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ORAL MICROBIOLOGY
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Human T-cell responses to oral streptococci in human PBMC-NOD/SCID mice

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We investigated cellular and humoral immune responses to oral biofilm bacteria, including *Streptococcus mutans, Streptococcus anginosus, Streptococcus sobrinus*, and *Streptococcus sanguinis*, in NOD/SCID mice immunized with human peripheral blood mononuclear cells (hu-PBMC-NOD/SCID mice) to explore the pathogenicity of each of those organisms in dental and oral inflammatory diseases. hu-PBMC-NOD/SCID mice were immunized by intraperitoneal injections with the whole cells of the streptococci once a week for 3 weeks. FACS analyses were used to determine the percentages of various hu-T cell types, as well as intracellular cytokine production of interleukin-4 and interferon-γ. Serum IgG and IgM antibody levels in response to the streptococci were also determined by enzyme-linked immunosorbent assay. *S. anginosus* induced a significant amount of the proinflammatory cytokine interferon-γ in $CD4^+$ and $CD8^+$ T cells in comparison with the other streptococci. However, there was no significant differences between the streptococci in interleukin-4 production by $CD4^+$ and $CD8^+$ T cells after inoculation. Further, *S. mutans* significantly induced human anti-*S. mutans* IgG, IgG_1, IgG_2, and IgM antibodies in comparison with the other organisms. In conclusion, *S. anginosus* up-regulated Th1 and Tc1 cells, and *S. mutans* led to increasing levels of their antibodies, which was associated with the induction of Th2 cells. These results may contribute to a better understanding of human lymphocyte interactions to biofilm bacteria, along with their impact on dental and mucosal inflammatory diseases, as well as endocarditis.

Key words: biofilm; cytokines; interferon-γ; NOD/SCID mice; oral streptococci; T cells

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Generation of effective and efficient cellular immune responses to foreign antigens is considered to be a key function in the elimination of most pathogens (35). Further, such immune responses are not only critical for eliminating intracellular pathogens, but also dictate the amounts and diversity of the antibodies produced (27, 49). Studies that have examined the complex series of events following exposure of human peripheral blood mononuclear cells (hu-PBMC) to intact microorganisms have generally been limited to examining the 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 that examine responses to whole organisms are still required. Due to recent technological advances (57) and increased understanding of T cell subsets (35), effective tools for examining those responses using human mixed lymphocyte populations, such as hu-PBMC, are now available. The purpose of the present study was to examine the complex cellular and humoral immune responses of hu-PBMC to biofilm bacteria including oral streptococci, as the results could provide valuable information regarding the control of infectious biofilm organisms. We chose hu-PBMC because they are often the first cells to encounter foreign pathogens in humans (35). In addition, nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice reconstituted with hu-PBMC (hu-PBMC-NOD/SCID) have been used as *in vivo* models for studying human-specific infectious pathogens, such as human immunodeficiency virus (HIV) (9, 15, 46, 47) and oral streptococci (4, 36), as that strain can support levels of hu-cell grafting that are 5- to 10-fold greater than those obtained in SCID mice (35).

Dental caries is one of the most common infectious diseases affecting humans (23) and several different types of bacteria, especially species of viridans streptococci found in dental plaque biofilm, have been shown to be associated with oral infections (2, 19) as well as dental caries (23, 25, 29). Oral streptococci are also important agents of infective endocarditis that are suspected to contribute to its etiology in over 50% of the cases encountered (42, 54). In addition, the viridans group of streptococci has been shown to be responsible for alpha-streptococcal shock syndrome in neutropenic patients (48). However, the mechanisms by which viridans streptococci cause pathogenic lesions and bacteremia associated with severe clinical manifestations have not been elucidated.

In the present study, we examined *Streptococcus mutans, Streptococcus sobrinus, Streptococcus sanguinis*, and *Streptococcus anginosus* as representative viridans oral biofilm streptococci. *S. mutans* are thought to be present in large numbers in dental plaque biofilm, and there is a strong correlation between the presence of cariogenic organisms in plaque and the occurrence of dental caries in humans (14, 29). *S. sobrinus* is also one of the most common species encountered in human dental plaque, and is considered to be a principal agent of dental caries and oral infections in humans (6, 16) as well as animals (59). In addition, *S. sanguinis* is among the first bacteria to adhere selectively to and colonize saliva-coated teeth (11, 22), while it also interacts with salivary film, which is known to contain α-amylase, secretary IgA (10, 26), mucin (50), and agglutinin (5). *S. anginosus*, another member of the oral viridans group of streptococci, is a common commensal organism found in the human oral cavity and on other mucosal surfaces, such as in the upper respiratory and gastrointestinal tracts (43). This organism is considered to have a low level of pathogenicity; however, it has been reported to cause serious purulent abscesses in various body sites (11, 40, 55) and subacute bacterial endocarditis (40, 56).

A critical advance in cellular immunology was the discovery of two types of functionally distinct T cell subsets, T-helper type 1 (Th1) and T cytotoxic type 1 (Tc1) cells, and type 2 (Th2 and Tc2) cells, which are classified on the basis of their cytokine expression. Th1 and Tc1 cells produce cytokines that are associated with inflammatory reactions, as well as cell-mediated immune response factors such as interferon-γ (IFN-γ) (41). In contrast, Th2 and Tc2 cells release cytokines, including interleukin (IL)-4, IL-5, IL-6, and IL-10, that promote antibody-mediated immune responses (1, 33, 41). Therefore, IFN-γ and IL-4 have important and cross-regulatory roles in infections (38, 52).

In the present study, we examined the effects of the oral viridans streptococci *S. mutans, S. sobrinus*, *S. sanguinis,* and *S. anginosus* on leukocyte recruitment, as well as their stimulation of cytokine and antibody production by mononuclear cells *in vivo* in hu-PBMC grafted NOD/SCID mice, an animal model recently shown to be effective for evaluating host responses to oral pathogens (13, 60). Our findings have important implications regarding the regulation of immunity to oral streptococci and the capacity of those pathogens to stimulate the host during oral infection.

Material and methods

Mice

Specific pathogen-free female NOD/SCID mice from 6 to 8 weeks old were used in the experiments and housed in polypropylene mouse cages with pine sawdust on the floor. Each experiment utilized six mice, including two controls, and each mouse was transplanted with hu-PBMC from a donor. Animal care was performed in accordance with the guidelines of the National Institute of Infectious Diseases.

Microorganisms

S. mutans MT8148, *S. anginosus* ATCC 33397, *S. sobrinus* 6715, and *S. sanguinis* ATCC 10556 bacterial strains were taken from our laboratory stocks and used as representative strains. The microorganisms were cultured in brain-heart infusion media in anaerobic gas jars (Type AZ-Hard, Hirasawa, Tokyo, Japan) with an atmosphere of 10% CO_2 and 10% H_2 in nitrogen, then harvested by centrifugation and washed twice with 40 mM of sterile phosphate buffered saline (PBS, pH 7.4).

Antibodies and chemicals

The following monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA): R-phycoerythrin-conjugated antihu-CD8 and antihu-IFN-γ; fluorescent isethionate (FITC)-conjugated antihu-CD4 (clone RPA-T4), antihu-CD8 (RPA-T8), antihu-CD45 (30-F11), and antimouse CD45 (30-FH); and antigen presenting cell-conjugated antihu-IL-4. Alkaline phosphatase-conjugated goat antihuman IgG (γ), IgG_1, IgG_2, and antihu-IgM (μ) were also obtained (Organo, West Chester, PA), as were mouse antihuman IgG_3 (5G12) and IgG_4 (HP6025) (Biogenesis, England, UK, and Chemicon International Inc., Temecula, CA). Alkaline phosphatase-conjugated goat antimouse IgG (H+L) antibody (Zymed Laboratory, South San Francisco, CA) was used as a third antibody to detect human IgG_3 and IgG_4. We also purchased cell separating medium Lympholyte-M (Cedarlane, Hornby, Canada), Eagle's minimal essential medium (Nissui, Tokyo, Japan), RPMI-1640 (Sigma, St. Louis, MO), FACS lysing solution and FACS permeabilizing solution (BD Pharmingen), fetal bovine serum (ICN, Aurora, OH), Saponin and DNase I (Sigma, St. Louis, MO), disodium *P*-nitrophenyl-phosphate hexahydrate (Wako, Osaka, Japan), and trypan blue (Chroma, Stuttgart, Germany).

Transplantation of hu-PBMC

hu-PBMC were isolated from 400 ml of peripheral blood taken from an individual healthy adult volunteer by separation using Ficoll-Hypaque gradients, with a gravity of 1.077 ± 0.001 (Immuno-Biological Laboratory, Gunma, Japan). The cells were washed three times in Hanks' balanced salt solution (Gibco Laboratories, Life Technologies, Paisley, UK) and adjusted to a concentration of 2.5×10^7 in 500 μl of Hanks' balanced salt solution. Suspensions of hu-PBMC were then administered intraperitoneally at 500 μl per mouse using the transplantation technique reported by Mosier et al. and Senpuku et al. (31, 44). The NOD/SCID model grafted intraperitoneally with hu-PBMC has been employed for long-term *in vivo* analysis of human lymphocyte activation and pathogens (44). For each experimental group, six female mice from a single litter were grafted with PBMC from a donor and used.

Injection of humanized NOD/SCID mice with oral streptococci

One day after the experimental groups, each consisting six NOD/SCID mice, were transplanted with hu-PBMC from a single donor, the mice were injected intraperitoneally with 100 μg of each bacterial strain (dry weight/mouse, 1×10^7 colony forming units) suspended in 300 μl of 40 mM PBS, pH 7.0. Intraperitoneal injections of *S. mutans, S. anginosus, S. sobrinus, S. sanguinis,* or PBS (control) were given to five mice in separate groups, with the remaining

noninjected mouse in each group used as a negative control. Intraperitoneal injection has been reported to be a useful method to induce specific immuno-responses by hu-lymphocytes to immunogens in the hu-PBMC-NOD/SCID system (34, 44). The injections were given once a week for 3 weeks to both the experimental and the control mice. Three weeks after the final injection, blood serum, spleen cells, and peritoneal exudate cells were sampled for the following experiments. Each experiment was performed independently three or four times.

Flow cytometric analysis

Peritoneal exudate cells were collected by washing the peritoneal cavities twice with RPMI-1640 containing 10% heat-inactivated fetal bovine serum. The spleens were removed, then teased and filtered through a stainless steel mesh (Ikemoto, Tokyo, Japan) in ice-cold Hanks' balanced salt solution, after which mononuclear cells were isolated by density gradient centrifugation and contaminating erythrocytes were lysed with 0.83% ammonium phosphate buffer solution (pH 7.6). After washing, the cells were counted using a hemacytometer. All cell suspensions were washed once in ice-cold Hanks' balanced salt solution as described below. The spleen cells and peritoneal exudate cells were stained with FITC-, phycoerythrin-, and antigen presenting cell-conjugated antihu-marker mAbs in PBS/1% bovine serum albumin, then washed with Hanks' balanced salt solution medium. At least 10^4–10^5 live spleen cells, including mouse and hu-lymphoid cells, were acquired in each run. For each mouse analyzed, cells were also stained with mouse IgG conjugated to FITC, phycoerythrin, and antigen presenting cell as an isotype control. Spleen cells and peritoneal exudate cells from a nontransplanted NOD/SCID mouse were stained in parallel as an additional negative control. Fluorescence levels that excluded greater than 98% of the cells in the negative controls were considered to be positive and specific for hu-staining. The cells were fixed in a 3% formalin/Hanks' balanced salt solution and stored at 4°C until flow cytometric analysis. Samples gated on the forward light scatter and side light scatter were used to identify viable lymphocytes. Proportions of the major subsets were determined by single and quadrant analyses. Single cell suspensions were stained with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated antimouse CD45, antihu-CD45, antihu-CD4, and phycoerythrin-conjugated antihu-CD8. The percentages of FITC- and phycoerythrin-positive cells were measured using a FACS with the CELLQUEST program (Beckton Dickinson, San Jose, CA). Negative control samples were incubated with irrelevant, isotype-matched antibodies in parallel with all of the experimental samples, and produced no more than 0.2% fluorescent $CD4^+$ or $CD8^+$ T cells.

In a detection assay of intracellular cytokine production, spleen cells and peritoneal exudate cells were incubated for 4 h at 37°C in brefeldin A (10 μg/ml), then 100 μl of each were incubated for 15 min at room temperature with either FITC-conjugated antihu-CD4 or antihu-CD8 monoclonal antibodies (mAbs). Next, the cells were lysed with FACS lysing solution and permeabilized using FACS permeabilizing solution according to the manufacturer's instructions. The cells were then washed with PBS containing 2% fetal bovine serum and incubated for 30 min at room temperature in the dark with either phycoerythrin-conjugated anti-IFN-γ or antigen presenting cell-conjugated anti-IL-4. The cells were washed and resuspended in 500 μl of 1% paraformaldehyde, and cell fluorescence was measured using a Becton Dickinson FACScan. Cell events were acquired using gates set by forward and side scatter plots to determine the proportion of each T cell subset, as well as by a side scatter plot and FL1 (FITC), FL2 (phycoerythrin), or FL3 (antigen presenting cell) to determine the proportion of cytokine-producing T cells for each subset ($CD4^+$ or $CD8^+$). To ensure that only intracellular proteins were quantified, the cells were fixed but not permeabilized, resulting in less than 0.2% fluorescence per subset of T cells ($CD4^+$ or $CD8^+$). The analysis gate was set on $CD4^+$ and $CD8^+$ T cells and invariant quadrant histograms were constructed for IL-4^+/$CD4^+$, IFN-γ^+/$CD4^+$, IL-4^+/$CD8^+$, and IFN-γ^+/$CD8^+$ T cells. Data are expressed as the percentage of positive cells.

Enzyme-linked immunosorbent assay (ELISA)

Three weeks after the final injection of streptococci, the mice were killed under anesthesia with diethyl ether and serum was collected from a retinal vein puncture. A conventional sandwich ELISA method was used to detect serum IgG and IgM antibodies against all of the tested streptococci. Briefly, 96-well microtiter H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated at 4°C with 20 μg of each of the streptococci per well in 50 mM of carbonate buffer (pH 9.6) after sonication. The plates were washed with PBS containing 0.1% (v/v) Tween 20 (PBST) and blocked with 1% (wt/vol) skim milk in PBST at 37°C for 1 h. Excess skim milk in PBST was removed by three washes with PBST, and 100 μl of a 1/200 dilution of the serum sample including serum/0.5% skim milk in PBST was added to the wells and the plates incubated at 37°C for 1 h. The wells were then washed five times with PBST and further incubated at 37°C for 1 h with 100 μl of a 1/1000 dilution of alkaline phosphatase-conjugated goat antihu-IgG, IgM, IgG_1, or IgG_2 antiserum, and mouse antihu-IgG_3 and -IgG_4 monoclonal antibodies. To detect IgG_3 and IgG_4 after washing five times with PBST, 100 μl of a 1/1000 dilution of alkaline phosphatase-conjugated goat antimouse IgG (H +L) antibody was added to the wells and incubated at 37°C for 1 h. After five washes with PBST, bound antibodies were detected by the addition of 100 μl of 3 mg/ml of *p*-nitro-phenyl phosphate hexahydrate (Wako) as a substrate and incubated at 37°C for 1 h. The absorbance at 405 nm (A_{405}) was measured with a Multiskan Bichromatic microplate reader (Labsystems, Helsinki, Finland).

Statistical analysis

Statistical analyses were performed using ANOVA. *P*-values of 0.05 or less were considered to indicate statistical significance.

Results

$CD45^+$, $CD4^+$, and $CD8^+$ cell responses to oral streptococci

Three weeks after the final injections of the oral streptococci, the mice were killed, and the hu-leukocyte contents and subset distributions in suspensions of spleen cells and peritoneal exudate cells were analyzed by flow cytometry. hu-$CD45^+$ cells represented up to 72.6 ± 9.3% (range 19.0 ± 4.3% to 72.6 ± 9.3%) and 75.6 ± 12.3% (range 40.0 ± 5.4% to 75.6 ± 12.3%) of the cells (2.5×10^7) recovered from the spleen and among the peritoneal exudate cells, respectively, in both streptococci-injected and PBS-injected hu-PBMC-NOD/SCID mice (Figs. 1A and 2). The percentages and total numbers of hu-$CD45^+$ cells were significantly increased among the spleen cells following immunization with *S. mutans* (72.6 ± 9.3% and 20.2 ± 4.3 × 10^6,

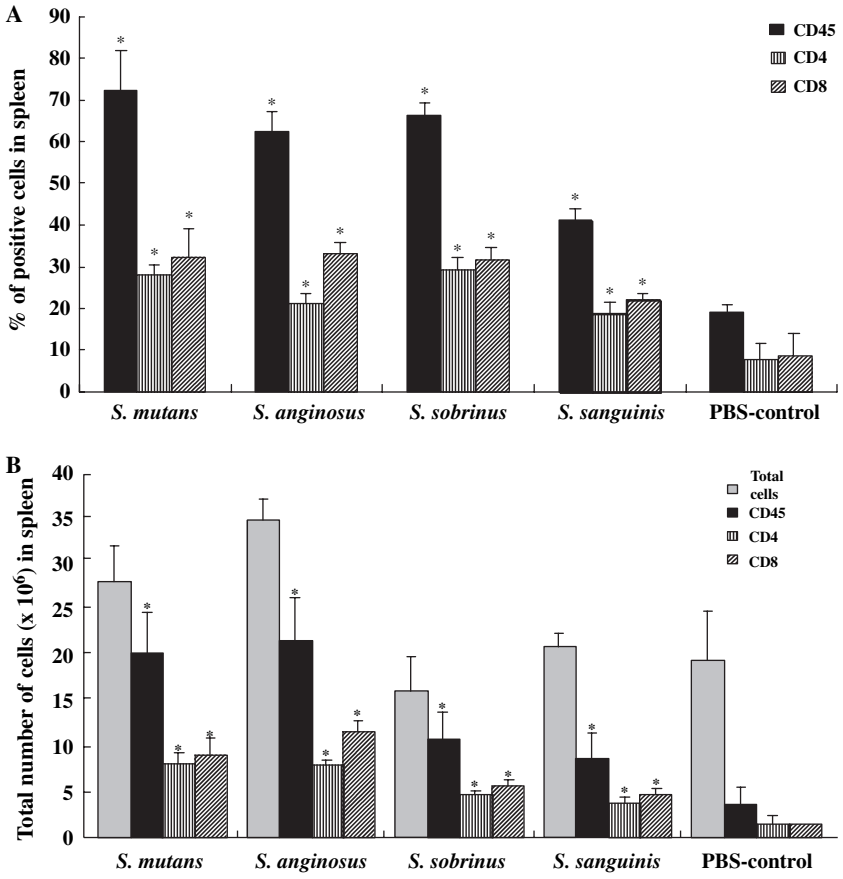

Fig. 1. Flow cytometric analyses of proportions of hu-CD45$^+$, CD4$^+$, and CD8$^+$ cells present in the spleens from grafted NOD/SCID mice injected with *Streptococcus mutans*, *Streptococcus anginosus*, *Streptococcus sobrinus*, *Streptococcus sanguinis,* and phosphate-buffered saline (PBS; control). The analyses were performed on gated lymphocytes with forward light scatter/side light scatter characteristics. Lymphocytes were distinguished in the quadrant analysis. Data are shown as proportions (A) and populations (B) of hu-CD45$^+$, CD4$^+$, and CD8$^+$ cells, and are expressed as the mean ± SD of four independent assays. Asterisks denote significant differences (*$P < 0.05$, vs. PBS).

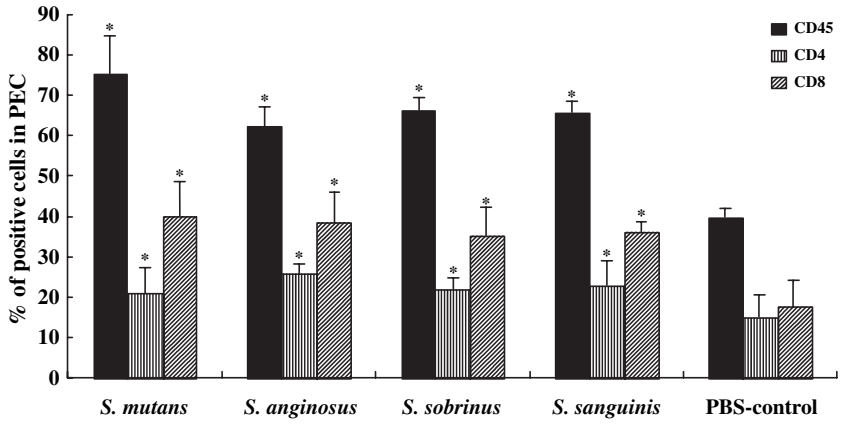

Fig. 2. Flow cytometric analyses of proportions of hu-CD45$^+$, CD4$^+$, and CD8$^+$ cells present in peritoneal exudate cells from grafted NOD/SCID mice injected with *Streptococcus mutans*, *Streptococcus anginosus*, *Streptococcus sobrinus*, *Streptococcus sanguinis,* and phosphate-buffered saline (PBS; control). The analyses were performed on gated lymphocytes with forward light scatter/side light scatter characteristics. Lymphocytes were distinguished in the quadrant analysis. Proportions of hu-CD45$^+$, CD4$^+$, and CD8$^+$ cells are shown as the mean ± SD of four independent assays. Asterisks denote significant differences (*$P < 0.05$, vs. PBS).

respectively), *S. anginosus* (62.5 ± 4.3% and 21.5 ± 4.7 × 10^6, respectively), *S. sobrinus* (66.4 ± 3.3% and 10.7 ± 4.3 × 10^6, respectively), and *S. sanguinis* (41.1 ± 5.8% and 8.6 ± 2.8 × 10^6, respectively) in comparison with the controls, which were injected with PBS (19.0 ± 3.6% and 3.6 ± 1.9 × 10^6, respectively) (Fig. 1A, B). The percentages of hu-CD4$^+$ and CD8$^+$ T cells were also significantly increased among the spleen cells following injection with *S. mutans* (28.1 ± 2.3% and 32.4 ± 6.6%, respectively), *S. anginosus* (21.2 ± 2.3% and 33.2 ± 2.6%, respectively), *S. sobrinus* (29.3 ± 3.3% and 31.7 ± 4.3%, respectively), and *S. sanguinis* (18.6 ± 2.8% and 21.9 ± 1.8%, respectively) in comparison with the control (7.5 ± 3.9% and 8.5 ± 5.4%, respectively) (Fig. 1A). In addition, the total numbers of CD4$^+$ and CD8$^+$ T cell subsets among the spleen cells from hu-PBMC-NOD/SCID mice were significantly increased following immunization, in comparison with the PBS-injected control mice (Fig. 1B). The relative percentages of CD45$^+$, CD4$^+$, and CD8$^+$ cells among peritoneal exudate cells were also significantly increased following immunization with *S. mutans* (75.6 ± 9.3%, 21.0 ± 6.6%, and 40.1 ± 8.6%, respectively), *S. anginosus* (62.5 ± 4.8%, 26.1 ± 2.2%, and 38.8 ± 7.3%, respectively), *S. sobrinus* (66.4 ± 4.3%, 21.9 ± 3.3%, and 35.31 ± 6.9%, respectively), and *S. sanguinis* (65.9 ± 6.8%, 22.9 ± 6.2%, and 36.3 ± 3.2%, respectively) in comparison with the PBS controls (40.0 ± 3.9%, 15.2 ± 5.4%, and 17.9 ± 6.4%, respectively) (Fig. 2). In contrast, the ratios of CD4$^+$/CD8$^+$ T cells from mice infected with *S. mutans*. *S. anginosus*, *S. sobrinus*, and *S. sanguis* were slightly reduced among spleen cells (0.76 ± 0.25%, 0.68 ± 0.12%, 0.66 ± 0.31%, and 0.76 ± 0.25%, respectively), as well as among peritoneal exudate cells (0.59 ± 0.18%, 0.57 ± 0.12%, 0.62 ± 0.25%, and 0.68 ± 0.19%, respectively) in comparison with PBS-injected mice (0.85 ± 0.32% and 0.91 ± 0.32%, respectively). These results demonstrate that the tested oral streptococci influenced human T cells engrafted in NOD/SCID mice to penetrate, activate, and proliferate.

Inductions of IFN-γ and IL-4 in CD4$^+$ and CD8$^+$ T cells by oral streptococcal stimulation

To explore the mechanisms involved with the differences in inflammatory

responses induced by *S. mutans, S. anginosus, S. sobrinus,* and *S. sanguinis* infection, we examined the effects of those bacteria on stimulation of the production of the proinflammatory cytokine IFN-γ and antiinflammatory cytokine IL-4 in hu-PBMC *in vivo*. Three weeks after the final immunization, all streptococci-injected hu-PBMC-NOD/SCID mice showed significantly increased levels of IFN-γ and IL-4 production by both $CD4^+$ and $CD8^+$ splenic and peritoneal cells in comparison with the PBS-injected control (less than 2.5%) (Fig. 3 and 4). hu-PBMC-NOD/SCID mice infected with *S. anginosus* showed significantly increased levels of IFN-γ production by both $CD4^+$ and $CD8^+$ splenic T cells in comparison with mice infected with the other streptococcal organisms (Fig. 3, $P = 0.001$), and *S. mutans, S. sobrinus,* and *S. sanguinis* appeared to have very similar effects, inducing the secretion of IFN-γ by both $CD4^+$ and $CD8^+$ T cells in mouse spleens (Fig. 3). However, there was no significant difference in IL-4 production by $CD4^+$ and $CD8^+$ T cells in the mouse spleens following stimulation between the tested oral streptococci, whereas the levels of IL-4 were lower than those of IFN-γ (Fig. 3).

IFN-γ production by $CD4^+$ and $CD8^+$ T cells among peritoneal exudate cells from hu-PBMC-NOD/SCID mice was significantly higher following injections with *S. anginosus* (26.9 ± 2.3% and 24.9 ± 4.2%, respectively) and *S. mutans* (16.4 ± 1.3% and 14.7 ± 3.5%, respectively), than with *S. sobrinus* (12.0 ± 2.6% and 11.5 ± 1.5%, respectively) and *S. sanguinis* (10.6 ± 3.9% and, 9.6 ± 4.2%) (Fig. 4). There were no significant differences in IL-4 production by $CD4^+$ and $CD8^+$ T cells among the different groups of streptococcal-infected mice (Fig. 4). The proportions of cells stained with mouse IgG conjugated FITC, phycoerythrin, and antigen presenting cell, used as an isotype control, were less than 0.1% in all of the experiments.

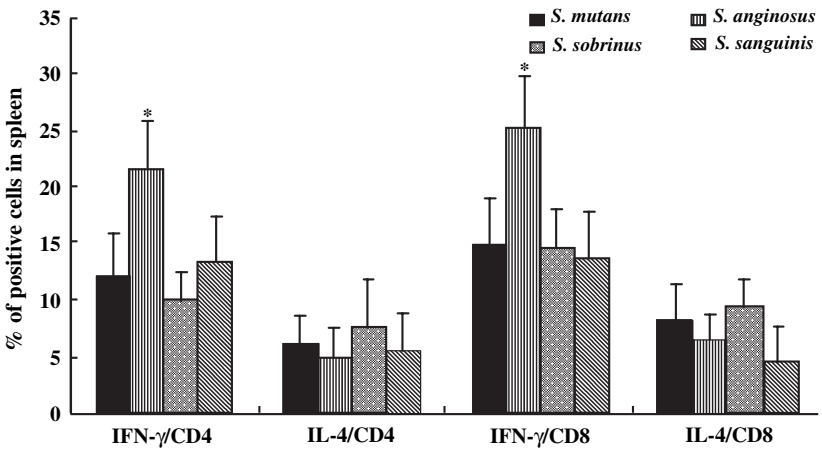

Fig. 3. Flow cytometric analysis of proportions of hu-$CD4^+$ and $CD8^+$ T-cell populations producing IFN-γ and IL-4 in the spleens from grafted NOD/SCID mice injected with *Streptococcus mutans*, *Streptococcus anginosus*, *Streptococcus sobrinus*, *Streptococcus sanguinis,* and phosphate-buffered saline (PBS; control). The analyses were performed on gated lymphocytes with forward light scatter/side light scatter characteristics. Lymphocytes were distinguished in the quadrant analysis. Results for interferon (IFN)-γ^+CD4^+, interleukin (IL)-4^+CD4^+, IFN-γ^+CD8^+, and IL-4^+CD8^+ are shown as the mean ± SD of four independent assays. Asterisks denote significant differences (*$P < 0.05$, vs. other streptococci).

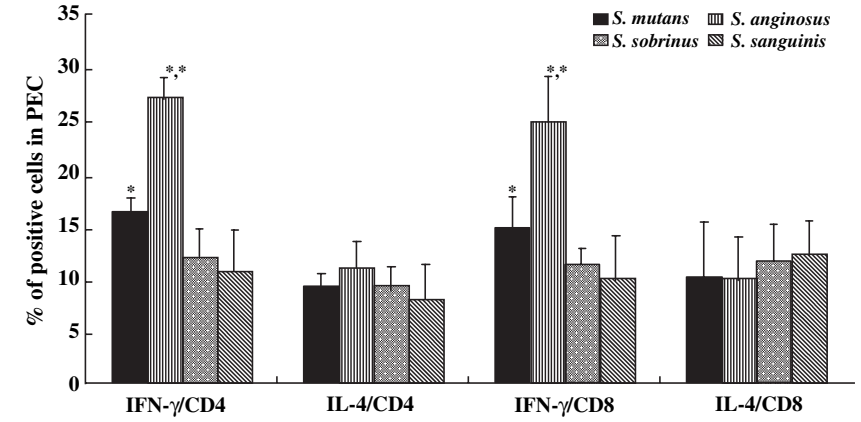

Fig. 4. Flow cytometric analysis of proportions of hu-$CD4^+$ and $CD8^+$ cells producing interferon (IFN)-γ and interleukin (IL)-4 present in peritoneal exudate cells from grafted NOD/SCID mice injected with *Streptococcus mutans*, *Streptococcus anginosus*, *Streptococcus sobrinus*, and *Streptococcus sanguinis.* The analyses were performed on gated lymphocytes with forward light scatter/side light scatter characteristics. Lymphocytes were distinguished in the quadrant analysis. Results for IFN-γ^+CD4^+, IL-4^+CD4^+, IFN-γ^+CD8^+, and IL-4^+CD8^+ are shown as the mean ± SD for four independent assays. Asterisks denote significant differences (*$P < 0.05$, vs. *S. anginosus* and *S. sobrinus*, **$P < 0.05$, vs. *S. mutans*, *S. sobrinus,* and *S. sanguinis*).

Production of antibodies to oral streptococci

Evaluation of serum antibody responses in hu-PBMC-NOD/SCID mice 3 weeks after the final immunization with oral streptococci showed significantly enhanced levels of hu-IgG anti-*S. mutans*, anti-*S. anginosus,* and anti-*S. sanguinis*, as well as hu-IgM anti-*S. mutans*, anti-*S. anginosus*, and anti-*S. sobrinus,* as compared to the control mice injected with PBS ($P = 0.001$) (Fig. 5). Further, serum IgG and IgM anti-*S. mutans* antibody levels were higher in the *S. mutans*-injected mice than the antigen specific serum IgG and IgM levels in mice injected with the other streptococci (Fig. 5, $P = 0.001$). Specific serum IgG and IgM anti-*S. anginosus* antibody levels were also higher in the mice injected with *S. anginosus* than in mice injected with *S. sobrinus* and *S. sanguinis*, and significantly higher than in mice injected with PBS (Fig. 5, $P = 0.001$). In contrast, the mean level of antibody response in the mice injected with *S. anginosus* was lower than that in the group injected with *S. mutans* (Fig. 5).

IgG subclass responses to oral streptococci

To better understand the nature of the immune responses to oral streptococci, serum samples from hu-PBMC-NOD/SCID mice were also assessed for IgG subclass distribution in response to *S. mutans* and *S. anginosus*. Mice injected

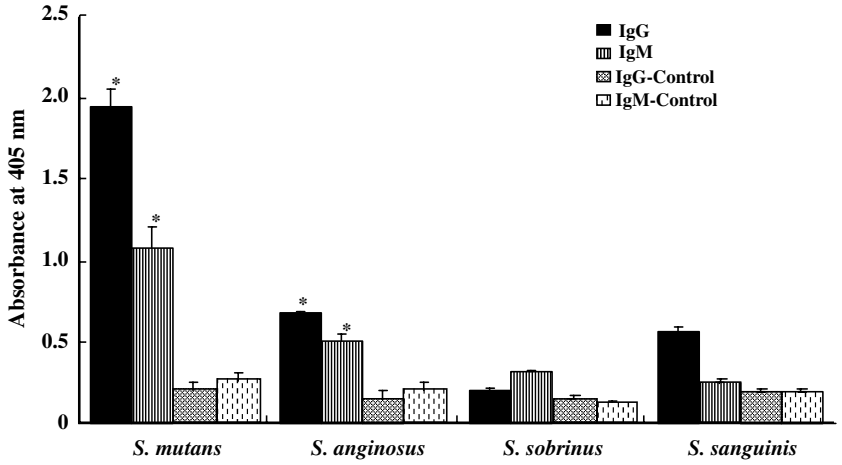

Fig. 5. Production of antihu-IgG and IgM antibodies in serum samples from grafted NOD/SCID mice injected with *Streptococcus mutans*, *Streptococcus anginosus*, *Streptococcus sobrinus*, *Streptococcus sanguinis,* and phosphate-buffered saline (PBS; control). Sera were collected 3 weeks after the last injection of bacteria. Anti-human IgG and IgM antibodies in the samples were analyzed by enzyme-linked immunosorbent assay. Results are expressed as the mean ± SD of absorbance at 405 nm in triplicate assays. The experiments were performed on three lots of serum antibodies from the injected hu-PBMC-NOD/SCID mice, with similar results obtained in each experiment. Asterisks denote significant differences (*$P < 0.01$, other streptococci).

with *S. mutans* showed increases in levels of IgG_1 and IgG_2 anti-*S. mutans* antibody responses following the third immunization, and the specific antibody levels in those mice were significantly different from the levels in the other groups injected with oral streptococci (Fig. 6, $P = 0.001$), as well as the *S. sobrinus* and *S. sanguinis* groups (data not shown). However, significant increases in serum IgG_3 and IgG_4 anti-*S. mutans* antibody levels were not observed in mice injected with *S. mutans* or in the other groups (data not shown).

Discussion

The hu-PBMC-NOD/SCID mouse model has been shown to be useful for *in vivo* studies of hu-immune functions under normal and pathologic conditions (3, 37), including oral streptococci and HIV infections (30, 34, 45). Further, optimization of hu-T cell engrafting in a suitable small animal model like the NOD/SCID mouse is of substantial importance for studies of oral pathogens, as well as AIDS and HIV infection (24, 30, 34), as T cells have been shown to play important roles in both cellular and humoral responses to intra- and extracellular pathogens (27, 35). Grafted hu-T cells are likely to have secondary immune responses to *S. mutans* in the NOD/SCID mouse system, because oral streptococci are common commensal bacteria and initialize lymphocytes at an early age in humans (45). In the present experiments, we found increased levels of engrafted hu-$CD45^+$, $CD4^+$, and $CD8^+$ cells among the spleen cells and peritoneal exudate cells from hu-PBMC-NOD/SCID mice 3 weeks after the final immunization with the tested oral streptococci (Fig. 1 and 2). *S. anginosus* induced higher levels than the other tested oral streptococci of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in the spleens of hu-PBMC-NOD/SCID mice, as well as among the peritoneal exudate cells from those mice. However, there were no significant differences in the numbers of $CD4^+$ and $CD8^+$ T cells that produced IL-4 in the spleens and among peritoneal exudate cells from hu-PBMC-NOD/SCID mice after infection with the tested streptococci, whereas IFN-γ production in mice injected with *S. anginosus* was higher than that of IL-4. These results show that among the oral streptococci tested in the present study, *S. anginosus* induced Th1 and Tc1 cell responses to a greater degree than Th2 and Tc2 cell responses in comparison with the other streptococci. *S. anginosus, Streptococcus constellatus*, and *Streptococcus intermedius* are known for purulent infections that occur following local disruption of the mucosal barrier, such as in cases of ulceration, perforation, inflammation, and surgery (17, 40). Our results indicate that stimulation and recruitment of $CD45^+$, $CD4^+$, and $CD8^+$ lymphocytes, as well as IFN-γ-producing $CD4^+$ and $CD8^+$ T cells, are involved in the pathogenesis of purulent infections caused by *S. anginosus*.

We also found that anti-*S. mutans* hu-IgG and IgM antibody responses in sera from mice immunized with *S. mutans* were significantly higher than those of specific antibodies in mice infected with the other streptococci. We observed higher levels of IgG_1 and IgG_2 antibody responses to the antigens in hu-PBMC-NOD/SCID mice immunized with *S. mutans* as compared to mice immunized with the other streptococci, though the level of IgG_1 was significantly higher than

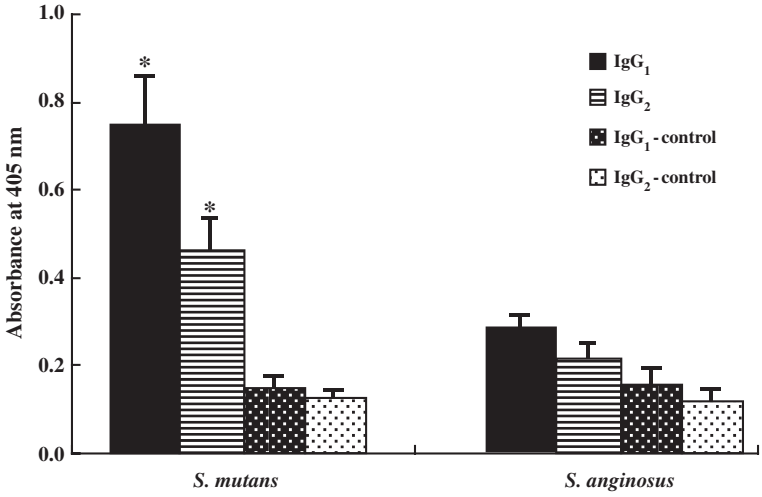

Fig. 6. Production of antihu-IgG_1 and IgG_2 in serum samples from grafted NOD/SCID mice injected with *Streptococcus mutans* and *Streptococcus anginosus*. Sera were collected 3 weeks after the last injection of bacteria. Anti-hu-IgG_1 and anti-IgG_2 antibodies were analyzed by enzyme-linked immunosorbent assay. Results are expressed as the mean ± SD of absorbance at 405 nm in triplicate assays. The experiments were performed on three lots of serum antibodies from the injected hu-PBMC-NOD/SCID mice, with similar results obtained in each experiment. Asterisks denote significant differences vs. *S. anginosus* (*$P < 0.01$, *S. mutans* vs. *S. anginosus*).

that of IgG_2. In mice, Th1 and Th2 cells mediate the production of IgG_{2a} and IgG_1 antibodies, respectively (20, 32, 49). In humans, Th2 cytokine enhances the switching to IgG_2 and IgG_4 in B cells (18). Whole cells of *S. mutans* induced specific IgG_1 and IgG_2, but not IgG_3 or IgG_4, production, as well as an increase in the average levels of Th1 and Tc1 type reactions in comparison with the other streptococci, except for *S. anginosus*. In addition, *S. mutans* did not induce significant IL-4 production in these mice (Fig. 4). It was previously reported that immune responses and susceptibility to IL-4 production are low in the NOD/SCID mouse system, whereas the responses and susceptibility to IFN-γ production are high (45). In addition, the production of CD8 and IFN-γ has been found to be dominant in long-term experiments with those mice (34, 44). Further, immunization with the peptide or protein antigen from *S. mutans* may induce antigen specific hu-antibodies in NOD/SCID mice (45, 53), and the noncorrelation between IL-4 production or stimulation and antibody production may indicate a unique *in vivo* immune response by hu-lymphocytes to *S. mutans* in these mice. The induction of antigen specific hu-IgG and IgM antibodies following immunization with *S. mutans* in the mice in the present study supports the results of various vaccine studies that used *S. mutans* antigens (28, 51).

S. sanguinis did not strongly induce $CD45^+$, $CD4^+$, and $CD8^+$ cells, intracellular cytokine-producing cells or antigen specific antibodies in comparison with the other streptococci tested in the present study. Generally, *S. sanguinis* together with *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus oralis,* and *Streptococcus parasanguis* (21) are prominent components of human oral microbiota and play a significant role as biofilm pioneer colonizers in the development of dental biofilm (8, 39). *S. sanguinis* is frequently found in the oral cavities of young children and is known to induce microbial oral flora. Therefore, it is possible that an immunologic tolerance to *S. sanguinis* may develop in human immune systems. Humoral antibody responses to *S. sanguinis*, which is consistently found in the subgingival plaque of periodontal patients, were reported to be depressed in periodontitis patients compared with plaque of healthy controls (7). In contrast, serum samples from patients with Behcet's disease, a chronic multisystemic inflammatory disorder, showed significantly higher antibody titers to *S. sanguinis* clinical strains than samples from healthy controls (58). It has been hypothesized that *S. sanguinis* is harmonized as a nonstimulator in normal immune systems, but not with lymphocytes in irregular immune systems. Further, the saliva IgA antibody to the surface protein antigen from *S. mutans* has been speculated to be a negative contributor as compared to the number of *S. mutans* organisms in the oral cavity (26), as the saliva antibody may regulate the attachment, colonization, and biofilm formation of *S. mutans* on the tooth surface. *S. sobrinus* produces insoluble and soluble glucans, and an antigenic surface protein, which is similar to *S. mutans*. However, neither *S. sobrinus* nor *S. sanguinis* induced significant stimulation of Th and Tc cells, or hu-IgG and IgM production in the present study. Taken together, *S. mutans* may act as a stimulator of specific antibody production in humans, which leads to immune reactivity that is different from other streptococci.

In conclusion, we found that *S. anginosus* infection induced significant levels of the proinflammatory cytokine IFN-γ in $CD4^+$ and $CD8^+$ cells, as well as T cells in hu-PBMC-NOD/SCID mice in comparison with other streptococci, and infection with *S. mutans* caused increased levels of hu-IgG, IgG_1, IgG_2, and IgM antibodies in serum samples. In contrast, *S. sanguinis* and *S. sobrinus* showed poor responses to stimulation by hu-PBMC. Our results suggest that biofilm bacteria have various properties and roles in the human immune system, such as the stimulation of Th1 and Tc1 by *S. anginosus* and antibody production by *S. mutans*. These findings may contribute to a better understanding of lymphocyte interactions among cytokines and immunoglobulins to biofilm bacteria, the control of oral flora by immune functions, and the impact on dental caries accompanied by pulpal infections, as well as inflammatory diseases in mucosal tissues and endocarditis.

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