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Oligoclonal CD4$^+$ T Cells Promote Host Memory Immune Responses to Zwitterionic Polysaccharide of *Streptococcus pneumoniae*$^\nabla$

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Zwitterionic polysaccharides of the normal flora bacteria represent a novel class of antigens in that they correct systemic CD4$^+$ T-cell deficiencies and direct lymphoid organogenesis during colonization of the host. Presentation of these polysaccharides to CD4$^+$ T cells depends on major histocompatibility complex class II- and DM-dependent retrograde transport from lysosomes to the cell surface. Yet the phenotype and clonality of the immune response to the polysaccharide in the mature host immune system have not been studied. Using the zwitterionic capsular polysaccharide Sp1 of *Streptococcus pneumoniae*, a transient member of the bacterial flora, in an experimental mouse model of cellular immunity, we demonstrated the accumulation of TH1- and TH17-polarized CD4$^+$ CD44^{high} CD62^{low} CD25$^-$ memory T cells. Subcutaneous immunization with Sp1 resulted in an increase of serum immunoglobulin G (IgG), predominantly of the IgG1 subclass, and suggested the presence of a humoral memory response to the polysaccharide. CD4$^+$ T cells stimulated with polysaccharide in vitro and in vivo showed a nonrestricted pattern for the T-cell receptor (TCR) β-chain variable region, as demonstrated by semiquantitative reverse transcription-PCR and flow cytometry. Clonotype mapping of in vivo and in vitro polysaccharide-activated CD4$^+$ T cells revealed clonotypic TCR transcripts. Taken together, the data show the induction of clonal expansion of CD4$^+$ T cells by polysaccharides of commensal bacteria. Cellular and humoral memory host responses imply the ability of these polysaccharides to mediate the expansion of T cells via recognition within the CDR3 region of the TCR.

Capsular polysaccharides of the human physiologic bacterial flora are immunogenic components that first encounter the human immune system during initial colonization and at the time that the immune system is developing and maturing. As opposed to common negatively charged polysaccharides, the biologic activities of certain commensal bacterial polysaccharides are unique in their ability to stimulate CD4$^+$ T cells in vivo and in vitro. They direct the development of the systemic cellular immune response by correcting CD4$^+$ T-cell deficiencies and TH1/TH2 imbalances toward a TH1 immune response. Responses to the polysaccharides are conferred by CD4$^+$ T cells, not B cells or other T cells (14, 18, 28, 31–33). Examples of such bacteria are the ubiquitous anaerobic member of the gut flora *Bacteroides fragilis*, *Staphylococcus aureus* as a temporary member of the skin and mucosal flora, and *Streptococcus pneumoniae* of the upper respiratory tract flora. CD4$^+$ T-cell activation induced by these polysaccharides depends on their unique electrical charge: each repeating unit has a minimum of one positive and one negative charge, leading to their common three-dimensional configuration characterized by a right-handed helix with repeating negatively charged grooves, with the positive charges being on the outer surface of the lateral boundaries (5, 14, 31, 36). Presentation of the so-called zwitterionic polysaccharide (ZPS) from *S. pneumoniae* serotype 1 (Sp1) by major histocompatibility complex (MHC) class II molecules requires its retrograde transport from lysosomes to the cell surface within tubules as a ZPS-MHC class II complex and also requires the DM molecule (12, 22). DM is known to catalyze and edit the exchange of the self-peptide CLIP with processed antigen in MHC class II compartments. The requirement of DM for Sp1 presentation via MHC class II suggests presentation within the antigen binding groove, which is supported by recent studies demonstrating that binding of the ZPS PS A1 from *B. fragilis* to MHC class II molecules can be competed by peptides known to be presented in the antigen binding cleft (7).

For protein-derived T-cell antigens, it is well established that their presence in the MHC class II binding groove leads to the recognition of their antigenic epitopes by the T-cell receptor (TCR), within the CDR3 antigen binding domain of the β-chain variable (BV) region, and to subsequent T-cell activation and oligoclonal T-cell proliferation. However, for ZPS, besides the requirement of engaging the MHC class II molecule with the αβ TCR for ZPS-induced CD4$^+$ T-cell activation (6, 29), little is known about the clonality of the T-cell response. So far, attempts to generate ZPS-specific T-cell clones have been unsuccessful. In rats, only two ZPS-cross-reactive T-cell hybridomas have been established (23). The ZPS-specific hybridomas responded to a variety of other ZPS but not to nonzwitterionic polysaccharides, indicating cross-reactivity between different ZPS.

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In the present study, we tested the hypothesis that ZPS induce host memory responses in the mature host immune system. We further studied the clonality of the immune response. Using the ZPS Sp1 in an experimental mouse model of cellular immunity, besides TH1-polarized cells, we found predominantly $CD4^+$ T cells of a TH17-polarized phenotype. They were characterized by their $CD44^{high}$ $CD62L^{low}$ $CD25^-$ phenotype as memory $CD4^+$ T cells. In an experimental mouse model of humoral immunity, subcutaneous immunization with Sp1 resulted in significant Sp1-specific immunoglobulin G (IgG) titers, predominantly of the IgG1 subclass. In vivo and in vitro stimulation of murine $CD4^+$ T cells with Sp1 resulted in oligoclonal T-cell expansion within numerous TCR BV families.

MATERIALS AND METHODS

Antigens. *Streptococcus pneumoniae* type 1 capsular polysaccharide complex was obtained from the American Type Culture Collection and further purified to obtain Sp1 as previously described (22). The average molecular size of Sp1 was 70 kDa. Sp1 was subjected to high-resolution (500 MHz) proton nuclear magnetic resonance spectroscopy (5). Sp1 was found to contain no detectable protein or nucleic acid by the bicinchoninic acid method (Pierce) and by UV absorbance at 280 and 260 nm. Endotoxin was not detectable by the *Limulus* test, with a sensitivity of <8 pg lipopolysaccharide/mg Sp1, which corresponds to <0.0028 endotoxin units/100 μg Sp1/ml cell culture medium. Chemical modification of Sp1 by neutralization of the free amino group on the 2-acetamido-4-amino-2,4,6-trideoxygalactose by N-acetylation, which creates a polysaccharide with a net negative charge, was performed as previously described (31). We used tetanus toxoid (TT) (a generous gift of Chiron Behring) as a proteinaceous antigen control, and as a superantigen control, we used *Staphylococcus aureus* enterotoxin A (SEA) (Sigma-Aldrich).

Antibodies. Purified fluorescein isothiocyanate-, phycoerythrin-, or allophycocyanin-labeled monoclonal antibodies specific to murine CD3 (clone 145-2C11), CD4 (clone RM4-5), CD25 (7D4), CD28 (37.51), CD44 (IM7), CD62L (clone MEL-14), CD69 (clone H1.2F3), interleukin-2 (IL-2) (JE S6-5H4), IL-4 (11B11), IL-10 (JE55-I6E3), IL-17 (TC11-18H10, BD), gamma interferon (IFN-γ) (MG1.2), and a panel of TCR BV regions and their respective isotype controls were obtained from BD Pharmingen.

Experimental mouse model of cellular response to Sp1. All animal experiments were performed in accordance with the guidelines of German animal protection legislation (license number 50.203.2-K 16, 3/02). Conventional 8-week old C57BL/6 specific-pathogen-free mice (Charles River Laboratories) were challenged by intraperitoneal injection with Sp1 (100 μg) mixed with sterile cecal content (SCC) adjuvant (0.68 μl/g of body weight; 200-μl total volume) (Sp1 challenge). Control groups were injected with chemically modified Sp1 mixed with SCC adjuvant and with SCC adjuvant or phosphate-buffered saline (PBS) alone. For $CD4^+$ T-cell depletion, C57BL/6 mice were treated intravenously with 500 μg of CD4-specific antibody (clone YTS 191.1.2) or PBS 24 h before intraperitoneal Sp1 challenge. Depletion of $CD4^+$ T cells was confirmed by flow cytometry analysis of whole blood and spleen cells 24 h and 48 h after depletion. The analysis showed depletion of >95% of the $CD4^+$ T-cell population (data not shown). At different time points following Sp1 challenge, mice underwent peritoneal lavage with 4 ml of ice-cold saline to assess cellular influx into the peritoneal cavity. A total cell count was performed by trypan blue staining using a hemocytometer, and cells were stained for flow cytometry. Six days after challenge, mice were examined at autopsy for the presence of abscesses within the peritoneal cavity by two blinded examiners. Abscesses were isolated, and their diameters were measured. For each experiment, four to six mice per group were tested, and the experiments were performed three times independently.

Analysis of humoral immune responses. For antibody studies, BALB/c mice received Sp1 (5 μg) emulsified in Quil A (12.5 μg) in a total volume of 100 μl in PBS subcutaneously on days 0 and 21. As negative controls, mice received dextran (5 μg) emulsified in Quil A (12.5 μg) or 12.5 μg Quil A alone in 100 μl saline subcutaneously at the same time points. Serum was collected by bleeding from the tail vein on days 9, 28, and 49 and was analyzed for Sp1-specific antibodies by enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with Sp1 (10 μg/ml; 100 μl/well) in 0.1 M sodium carbonate buffer with 0.02% sodium azide (pH 9.6) for 6 h at 30°C, followed by five washes with 10 mM Tris buffer with 154 mM NaCl and 0.1% Brij (Sigma). Mouse serum was added at 1:2 stepwise dilutions, with a starting dilution of 1:100, and incubated overnight at 4°C. Plates were washed five times. Alkaline phosphatase (AP)-conjugated anti-mouse IgG (Sigma) was added as a secondary antibody at a concentration of 1.8 μg/ml for 2 h at 30°C, followed by five washings. AP solution (Sigma) was then added (100 μl/well). For IgG subtyping, biotin-conjugated anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, or the respective isotype controls were added at 0.05 μg/ml (all from Southern Biotech) and incubated for 2 h at 30°C, and AP-conjugated streptavidin (Pierce) was added for 2 h at 30°C, followed by three washings. Color was read at 405 nm after 60 min, using an ELISA reader (Dynex).

Immunohistochemistry. Frozen sections of abscesses were fixed in cold acetone for 10 min, followed by blocking of endogenous peroxidase with peroxidase blocking solution (Dako, Hamburg, Germany) for 10 min at room temperature. Anti-CD4 antibody was then used as an overlay at predetermined dilutions, and the slides were incubated in a humid chamber for 45 min. With Tris washes between every step, a biotinylated link antibody (BD Pharmingen) was applied for 45 min, followed by streptavidin-AP (Dako) for 10 min. After another wash, the substrate (Vector NovaRed; Vector Labs, Burlingame, CA) was added, and the slides were incubated in the dark for 20 min. After a Tris wash, the slides were counterstained, mounted, and viewed using a Zeiss Axiophot microscope with photographic capabilities.

Flow cytometry. For intracellular cytokine staining, cells were stimulated with anti-CD3 (5 ng/ml) and anti-CD28 (500 ng/ml) for 6 h at 37°C and 5% CO_2. After the first hour of incubation, Golgi Stopp (BD Pharmingen) was added. Cells were stained for surface markers for 30 min on ice with specific antibodies and then washed. Cells were fixed with Cytofix/Cytoperm (BD Pharmingen) for 20 min on ice and then permeabilized with Perm/Wash solution (BD Pharmingen). For interleukin staining, cells were treated with antibodies for 30 min at 4°C. For surface marker staining without intracellular cytokine staining, cells were stained with specific antibodies for 30 min on ice and then washed. Cells prepared for flow cytometry were analyzed—after gating for viable cells by forward and side scatter—by a FACScan flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson). The results were expressed as percentages of fluorescently labeled cells in a population. Experiments were performed a minimum of three times in an independent manner.

T-cell proliferation assay. $CD4^+$ T cells were isolated by centrifugation in Ficoll-Hypaque gradients (density, 1,088 g/ml) and purified with immunomagnetic beads. The purity of the $CD4^+$ cell populations was confirmed by flow cytometry (≥95%). T cells (5×10^4 per well) were incubated in triplicate with mitomycin C-treated spleen cells as antigen-presenting cells (APCs) (1×10^5 per well), with antigens, or with medium alone (37°C, 5% CO_2) in a 96-well plate in RPMI 1640 supplemented with 1% L-glutamine, sodium pyruvate, penicillin-streptomycin, nonessential amino acids, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum (Life Technologies, Gaithersburg, MD). $CD4^+$ T-cell proliferation was quantified by [^{3}H]thymidine incorporation (1 μCi/well) for 6 h. Assays were performed at least three times at independent time points. The results were expressed in counts per minute or as stimulation indexes.

Semiquantitative RT-PCR studies and clonotype mapping. For semiquantitative reverse transcription-PCR (RT-PCR) and clonotype mapping, $CD4^+$ T cells were enriched from spleen and lymph nodes of C57BL/6 mice by immunomagnetic bead negative selection according to the manufacturer's instructions (Miltenyi Biotec). $CD4^+$ T cells (5×10^6/ml) and mitomycin C-treated APCs (5×10^6/ml) from naïve C57BL/6 mice were cultured with Sp1 (50 μg/ml) or SEA (20 ng/ml) in RPMI 1640 supplemented with 1% L-glutamine, sodium pyruvate, penicillin-streptomycin, nonessential amino acids, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum (Life Technologies, Gaithersburg, MD). Cells were cultured for 1 week. Fresh medium and recombinant murine IL-2 (500 U/ml; Sigma) were added every third day. For the analysis of abscesses, cells were isolated and emulsified before RNA extraction. Total RNA was extracted using an RNeasy plant mini kit (Qiagen). Synthesis of cDNA was carried out using 1 to 3 μg of total RNA with oligo(dT) and Moloney murine leukemia virus SuperScript II reverse transcriptase (Invitrogen) in a total volume of 50 μl of 1× Tris-acetate-EDTA buffer containing 10 mM dithiothreitol. Incubations were performed at 42°C for 50 min and at 72°C for 5 min. Semiquantitative PCR was performed with 18 primers specific for TCR BV families 1 to 18 as forward primers (24, 26). A reverse primer specific for the β-chain constant region (GAT GGC TCA AAC AAG GAG ACC TT) and a minor-groove-binding TaqMan probe (6-carboxyfluorescein-AGT CAC ATT TCT CAG ATC C-6-carboxytetramethylrhodamine) were used. The percentage of each family was calculated using a standard curve for the β-chain constant region, with the forward primer CCC AAA CCT GTC ACA CAG AAC AT, the reverse primer TCA TAG AGG ATG GTG GCA GAC AA, and the minor-groove-binding TaqMan probe 6-carboxyfluorescein-TTA CCT CAG CAT CCT ATC AA-6-carboxytetramethylrhodamine. Analysis was performed on a 5700 SDS system (Applied Biosystems).

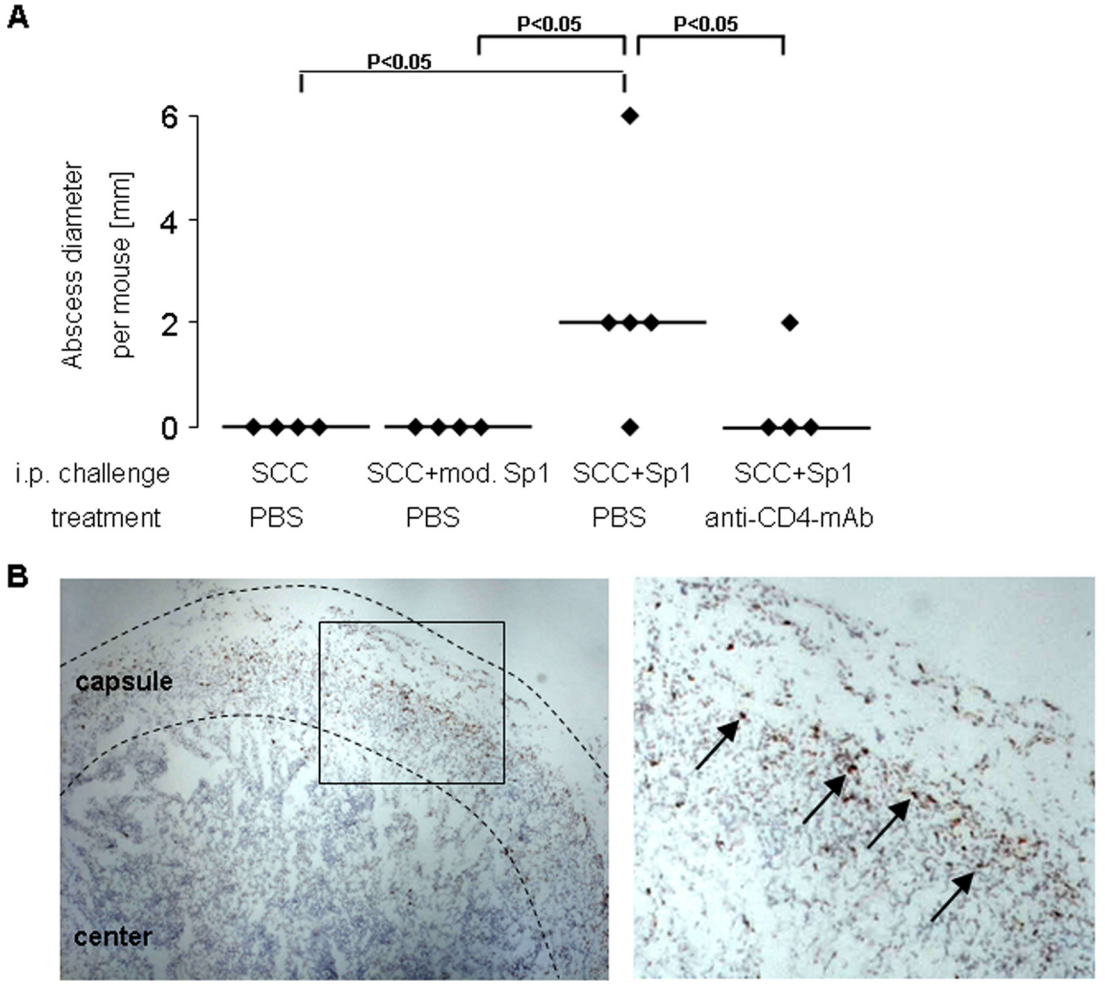

FIG. 1. $CD4^+$ T-cell-dependent immune response to intraperitoneal Sp1 application in mice. (A) C57BL/6 mice received either anti-CD4 monoclonal antibody or saline intravenously 24 h prior to intraperitoneal (i.p.) Sp1 challenge or challenge with chemically modified Sp1 (mod. Sp1) and SCC adjuvant alone as controls. Intraperitoneal abscess formation was examined at autopsy after 6 days. One dot represents the total abscess diameter per mouse, and each bar indicates the median abscess size per group. (B) Immunohistochemistry of Sp1-induced abscesses was performed with an anti-CD4 monoclonal antibody. The left panel shows an overview of an abscess. The right panel shows a magnified detail of the abscess wall within the rectangular box in the left panel. Some $CD4^+$ T cells are labeled with black arrows. The figure shows one representative result for three experiments.

Amplifications were conducted in a total volume of 20 μl containing 5 pmol of each BV primer and TaqMan master mix (Eurogentec). The parameters used for amplification were 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s for 40 cycles. PCR for clonotype mapping was performed as described before (26).

For denaturing gradient gel electrophoresis (DGGE) analysis, 10-μl aliquots were loaded into a denaturing gradient gel containing 6% polyacrylamide and a gradient of urea and formamide from 20% to 80%. Gels were run at 160 V for 4.5 h in 1× Tris-acetate-EDTA buffer kept at a constant temperature of 58°C. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV transillumination.

Statistical analysis. Comparisons of groups regarding abscess formation were made by the chi-square test. Results for the various groups for peritoneal cellular influx and memory $CD4^+$ T-cell phenotyping were compared by Student's *t* test.

RESULTS

Experimental mouse model of Sp1-induced $CD4^+$ T-cell-dependent peritoneal abscess formation. To investigate the phenotype and clonality of the immune response to Sp1 in the mature host immune system, we applied a modification of an experimental model for abscess formation which had been performed with rats, using commensal bacteria, PS A1, and Sp1, to 8-week-old C57BL/6 mice held under specific-pathogen-free conditions (29, 30). The model reflects reexposure to ZPS after maturation of the host immune system by a complex microbial flora. Challenging these mice with intraperitoneal Sp1 resulted in the formation of sterile abscesses 6 days later. Chemically modified Sp1, saline (not shown), and SCC adjuvant alone (controls) did not induce abscess formation. The $CD4^+$ T-cell dependency of this immune response was evident, as $CD4^+$ T-cell depletion led to a significant inhibition of abscess formation (Fig. 1A). $CD4^+$ T cells participate in forming the organized wall of abscesses, as shown by immunohistochemistry (Fig. 1B). These data indicate that in mice, Sp1 leads to $CD4^+$ T-cell-dependent abscess formation, establishing a valuable model for further investigation of the cellular immune response to Sp1.

Intraperitoneal Sp1 application induces a TH1 and a TH17 memory response. We performed a phenotype characteriza-

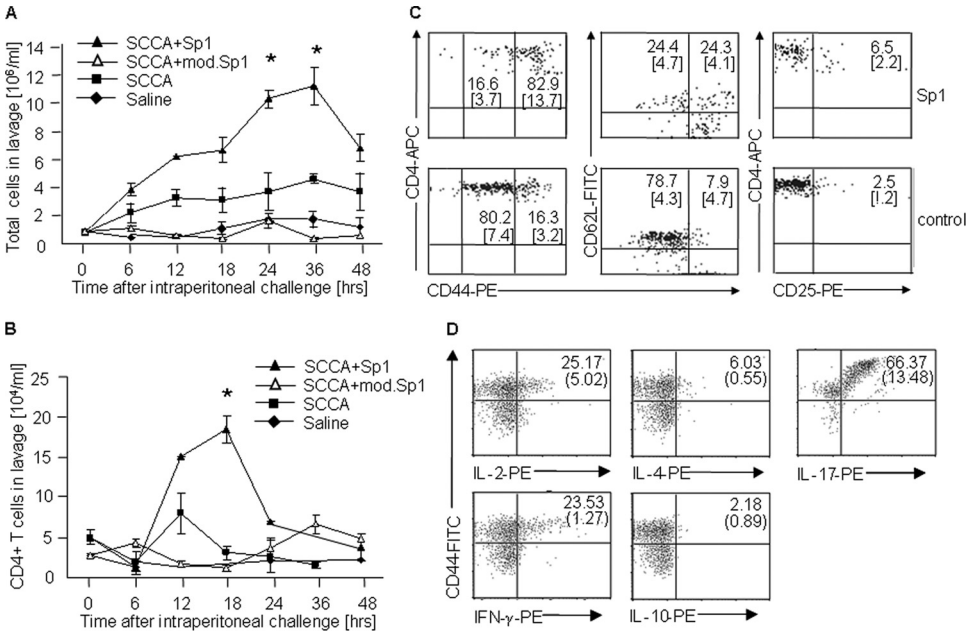

FIG. 2. Influx of TH1 and TH17 memory $CD4^+$ T cells into the peritoneal cavity upon Sp1 challenge. The influx of total cells (A) and $CD4^+$ T cells (B) into the peritoneum was measured in C57BL/6 mice (six per group) challenged with Sp1 plus SCC adjuvant or with chemically modified Sp1 plus SCC (mod. Sp1), SCC adjuvant alone, or saline as a control. Peritoneal lavage was performed at different time intervals (*x* axis) after challenge. Cell numbers were determined by cell counting and flow cytometry. Bars indicate standard deviations within a group tested. *, $P < 0.05$. (C) Intraperitoneal Sp1-induced $CD4^+$ T cells have a $CD44^{high}$ $CD62L^{low}$ $CD25^-$ memory cell phenotype. Peritoneal or spleen (control) cells were obtained 18 h after intraperitoneal Sp1 or PBS challenge, respectively, stained with surface markers, and analyzed by flow cytometry. The numbers in the dot plots represent percentages of $CD4^+$ T cells positive for both markers. The figure shows one representative result for 10 experiments performed independently. Standard deviations are given in brackets. (D) Intraperitoneal $CD4^+$ $CD44^{high}$ T cells produce TH1 and TH17 cytokines. Peritoneal cells from mice challenged with Sp1 and spleen cells from PBS-challenged animals, as controls, were restimulated with anti-CD3 and anti-CD28 for 6 h and subjected to intracellular cytokine staining. In the right upper quadrants of the dot plots, percentages of cytokine-producing $CD4^+$ $CD44^{high}$ T cells in Sp1-challenged mice are given, with values for control mice in parentheses. The figure shows one representative result for at least three experiments performed independently.

tion of $CD4^+$ T cells migrating into the peritoneum after Sp1 application. Intraperitoneal Sp1 challenge led to an increase of the total cell count in the peritoneal cavity. Cell influx reached its maximum level 36 h following polysaccharide administration and differed significantly from that after application of chemically modified Sp1, SCC adjuvant, or PBS ($P < 0.05$) (Fig. 2A). The infiltrate consisted mainly of polymorphonuclear cells, which migrated with rapid kinetics into the peritoneum and peaked 24 h and 36 h following Sp1 challenge (data not shown). The influx of $CD4^+$ T cells in Sp1-challenged mice was significantly higher than that in mice 18 h after challenge with chemically modified Sp1, SCC adjuvant, or PBS ($P < 0.05$) (Fig. 2B). Phenotyping of peritoneal $CD4^+$ T cells was performed 18 h after Sp1 challenge. In contrast to spleen $CD4^+$ T cells in PBS-challenged mice (control), 82.9% of the peritoneal $CD4^+$ T cells expressed CD44 with a high fluorescence intensity, about 48.7% of the $CD4^+$ T cells were positive for CD62L, and 2.5% were positive for CD25 (Fig. 2C). This result characterizes the $CD4^+$ T cells as $CD44^{high}$ $CD62L^{low}$ $CD25^-$ and classifies them as memory $CD4^+$ T cells (10, 17). In contrast, the few $CD4^+$ T-cells attracted by the application of modified Sp1 were $CD44^{high}$ (31.5%), $CD62^{low}$ (10.6%), and $CD25^+$ (5.0%) (not shown). Evaluation of the functional polarization of the $CD4^+$ T cells was performed by intracellular cytokine staining of prototypical TH1, TH2, TH17, and T_{reg} cytokines. In contrast to spleen $CD4^+$ T cells of PBS-challenged mice, in peritoneal $CD4^+$ T cells of Sp1-challenged mice the overexpression of IFN-γ (23.5%), IL-2 (25.2%), and IL-17 (66.37%) was observed. Individual T cells showed exclusive production of either IL-17 or IFN-γ (not shown). Cytoplasmic expression of IL-4 and IL-10 was not detected (Fig. 2D). Together, our data demonstrate the recruitment of TH1- as well as TH17-polarized memory $CD4^+$ T cells.

IgG humoral immune response to Sp1. Protein antigens activate T-cell-dependent cellular immune responses and consequently enable the humoral branch of the immune system to generate antibodies of IgG subclasses as a result of T-cell-mediated isotype switching. Here we showed that Sp1 also induces T-cell-dependent immune responses. Therefore, we tested whether Sp1 induces IgG antibodies. Subcutaneous application of Sp1 (5 μg) plus the adjuvant Quil A (12.5 μl) on day 0 and a second immunization with Sp1 (5 μg) plus Quil A (12.5 μl) on day 21 were administered. Serum was obtained on days 9, 28, and 49, and Sp1-specific IgG titers were measured by ELISA. A 24- to 48-fold titer increase of the IgG antibody response was observed on day 49 (Fig. 3A). The application of dextran with the Quil A adjuvant and the adjuvant alone did not induce an Sp1-specific antibody response. In contrast to the case for mice immunized with dextran or the adjuvant alone, IgG subclass analysis of sera from Sp1-immunized mice revealed significant inductions of Sp1-specific IgG1 and IgG3 (Fig. 3B). The relative titers of subclasses showed an 18.2-fold

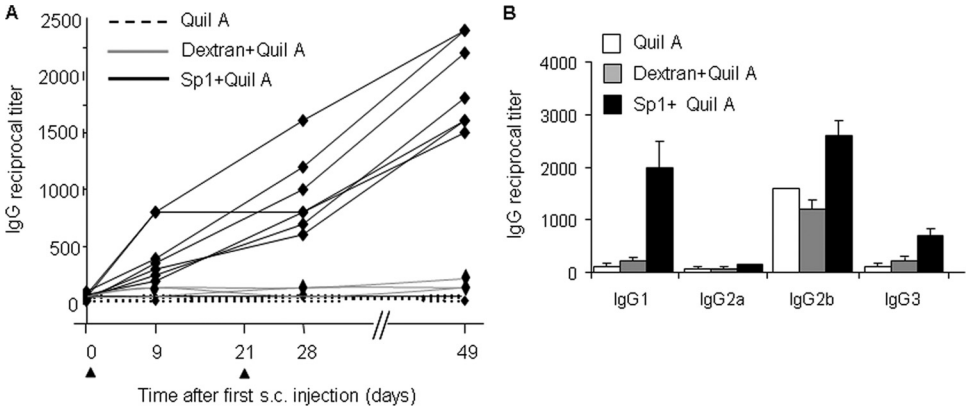

FIG. 3. Sp1-induced IgG antibody response. (A) BALB/c mice ($n = 7$ per group) were injected subcutaneously (s.c.) with Sp1 (5 μg) and Quil A adjuvant (12.5 μg) (black lines), with dextran (5 μg) and Quil A adjuvant (12.5 μg) (gray lines), or with Quil A (12.5 μg) alone (dotted lines) on day 0 and day 21 (triangles below the *x* axis). Serum was obtained on days 9, 28, and 49, and Sp1-specific reciprocal IgG titers (*y* axis) were determined by ELISA (*x* axis). (B) For IgG isotype analysis, serum was obtained 49 days after subcutaneous immunizations on days 0 and 21, and Sp1-specific titers of IgG1, IgG2a, IgG2b, and IgG3 were determined by ELISA.

increase for IgG1, a 6.4-fold increase for IgG3, a 2-fold increase for IgG2a, and a 1.6-fold increase for IgG2b. Our observation of an IgG humoral response to Sp1 suggests $CD4^+$ T-cell-dependent isotype switching.

Sp1-meditated $CD4^+$ T-cell proliferation with a nonrestricted BV repertoire in vivo and in vitro. According to the immunology memory paradigm, responses to processed proteins are based on rapid clonal expansion of antigen-experienced T cells that are not restricted to specific BV families of the TCR. In contrast, superantigens induce a polyclonal response within a restricted number of specific TCR BV families. To evaluate the effect of Sp1-induced $CD4^+$ T-cell activation on the repertoire of TCR BV families in vitro, we first tested $CD4^+$ T cells for proliferation in the presence of Sp1 and APCs. As further controls, we used medium alone, dextran, TT as a processed proteinaceous antigen control, and SEA as a superantigen control in the presence or absence of APCs and $CD4^+$ T cells. Sp1-induced T-cell proliferation was maximal on days 4 and 5 of culture, with an average 3.5-fold increase (Fig. 4A). $CD4^+$ T-cell proliferation was dependent on the presence of APCs. Semiquantitative RT-PCR using specific primers for BV families of the TCR was performed with mRNAs derived from $CD4^+$ T cells that were stimulated for 1 week with Sp1, SEA, or medium alone in the presence of APCs and IL-2. The Sp1-stimulated $CD4^+$ T cells were found to express a nonskewed TCR BV repertoire (Fig. 4B). In contrast, stimulation with SEA resulted in selective expansion and overexpression of $CD4^+$ T cells expressing TCR BV3 and BV11. To assess the prevalence of TCR BV families within the $CD4^+$ T-cell population that responded to Sp1 in vivo, we performed fluorescence-activated cell sorter analysis of the Sp1-induced $CD4^+$ T-cell influx and $CD4^+$ T cells obtained from abscess walls. Neither $CD4^+$ T cells migrating into the peritoneal cavity upon Sp1 application (Fig. 4C) nor $CD4^+$ T cells localizing in the abscess capsule (not shown) expressed a nonskewed TCR BV repertoire. Thus, Sp1 proliferation in vivo and in vitro is not restricted to $CD4^+$ T cells with a specific BV region.

Oligoclonal $CD4^+$ T-cell expansion in vivo and in vitro in response to Sp1. The relative shift within the BV repertoire supports the hypothesis of Sp1-induced oligoclonal $CD4^+$ T-cell activation. Since clonotypic TCR transcripts may be present in nonoverexpressed as well as overexpressed BV families, as described for proteinaceous antigens (21, 27), we tested for clonal transcripts within the TCR BV families by clonotype mapping of in vivo and in vitro Sp1-stimulated $CD4^+$ T cells. Clonotype mapping that combines RT-PCR for amplifying the respective TCR BV families with DGGE allows nucleotide sequence-specific discrimination (24, 26). Clonotypic transcripts are detected as specific bands, whereas polyclonal expansion products settle diffusely in the gel.

After in vitro stimulation of $CD4^+$ T cells with Sp1, SEA, or medium alone in the presence of APCs and IL-2 for 1 week, DGGE analysis of the respective TCR BV families revealed several distinct bands in the gel (Fig. 5A, left panel). In contrast to polysaccharide-stimulated T cells, $CD4^+$ T cells cultured in medium without antigen exhibited significantly fewer clonotypic bands (Fig. 5A, center panel). In contrast, clonotype mapping of $CD4^+$ T cells stimulated with the superantigen SEA showed a smear for TCR BV3 and BV11 as a result of the BV-specific polyclonal expansion (Fig. 5A, right panel). The experiment was performed three separate times with pooled $CD4^+$ T cells. However, a common clonal band for a specific TCR BV region was not detected. Therefore, sequencing of a specific CDR3 transcript was not performed. To assess the prevalence of clonotypic $CD4^+$ T-cell transcripts in the organized wall of intraperitoneal abscesses, we performed clonotype mapping of the $CD4^+$ T cells retrieved from Sp1-induced abscesses. Within the abscess capsule, few clonotypic bands were detected by DGGE (Fig. 5B). A common band was not observed in six abscesses investigated by clonotype mapping. In summary, we provide evidence for oligoclonal expansion of Sp1-activated $CD4^+$ T cells, without restriction to certain TCR BV families, both in vivo and in vitro.

DISCUSSION

In this study, we sought to answer the question of whether ZPS application to immune-mature hosts results in memory

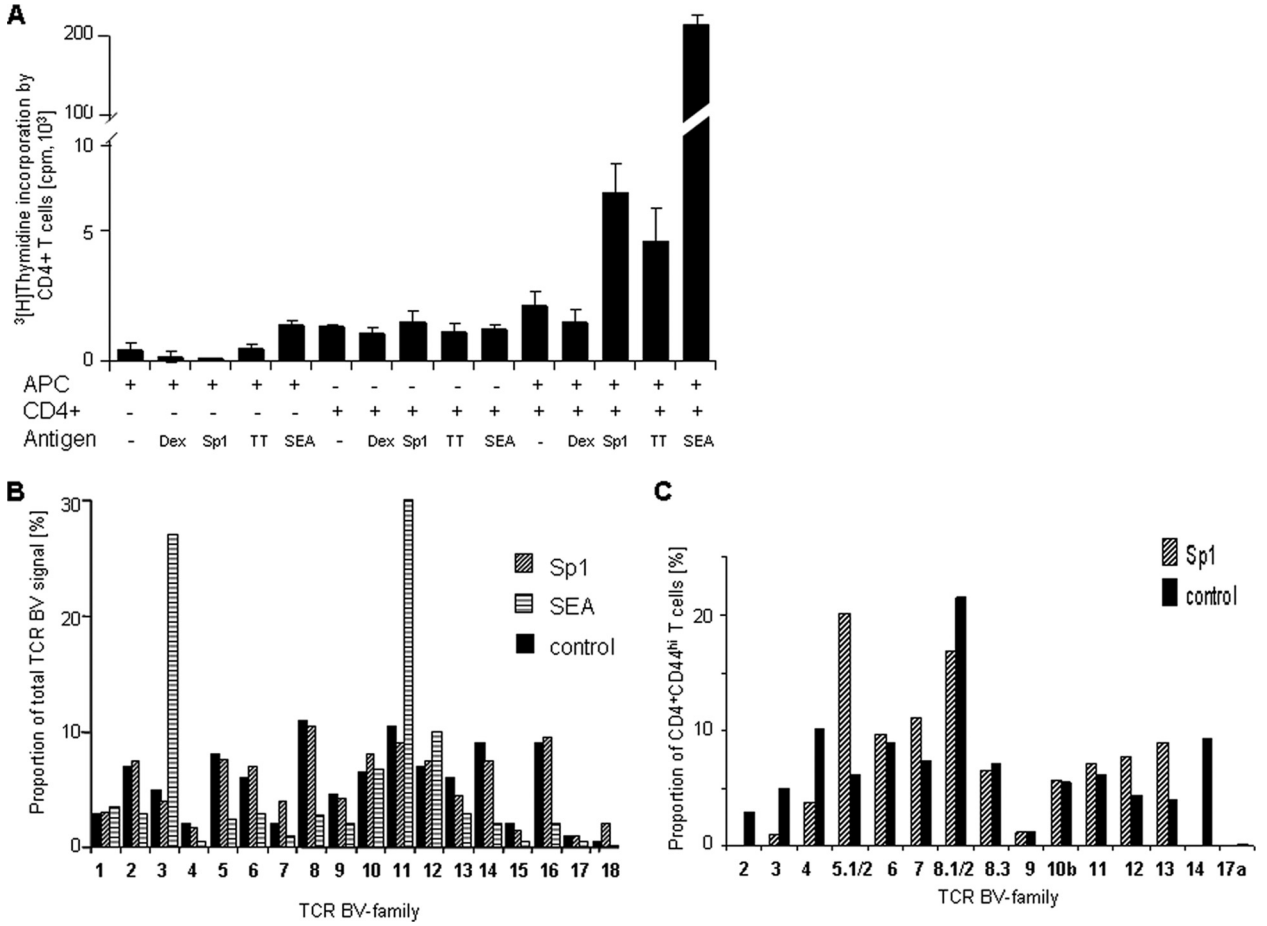

FIG. 4. $CD4^+$ T-cell proliferation with a nonrestricted TCR BV repertoire in vivo and in vitro. (A) T-cell proliferation assays with $CD4^+$ T cells as responders were performed in the absence or presence of APCs incubated for 5 days with dextran (Dex; 50 μg/ml), Sp1 (50 μg/ml), TT (2 flocculation units/ml), or SEA (10 ng/ml) or in medium alone as described in Materials and Methods. $CD4^+$ T-cell proliferation was measured by [^{3}H]thymidine incorporation (*y* axis). The T-cell proliferation assay results are representative of three independent experiments. (B) Distribution of TCR BV transcripts of Sp1-activated $CD4^+$ T cells in vitro, determined by semiquantitative RT-PCR. $CD4^+$ T cells were cultured in the presence of APCs and IL-2 with Sp1, SEA, or no antigen (control) for 1 week. The respective TCR BV family expression (*x* axis) is given as the mean percentage of the total TCR BV signal detected in the gel after 1 week of culture (*y* axis). The figure shows one representative result for six experiments. (C) The TCR BV repertoire of Sp1-induced $CD4^+$ $CD44^{high}$ T cells in vivo was determined by flow cytometry 18 h after intraperitoneal Sp1 challenge or saline application as a negative control. The respective TCR BV family expression (*x* axis) is given as the percentage of BV-positive $CD4^+$ $CD44^{high}$ T cells (*y* axis). Results for one experiment of three total is shown.

responses. So far, abscess formation has been discussed controversially, either as a suppressive immune response to prevent intra-abdominal sepsis or as a specific pathological inflammatory immune response. Using the model ZPS Sp1, we demonstrated with an experimental cellular mouse model simulating the leakage of intestinal bacterial flora into the sterile peritoneal cavity that memory $CD4^+$ T cells mediate the immune response resulting in abscess formation. Characterization of the memory cells revealed proinflammatory TH1 as well as TH17 cells. It had been shown that bacterial ZPS are unique in their ability to direct the development of the systemic cellular immune response by correcting TH1/TH2 imbalances toward a TH1 immune response (18). In that study, ZPS-mediated expansion of TH17 cells in the maturing host was not addressed. Although TH1 cells target naïve T cells and inhibit their TH17 development through IFN-γ, we and others showed both TH1 and TH17 memory cells colocalizing in pathological inflammatory environments (2, 3, 9, 11, 15, 25, 34, 38). Recently, the following novel dynamic between TH1 and TH17 in the course of inflammation was proposed: TH1-mediated inflammation is attenuated by IFN-γ-mediated modulation of APCs and is evolved toward TH17-mediated chronic inflammation (16). This model explains the observation that acute inflammation may evolve toward chronic inflammation where TH17 memory cells are largely found. The explanation also might be applied to intraperitoneal abscess formation, which certainly starts as an acute inflammatory response to the translocated intestinal flora and continues as a chronic inflammatory process.

In a mouse model of humoral immunity, we observed an IgG-specific humoral response to Sp1. After subcutaneous immunization in the presence of the TH1-promoting adjuvant Quil A, we observed a low but significant IgG response. Detection of not only IgG3, the predominant isotype produced in

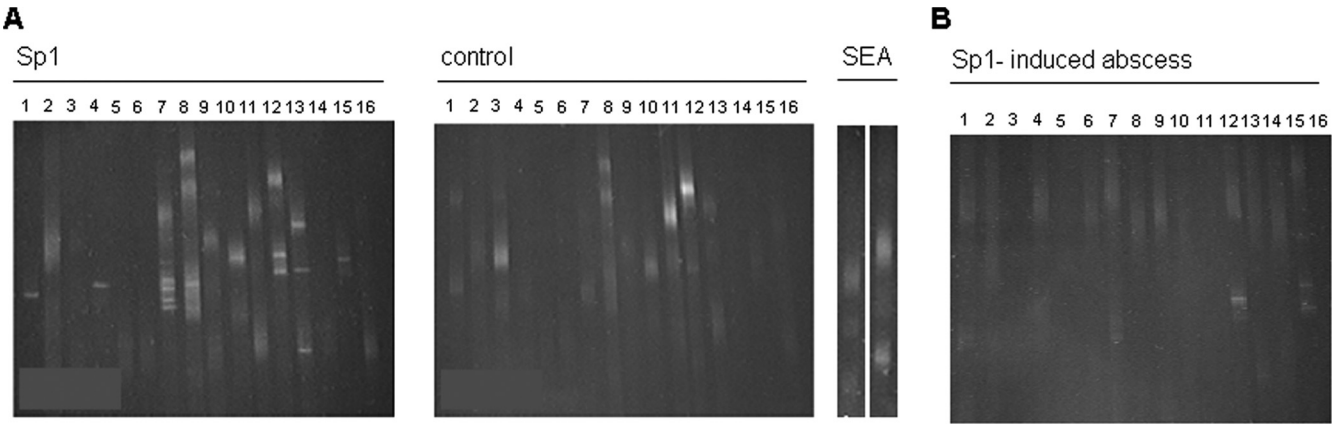

FIG. 5. Clonotypic Sp1-induced $CD4^+$ T-cell activation in vitro and in vivo. (A) Clonotype mapping of in vitro-stimulated $CD4^+$ T cells. $CD4^+$ T cells were cultured in the presence of APCs and IL-2 with Sp1 (left), in the absence of antigen (center), or in the presence of SEA (right) for 1 week. Transcripts of clonally expanded $CD4^+$ T cells within TCR BV families appear as distinct bands. Transcripts of polyclonally expanded $CD4^+$ T cells within TCR BV3 and BV11 of SEA-treated cells appear as smears. Results for one experiment of three are shown. (B) Clonotypic $CD4^+$ T-cell transcripts within intraperitoneal abscesses. C57BL/6 mice (6 per group) were challenged with Sp1, and abscesses were isolated after 6 days. Clonotype mapping was performed with isolated abscesses. Clonotypic transcripts appear as distinct bands for the TCR BV family. The figure shows one representative result of analyses of six abscesses.

response to T-cell-independent TI-2 polysaccharide antigens (19), but also a significant IgG1 titer promotes our hypothesis of T-cell-mediated isotype switching and suggests a humoral memory response. Subcutaneous ZPS application without adjuvant has been shown to induce regulatory IL-10-secreting $CD4^+$ $CD45Rb^{low}$ $CD25^-$ T cells that protect from disease (20). Thus, it is conceivable that subcutaneous Sp1 administration in the presence of adjuvant induces regulatory $CD4^+$ T cells that contribute to the low IgG titer and the shift to a predominant IgG1 (TH2) isotype. An interplay of TH17 and regulatory cells has been described for various immune reactions (1). The potential role of Sp1-induced TH17 cells in the induction of regulatory $CD4^+$ T cells and the modulation of humoral immunity remains to be determined. Cross-reactivity of the humoral response to Sp1 with other ZPS, such as PS A1 from *B. fragilis*, needs to be studied in the future because primary Sp1 immunization might also boost an already existing cross-reactive humoral immunity induced by ZPS of the gut flora.

Recent evidence indicates processing and MHC class II-dependent presentation of ZPS prior to activation and proliferation of $CD4^+$ T cells (8, 22). The requirement of DM suggests polysaccharide presentation within the MHC class II antigen binding groove, which was confirmed by recent in vitro studies (7, 35). These observations imply binding to and recognition by the CDR3 TCR antigen binding domain, which results in activation and oligoclonal expansion of specific $CD4^+$ T cells. However, for mice, the clonality of the $CD4^+$ T-cell response had not been addressed. Here we demonstrate a nonrestricted TCR BV repertoire of Sp1-activated $CD4^+$ T cells and oligoclonal expansion of $CD4^+$ T cells within the TCR BV families, supporting the hypothesis of Sp1 recognition by the CDR3 TCR antigen binding domain. Notably, in contrast to peptide antigens presented by MHC class II molecules, with average molecular masses ranging from 1.6 kDa to 3.0 kDa (4), polysaccharide fragments require an average 15-kDa molecule for the induction of T-cell-dependent immune responses (13). The MHC class II binding groove is open ended at both sides and therefore may accommodate antigens as large as 15 kDa. Given the repetitive highly charged α-helical structure of polysaccharide molecules, it is possible that protruding parts additionally stabilize the trimeric complex by displaying different binding sites at the outer MHC class II molecule and the TCR. In this scenario, polysaccharide processing might be crucial to achieve optimal antigen anchoring at both MHC class II molecules and the TCR. For a definite elucidation of this question, crystallography studies need to be performed.

Antigen recognition by the CDR3 binding domain of the TCR resembles the mechanism of presentation and T-cell activation by peptide antigens (37). Yet ZPS-specific clones in mice have not been established. The difficulties observed so far in obtaining and culturing polysaccharide-reactive T cells might be explained by complex immunomodulatory features, such as the polysaccharide-mediated T-cell anergy and the induction of IL-10-producing regulatory T cells, which may inhibit clonal expansion (20, 23). In light of these difficulties, clonotype mapping, which is a highly sensitive method to detect clonotypic TCR transcripts making up as little as 0.1% of a mixed T-cell population, seems to be a suitable method for analysis of the clonality of in vitro ZPS-activated $CD4^+$ T cells without the need for long-term expansion (21, 26). Notably, in Sp1-induced intraperitoneal abscesses, we were able to detect some, but not many, clonotypic transcripts. One limiting factor of clonotype mapping with this type of specimen may be the limited number of $CD4^+$ T cells in abscesses. The maximum influx of 5×10^6 $CD4^+$ T cells into the peritoneal cavity upon Sp1 challenge does not reflect the significantly smaller number of $CD4^+$ T cells present in the abscess wall. Another limiting factor in the investigation of clonal transcripts in vivo is the difficulty in extracting intact RNA material from abscess specimens that consist mainly of polymorphonuclear leukocytes.

Our study demonstrates that in mice, intraperitoneal and subcutaneous applications of Sp1 induce cellular and humoral

memory host responses, respectively. Oligoclonal expansion of polysaccharide-activated $CD4^+$ T cells highly suggests recognition of Sp1 by the CDR3 binding domain of the TCR. These observations open new opportunities for the design of vaccines against encapsulated microbial pathogens.

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