulated PBMC were separated by a semipermeable membrane through which whole *S. mutans* could not pass, IL-2 detection in supernatants generated by PBMC stimulated with TT was again significantly reduced (Fig. 2). This suggests that the factor which is inhibiting IL-2 is soluble in nature and less than 2 µm in size, possibly a

Table 1. Cytokines produced in response to *S. mutans* and TT

	Th1-like						Th2-like					
	IFN-γ		TNF-β		IL-2		IL-4		IL-5		IL-10	
Donor	SM	TT	SM	TT	SM	TT	SM	TT	SM	TT	SM	TT
1	20250	840	40	70	<5	289	<5	<5	38	108	480	<5
2	24500	>1250	320	440	<5	250	52	113	48	108	240	<5
3	24750	250	130	270	<5	220	<5	<5	<1	28	800	<5
4	19400	863	50	80	<5	217	<5	52	<1	10	140	<5
5	24750	638	170	140	<5	250	35	<5	<1	>1000	<5	<5
7	8750	113	5	240	<5	21	<5	30	<1	225	1200	700
8	250	430	<7	140	<5	125	<5	30	<1	3	<5	<5
9	200	713	<7	560	18	975	<5	<5	<1	202	<5	<5
10	4250	100	40	40	<5	95	<5	<5	5	3	110	<5

Results represent cytokine levels in pg ml detected in supernatants generated by PBMC in the presence of *S. mutans* (SM) or TT for 3 days. The > or < symbols indicate that the value obtained was not within the level of detectability of the assay.

Table 2. Inhibition of TT-induced IL-2 in the presence of *S. mutans*

Donor	TT alone	*S. mutans* TT	*S. mutans* alone
1	260	13	<5
2	>1000	69	<5
3	330	105	<5
7	129	50	<5

TT and *S. mutans* were incubated either alone or together for 3 days in the presence of PBMC. Supernatants were assayed for IL-2 and results are expressed in pg/ml. Statistical analysis was performed using a paired *t* test which compared the values for TT alone and *S. mutans* TT and were determined to be statistically different with $P<0.03$.

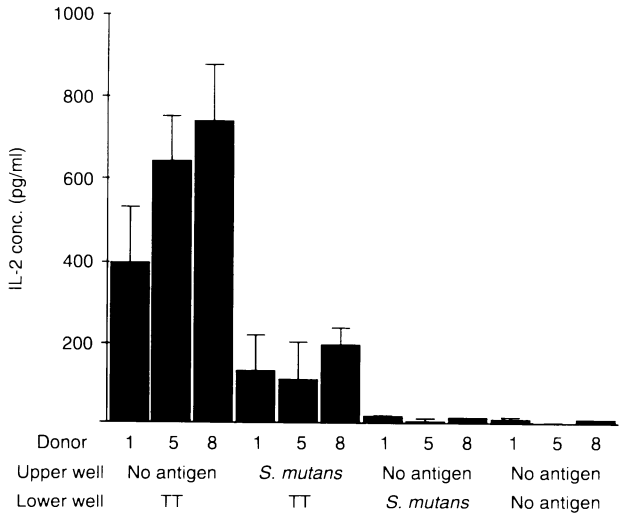

Figure 2. A soluble factor induced by *S. mutans* mediates suppression of IL-2 in PBMC responding to TT. PBMC and antigen were incubated for 3 days in transwell plates in the following manner: *S. mutans* in the top well and TT in the bottom well, each antigen alone and a no antigen control. Supernatants were then assayed for IL-2. Data is expressed as IL-2 concentration in pg ml of culture supernatant. SM = *S. mutans*, NA = no antigen. Each bar represents the average of the IL-2 detected in the top and bottom wells.

cytokine or bacterial component which has been processed and released by macrophages.

S. mutans generates a mixed cellular response consisting of CD4 T, CD8 T, NK and B cells

In order to more clearly define whether the cells proliferating in response to intact *S. mutans* were in fact T cells, or other cell populations present in peripheral blood, we employed a novel proliferation assay which allows one to identify the specific cell types responding. Using ModFit analysis as previously described,[4] it was apparent that only a small percentage of the total PBMC population (10 20%) proliferated in response to *S. mutans*, and that the responding cells underwent multiple divisions (Fig. 3 and data not shown). Surprisingly, within the population of cells proliferating in response to *S. mutans*, we observed proliferation by CD8 as well as CD4 T cells. In addition, NK cells and B cells also responded, although B cells were a minor portion of the responding

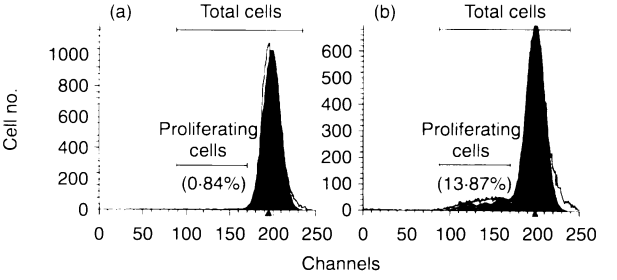

Figure 3. Identification of cells proliferating in response to *S. mutans* using the 'cell census plus' assay and flow cytometry. Cells were treated with a fluorescent membrane dye, PKH26, followed by incubation for 7 days in the absence (a) or presence (b) of *S. mutans*. Cells were harvested on day 7, stained for surface marker expression and analysed by flow cytometry. PKH26 data was analysed using ModFit software as previously described.[4] Each cell division produces an approximate 50% reduction in fluorescence. Thus, each peak within the proliferating population represents an individual generation. Data is expressed as the percentage of proliferating cells within the total cell population. This histogram is representative of four separate experiments.

Table 3. Cells proliferating in response to *S. mutans*

Donor	1 (13·87)*	5 (19·6)	8 (19·6)	10 (16·6)
Cell type	% of proliferating cells†			
CD4 T cells	8·9±0·1	35·1±2·7	49·5±7·0	18·4±3·3
CD8 T cells	11·1±0·05	19·5±0·5	27·5±0·8	19·9±0·8
NK cells	25·5±1·0	21·2±0·7	14·7±3·8	25·9±0·6
B cells	2·3±0·007	4·0±0·01	4·7±0·3	3·5±0·2
Other‡	52·2	20·2	3·6	32·3

*Numbers in parentheses indicate the percentage of cells within the total PBMC population proliferating in response to *S. mutans*. Percent of cells proliferating in the absence of antigen was generally less than 1% (see Fig. 3).

†Results represent the percentage of a specific cell type within the proliferating population (Fig. 3). PKH26-stained PBMC and *S. mutans* were incubated for 7 days, followed by staining with mAb specific for the indicated surface markers and flow cytometric and ModFit analyses.

‡The 'other' population refers to the portion of cells which did not stain with mAb specific for CD4, CD8, CD56(NK cells) or CD19(B cells).

population (Table 3). Donor to donor variation was also observed, and a significant percentage of the proliferating cells could not be identified using T-, NK-, and B-cell markers.

S. mutans releases factors which suppress IL-2

Because our proliferation studies indicated that *S. mutans* induces a mixed cellular response, we initially hypothesized that *S. mutans* may induce responding cells to produce a factor which suppresses IL-2. Therefore, we attempted to identify the source of the suppressive factor by comparing the ability of purified monocytes versus PBMC to inhibit IL-2 in the presence of live or fixed *S. mutans*. As part of this analysis, we also placed the bacteria in media without cells, assuming the presence of immune cells would be required to obtain suppressive activity. However, we observed that in the presence of fixed *S. mutans*, and the absence of monocytes (data not shown) or lymphocytes, suppressive activity was still present (Fig. 4). In contrast, when using live bacteria, the presence of PBMC was required to generate the suppressive activity (Fig. 4).

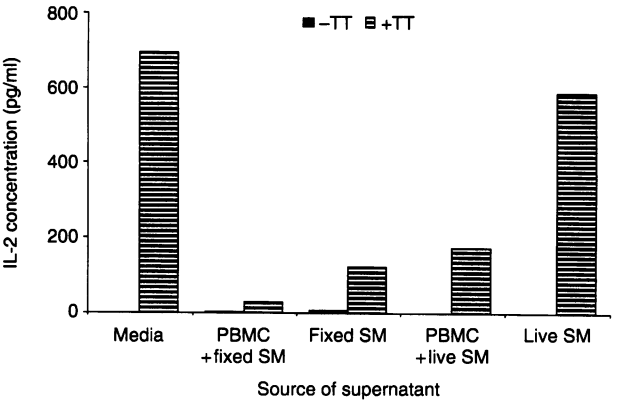

Figure 4. *S. mutans* releases factors which suppress IL-2. Live and fixed *S. mutans* were incubated either alone or in the presence of lymphocytes for 3 days. Supernatants were collected on day 3 and passed through a 0·2 μm filter. These supernatants were then incubated with TT and PBMC for 3 days and the resultant supernatants assayed for IL-2. Data represents the amount of IL-2 in pg/ml present in culture supernatants.

DISCUSSION

These are the first studies to provide a comprehensive examination of the cellular arm of the immune response to an intact Gram-positive bacterium, *S. mutans*. To accomplish this, we have used a novel flow cytometric assay which permits identification of responding cells, as well as cytokine assays to monitor the presence or absence of cytokines characteristic of specific T-cell subsets.

As previously observed using purified *S. mutans* antigens,[15] we saw a potent proliferative response to whole *S. mutans* by PBMC from all donors tested (Fig. 1). To address the possibility of superantigenic activity by *S. mutans*, we conducted preliminary studies to test whether the proliferative response to *S. mutans* was non-specific in nature. Despite reports that other Gram-positive organisms exhibit superantigenic activity,[16–18] to date, there is no evidence in the literature to suggest that *S. mutans* does. We found that Epstein–Barr virus (EBV)-transformed B cells, although able to stimulate TT-specific T cells in the presence of TT, were unable to stimulate *S. mutans*- or TT-specific T cells in the presence of *S. mutans* (data not shown). Furthermore, *S. mutans* was unable to induce proliferation of TT-specific T cells in the presence of macrophages which do process and present *S. mutans* to *S. mutans*-specific T cells (data not shown). These studies indicate that neither whole *S. mutans* nor its processed components stimulate T cells independent of antigen processing or antigen-specific recognition, suggesting that superantigens are not involved.

Having observed a significant proliferative response to *S. mutans*, we attempted to identify Th subsets responding to *S. mutans* by screening supernatants for the cytokines characteristic of each subset (Table 1). CD4 T cells have been divided into a number of major subsets based on their expression of CD4 and CD8, and the cytokines they secrete (Th0: IL-2, IFN-γ, IL-4, IL-5 and IL-10; Th1: IL-2, IFN-γ, and TNF-β; and Th2: IL-4, IL-5 and IL-10). Depending on the subset involved, T cells have been shown to play an important role in both the cellular and humoral response to both intracellular and extracellular pathogens.[2,3] Surprisingly, we failed to detect IL-2 in response to *S. mutans* in the majority of donors tested (Table 1). Initially, we sought to explain the absence of IL-2 in the presence of *S. mutans* by examining whether *S. mutans* blocked detection of IL-2 in our ELISA assays (data not shown). In fact, *S. mutans* did not appear to block IL-2 detection by ELISA; however, when *S. mutans* was added to PBMC stimulated with TT, IL-2 levels were significantly reduced (Table 2). This suggested active suppression of IL-2 by *S. mutans*. Furthermore, transwell experiments indicated that this suppression was mediated by a soluble factor derived either from responding cells or *S. mutans* itself (Fig. 2). In testing this hypothesis, we found that while fixed *S. mutans* spontaneously releases a factor that suppresses IL-2, live organisms require monocyte/macrophage processing (Fig. 4). Evidence suggesting that fixation can lead to changes in the

composition of the bacterial cell wall,[24] could explain the apparent dichotomy between fixed and live organisms. In addition, it is known that phagocytic cells are capable of releasing components of internalized organisms and antigens, once degraded.[26,27] Therefore, we believe that processing of live bacteria by phagocytic cells, specifically monocyte/macrophages in our system, could lead to the release of bacterial components, suppressive in nature, which are not normally available in the intact, unfixed organism. Combined, these data suggest that a component of *S. mutans* is the source of the suppressive activity, and that monocytes or macrophages are required to generate such activity under normal conditions where *S. mutans* is actively replicating. Several explanations exist which may explain the absence of IL-2. First, the T-cell subset responsible for secreting IL-2 may be absent.[19] Second, IL-2 production by Th1 cells may be inhibited. This issue is important because IL-2 is an autocrine growth factor for T cells and, in limiting amounts, could constrain T-cell responses. Third, the IL-10 that we detected in response to *S. mutans*, may be involved in the decrease of IL-2 since IL-10 favours the development of the Th2 helper T-cell subset. However, if IL-10 was involved in lessening the Th1 response, one would not expect to detect other cytokines traditionally associated with a Th1-type response such as TNF-β and IFN-γ. We detected both of these cytokines in the presence of *S. mutans*. In addition, the actions of IL-10 are reported to be antagonized by IFN-γ.[28] Finally, it is also possible that components of *S. mutans* interact with IL-2 and block its interaction with the IL-2 receptor on T cells. In fact, we have preliminary data to suggest that this is likely the case (data not shown).

The apparent lack of defined CD4 T-cell subsets responding to *S. mutans*, limited quantities of T-cell growth factors (IL-2 and IL-4), and high levels of IFN-γ (Table 1), left the possibility that perhaps cell populations other than CD4 T cells might account for much of the response observed when using [^{3}H] thymidine incorporation to measure proliferation in the presence of *S. mutans*. This was confirmed using the 'cell census plus' assay in which it was apparent that the proliferative response to *S. mutans* was in fact composed of not only CD4 T cells, but CD8 T cells, NK cells and B cells (Table 3). Although we have no specific data to suggest that the CD8 T-cell response we observed is antigen specific, recently data has appeared suggesting that CD8 T cells have the capacity to respond to exogenously derived antigens as a consequence of monocyte/macrophage processing.[8] In addition, the fact that *S. mutans* is present on the smooth surfaces of teeth in the oral cavities of most individuals above the age of 3,[5,6] and is therefore introduced through the oral route, may also help to explain the presence of CD8 T-cell responses in the periphery. Numerous studies with soluble antigens suggest that chronic low-dose oral immunization, like that which occurs as a consequence of natural immunization with *S. mutans*, can lead to immune suppression.[20] Such suppression has been attributed to cytokines induced by CD8 T cells.[21] Furthermore, data exists suggesting a similar situation could apply in the case of *S. mutans*. Studies in which humans were fed capsules containing *S. mutans* demonstrated that while salivary IgA titres were increased following oral immunization, peripheral IgG responses to *S. mutans* were absent.[22] Also, when rhesus monkeys were immunized with high doses of *S. mutans*, suppressor cells were present and IgG titres were depressed.[23] Furthermore, our studies have shown that antibody titres to *S. mutans* in human peripheral blood are 10-fold lower than to TT (data not shown), an intramuscular immunogen recognized for its ability to generate high titres of antigen-specific IgG in human peripheral blood.

We have preliminary evidence to suggest that the suppression of IL-2 is at least in part caused by the surface molecule lipoteichoic acid (LTA) (data not shown). Studies in this regard are ongoing. Previous studies have shown that LTA[29] and teichoic acid,[30] which is similar to LTA but lacks the lipid portion, are involved in immune suppression in mice. In addition, LTA shares many properties in common with lipopolysaccharide, which has been shown to be suppressive in mice[31,32] as well as humans.[33,34] In fact, this is not the first report to suggest that the levels of important cytokines may be decreased as a result of Gram-positive or -negative bacterial components.[16,35] Such components have been shown to interfere with the production as well as release of specific cytokines.[35] In fact, we have preliminary evidence to suggest that after a period of 3 days at 37°, LTA physically binds to IL-2 (data not shown). Therefore, we hypothesize that LTA is involved in the specific decrease of IL-2 in our system and that perhaps the mechanism whereby this suppression occurs lies at the level of a physical interaction between the two molecules which interferes with the binding of IL-2 to its receptor.

In summary, these are the first studies, of which we are aware, that examine the complex human cellular response to an intact Gram-positive organism by simultaneously identifying the cytokines produced, and specific cell types responding within a mixed population of human lymphocytes, PBMC. We demonstrate for the first time, that CD8 T cells and NK cells comprise a significant portion of the human response to this gram positive organism. We also demonstrate that *S. mutans* actively inhibits IL-2, and fails to induce substantial levels of IL-4 (Table 1 and Figs 2 and 4). The fact that proliferation is still observed in the apparent absence of these cytokines suggests that IL-2 and IL-4 may be present in limiting amounts, or that another cytokine, perhaps IL-15,[25] may replace IL-2 and/or IL-4.

Thus far, the mechanisms by which *S. mutans* stimulates CD8 T cells, and inhibits IL-2 are not entirely clear and need further investigation. However, the latter observation in particular could represent an additional important mechanism by which Gram-positive microorganisms evade host immunity.

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