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Streptococcus sanguis Modulates Type II Collagen-Induced Arthritis in DBA/1J Mice

Massimo Costalonga,‡ James S. Hodges, and Mark C. Herzberg

Native type II collagen is tolerogenic when given orally or i.p. to DBA/1J mice and induces autoimmune arthritis when given s.c. in CFA. The tolerogenic epitope is contained in cyanogen bromide fragment 11 (CB11) and is structurally mimicked by PGEQGPK within the platelet aggregation-associated protein (PAAP) on Streptococcus sanguis. To learn whether S. sanguis modulates transmucosally the Ag-specific development of autoimmune arthritis, DBA/1J pups were given live S. sanguis, CB11, or type II collagen intragastrically. Feeding S. sanguis at 6 days postpartum delayed the onset of arthritis, and reduced the rate, final severity, and percentage of affected limbs. Next, PAAP± S. sanguis and type II collagen were tested for T cell cross-reactivity. T cells primed with the tolerogenic epitope of type II collagen proliferated more when incubated with PAAP± S. sanguis than with PAAP± Streptococcus gordonii or type II collagen, suggesting an Ag-specific transmucosal tolerogenic effect. In neonatal mice, therefore, bacterial surface Ags that mimic self can transmucosally stimulate Ag-specific inhibitory T cells. In adult mice immunized with type II collagen, these Ag-specific inhibitory T cells manifest later as attenuated arthritis. The PAAP± S. sanguis appear to activate adult memory, rather than naive, type II collagen-specific T cells, suggesting that systemic challenge with commensal self-mimicking microorganisms may perpetuate existing autoimmunity, but not initiate autorecognition. The Journal of Immunology, 2002, 169: 2189–2195.

The immune system generates a highly specific response against most pathogens while ignoring self Ags. After encountering self Ags, almost all autoreactive T cell clones are eliminated in the thymus medulla (negative selection) (reviewed in Ref. 1). In contrast, developmentally regulated self Ags are not presented to maturing thymocytes. Some self-specific T cells, therefore, may survive during the negative selection process (reviewed in Refs. 1 and 2). In the absence of autoimmune diseases, circulating self-specific T cells must either ignore tissue-specific self peptides, become functionally inactive, develop self-tolerance through a suppressive mechanism, or be physically eliminated outside the thymus.

Molecular structures that mimic self epitopes can be expressed by pathogenic and commensal microorganisms. Upon acute infection, such pathogens may activate undeleted self-reactive T cell clones promoting autoimmunity (3). Activation is mediated by APCs expressing high levels of class II MHC and costimulatory molecules (4). In contrast, it is unclear whether self-mimicking commensal microbes that chronically colonize mucosal compartments activate autoimmune T cells or inhibit activation to suppress autorecognition.

In experimental models, autoimmune diseases appear to be modulated by the presence or absence of commensal microbes colonizing the gut (5–8). Germfree animals fail to develop a state of tolerance that is typical of conventional animals (9). For example, germfree Fisher rats (strain F344) are extremely susceptible to streptococcal cell wall-induced arthritis (10). Conversely, conventional F344 rats are resistant to bacterial-induced arthritides, and their T cells do not respond to streptococcal cell wall Ags after immunization (6). Because the gastrointestinal tract is the most heavily colonized tissue, these observations suggest that the commensal microflora of the gastrointestinal tract induces a state of tolerance or unresponsiveness in cell-mediated responses.

The predominant oral commensal bacterium, Streptococcus sanguis, colonizes the oral cavity of human infants (11) and is swallowed at 2.3 × 10⁶ CFUs/min/ml saliva in dentate adults (12). Of limited pathogenicity in healthy individuals, S. sanguis can enter the blood periodically through breaches in the dento-gingival junction (13) or the oral mucosa (14). S. sanguis strain 133–79 also expresses a collagen-like epitope (accession A44428) within the platelet aggregation-associated protein (PAAP) (15). The PAAP epitope is partially homologous to residues 260–270 of the arthritogenic epitope of type II collagen (16). In rodent or primate animal models (10) and in rheumatoid arthritis patients (17, 18), type II collagen is a candidate Ag in autorecognition. Collectively, these data support the hypothesis that persistent swallowing of S. sanguis and lesions in the periodontal tissues can expose a susceptible individual to mucosal and systemic challenges with a self-like Ag expressed on microbial surfaces.

To study the effects of mucosal exposure early in life, S. sanguis was administered intragastrically in neonatal DBA/1J mice and then tested for its ability to modify experimental autoimmune arthritis in adult mice. Mucosal exposure of S. sanguis (PAAP+ ) in the neonate inhibited the development of autoimmune arthritis in the adult. Streptococcus gordonii (PAAP+ ) was without effect. S. sanguis could not activate naive type II collagen-specific T cells, but could stimulate primed type II collagen-specific T cells in vitro. In vivo, S. sanguis infection did exacerbate arthritis in type

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II collagen-primed mice. These results show that mice are susceptible to oral tolerance induced by commensal bacteria early in life and that the same bacteria can trigger autorecognition when primed autoreactive clones are present. To extrapolate from these conclusions to rheumatoid arthritis-susceptible individuals, early colonization by PAAP+ strains may be protective, while colonization or infection after the window of tolerogenicity could exacerbate arthritic episodes.

Materials and Methods

Neonatal oral tolerance

**Animals.** Male and female DBA/1J mice (4-A haplotype) at 8 wk of age (The Jackson Laboratory, Bar Harbor, ME) were fed bone/cartilage-like mouse chow (PMI Nutrition International, Richmond, IN) and water ad libitum. To obtain newborn pups, mice were bred in our facility. The animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee at University of Minnesota.

**Protein Ags.** All peptides and proteins were reconstituted to 2 mg/ml. Native chick type II collagen (Sigma-Aldrich, St. Louis, MO) was solubilized in 0.01 M acetic acid, pH 3.4, by tumbling overnight at 4°C (19). Cyanogen bromide (CNBr)-cleaved type II collagen (Chondrex, Redmond, WA) was solubilized in PBS, pH 7.4 (20). CB11 peptide was kindly provided by L. K. Myers and A. H. Kang (University of Tennessee, Memphis, TN). The arthritogenic epitope sequences in human (GenBank accession 115287), chick (21), and bovine (GenBank accession 214484) type II collagens are identical.

**Bacterial strains, growth, and Ags.** *S. sanguis* strain 133–79 was originally provided by R. R. Facklam, Center of Disease Control (Atlanta, GA), as a biotyped blood culture isolate from a confirmed case of bacterial endocarditis (22). S. gordonii strain M5 was originally isolated from dental plaque and kindly provided by C. F. Schachtele, University of Minnesota. The PAAP+ *S. sanguis* strain 133–79 and PAAP+ *S. gordonii* strain M5 were compared in these studies. Originally classified as *s. sanguis*, the M5 strain was taxonomically reclassified as *S. gordonii* during these studies (23). *S. gordonii* strain M5, *S. sanguis* 133–79 and can adhere to and induce platelets to aggregate when the collagen-like domain of PAAP interacts with platelet receptors (25).

**T cell cross-reactivity between *S. sanguis* and type II collagen Immunization.** Native chick type II collagen (Chondrex-LLC, Redmond, WA) was solubilized at 2 mg/ml in 0.01 M acetic acid, pH 3.4. A synthetic 19-mer peptide (Boston Biomolecules, Woburn, MA) containing the arthritogenic sequence (residues 260–270) from type II collagen (GELGIAGFKGEQGPKGETG) (31, 32) and OVA (98% pure) (Sigma-Aldrich) was solubilized at 2 and 4 mg/ml, respectively, in PBS, pH 7.4. Bacterial strains *S. sanguis* 133–79 and *S. gordonii* M5 were cultured and washed, as described above, and resuspended at 4 × 10^6 cells/ml in PBS.

**T cell proliferation assay.** Spleen cells from naive DBA/1J mice were treated with ammonium chloride buffer (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM EDTA) for 3 min to lyse RBCs. Splenocytes were washed and resuspended in Eagle’s Hanks’ amino acids (EHAA) medium (Irvine Scientific, Irvine, CA) supplemented with 4 mM l-glutamine (Life Technologies), 55 µM 2-mercaptoethanol, and 50 µg/ml gentamicin sulfate, and 50 µg/ml amphotericin-B (Clontech, Walkersville, MD) at 2 × 10^7 cells/ml and irradiated at 85.4 rad/min for 23.5 min (1940 rad). After adding fresh autologous mouse serum (5% v/v), the irradiated cells were plated on flat-bottom microtiter plates (Corning, Corning, NY) at 2 × 10^5 cells/100 µl/well. Denatured chick type II collagen (Sigma-Aldrich or Chondrex, Redmond, WA), 19-mer peptide of type II collagen, and OVA (Sigma-Aldrich) were solubilized at 0.5 mg/ml in serum-free EHAA medium and plated in triplicate at 50 µg/100 µl/well. *S. sanguis* strain 133–79 and *S. gordonii* strain M5 were grown and harvested, as described above, heat killed at 60°C for 25 min, and plated at 5 × 10^5 cells/100 µl/well in serum-free EHAA medium. Con A (Sigma-Aldrich) at 1 µg/ml was used as a positive control for T cell proliferation. Either bacteria or soluble Ags were preincubated with irradiated splenocytes at 37°C in 5% CO₂. Twenty-four hours later, peripheral lymph node T cells from mice immunized 13 days earlier were added to the culture. The T cells were enriched by negative selection with an affinity column (mouse T cell kit; Biotek Labora-
tory Products, Mountain View, CA), the cells eluted from the affinity column were 95%–98% CD3+ CD4− CD8− CD19− (B220) when stained with FITC- or PE-labeled mAbs, respectively (BD PharMingen, San Diego, CA).

Measurement of the incidence and severity of arthritis.** Starting 25 days after immunization, mice were lightly anesthetized with methoxyfluorane vapors, and limb joints were evaluated for signs of arthritis. Assessments were repeated every 4 days up to 92 days and every week up to 138 days postimmunization (194 days of age).

A blinded examiner (M. Costalonga) scored each paw on a scale 0–3, as previously described (19). The absence of arthritis was scored 0; mild swelling and erythema of one or more toes, or of the entire paw, 1; severe swelling and erythema of both tarsus and ankle, 2; and ankylosis (joint rigidity) and bony deformity, 3. The arthritic index (AI) is the sum of the score recorded for each limb in one mouse (0, no disease, to 12, most disease). The average arthritic index (AAI) for each group is the sum of the AI for each mouse divided by the number of mice in the group (modified from Ref. 19). A mouse was considered arthritic when it scored at least 1 at three or more consecutive time points. A preliminary calibration trial established that intraexaminer percentage of agreement was 99% for score of 1, 94% for score of 2, and 98% for score of 3. The rate of arthritis development was estimated by dividing the AAI by the time postimmunization and tested in a mixed linear model. The final severity of arthritis was the AAI at 138 days postimmunization. To corroborate the AI values, tarsus thickness was measured in 0.1-mm increments with a modified constant tension caliper calibrated to 15 g force (S/N P0120; Florida Probe, Miami, FL). Measurements were taken between the four walking pads (30) of the plantar surface before immunization (at 56 days of age; baseline) and at each time point after immunization. A calibration trial showed intraexaminer reproducibility of 98% within ±0.1 mm. The normal growth of paws in DBA/1J mice was essentially completed at 56 days of age because measures of untreated control mice taken at 194 days of age were not statistically different.

**Cell surface markers**

**Flow cytometry.** Spleen cells from naive DBA/1J mice were treated with ammonium chloride buffer (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM EDTA) for 3 min to lyse RBCs. Splenocytes were washed and resuspended in Eagle’s Hanks’ amino acids (EHAA) medium (Irvine Scientific, Irvine, CA) supplemented with 4 mM l-glutamine (Life Technologies), 55 µM 2-mercaptoethanol, and 50 µg/ml gentamicin sulfate, and 50 µg/ml amphotericin-B (Clontech, Walkersville, MD) at 2 × 10^7 cells/ml and irradiated at 85.4 rad/min for 23.5 min (~200 rad). After adding fresh autologous mouse serum (5% v/v), the irradiated cells were plated on flat-bottom microtiter plates (Corning, Corning, NY) at 2 × 10^5 cells/100 µl/well. Denatured chick type II collagen (Sigma-Aldrich or Chondrex, Redmond, WA), 19-mer peptide of type II collagen, and OVA (Sigma-Aldrich) were solubilized at 0.5 mg/ml in serum-free EHAA medium and plated in triplicate at 50 µg/100 µl/well. *S. sanguis* strain 133–79 and *S. gordonii* strain M5 were grown and harvested, as described above, heat killed at 60°C for 25 min, and plated at 5 × 10^5 cells/100 µl/well in serum-free EHAA medium. Con A (Sigma-Aldrich) at 1 µg/ml was used as a positive control for T cell proliferation. Either bacteria or soluble Ags were preincubated with irradiated splenocytes at 37°C in 5% CO₂. Twenty-four hours later, peripheral lymph node T cells from mice immunized 13 days earlier were added to the culture. The T cells were enriched by negative selection with an affinity column (mouse T cell kit; Biotek Labora-
tory Products, Mountain View, CA), the cells eluted from the affinity column were 95%–98% CD3+ CD4− CD8− CD19− (B220) when stained with FITC- or PE-labeled mAbs, respectively (BD PharMingen, San Diego, CA).

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4 GEGKPQK, PAAP homology; IAGFKGEQGPK, minimal epitope presented by I-Aq molecules.
ICN, Costa Mesa, CA) was added to each well. At 110 h, cells were harvested on a glass-fiber mat and analyzed with a beta-liquid scintillation counter (1214 RackBeta; Wallak Oy, Turku, Finland).

**Bacterial infection and arthritis**

**Bacterial infection and experimental arthritis.** Seven- to ten-week-old, age-matched, DBA/1J mice were primed by immunizing with type II collagen in CFA at the base of the tail, as described above, or injected s.c. at four separate sites in the back with live \textit{S. sanguis} (2 × 10^6 CFU/mouse). Twenty days later, the type II collagen-primed mice were boosted as above with \textit{S. sanguis}, or PBS and the \textit{S. sanguis}-primed mice were challenged with type II collagen in CFA. To assess persistence of infection, purulent exudates were plated on Todd Hewitt Broth-blood agar plates 20 days after s.c. injection. AI was measured at 0, 25, 44, 60, 90, and 120 days after initial priming.

**Results**

**Arthritis induction**

To establish the murine model (Fig. 1), arthritis resulting from a single immunization with type II collagen in CFA was compared with single immunization followed by three boosts in CFA. Mice immunized once at 56 days postpartum all developed arthritis 41 ± 4 days later. When immunization was followed by additional boosts, arthritis onset was significantly delayed to 55 ± 6 days (Weibull, \( p = 0.019 \)) and signs eventually appeared in only 77.8% of mice. The rate of arthritis development and the final severity after a single immunization was significantly higher than after multiple boosts (Fig. 2A) (mixed linear model, \( p = 0.001 \); ANOVA, \( p = 0.003 \), respectively). Sham-immunized and unimmunized mice did not develop significant spontaneous arthritis (Figs. 2A and 3), in contrast to previous reports (33, 34). Because a single immunization effectively induced maximal arthritis, all other mice were immunized once with type II collagen at 56 days postpartum (Fig. 1).

**Inhibition of arthritis by systemic tolerance**

To determine that systemic administration of soluble Ag reduces the expression of arthritis, some 6-day-old mice were injected i.p. with native type II collagen in IFA (Fig. 2B). After immunization with type II collagen at day 56, these mice showed delayed onset (\( p < 0.001 \)), lower frequency of affected limbs, and reduced severity of arthritis (Student’s \( t \) test, \( p < 0.05 \)) when compared with unimmunized mice (Fig. 2A). Inoculation with denatured type II collagen at day 6 was less effective in reducing the occurrence of arthritis (data not shown) and consistent with other reports (20).

**Oral tolerance**

To compare with the apparent tolerance induced by i.p. inoculation of native type II collagen at day 6, mice were fed intragastrically with potential tolerogens daily from days 6–10 postpartum (Fig. 1). Mice fed type II collagen (Fig. 2B) or CB11 (data not shown) showed delayed onset (\( p = 0.01 \)) and reduced the final severity of arthritis when compared with untreated controls (\( p < 0.05 \)) (Fig. 2A). CB11-fed mice showed significantly slower initial rate (\( p = 0.002 \)) and severity of arthritis at days 44, 80, and 103 postimmunization (\( p < 0.05 \)) than untreated mice (data not shown).

After feeding PAAP+ \textit{S. sanguis} strain 133–79 (Fig. 2C), the initial rate (\( p = 0.004 \)), prevalence, and final severity (\( p < 0.05 \))
of arthritis were significantly reduced and onset was delayed \((p = 0.006)\) when compared with untreated mice, and were similar to CB11 (not shown) and type II collagen (Fig. 2B).

In OVA- and \emph{S. gordonii}-fed mice, the initial rate; AI at 44, 80, and 103 days postimmunization; and final severity were similar to untreated mice (Fig. 2, B and C) \((p > 0.05\) for all comparisons). The onset, rate of development, and severity of arthritis differed significantly between OVA- and \emph{S. gordonii}-fed mice (negative controls for tolerance), and mice tolerized i.p. with native type II collagen (positive control for tolerance) that such groups were considered the boundaries of the experimental arthritis model. Descriptive statistics for all groups are shown in Table I.

In \emph{S. sanguis}-fed mice, the average tarsus thickness and the initial rate of increase \((day 0–40\) postimmunization) were less than OVA \((p < 0.05)\) or \emph{S. gordonii}-fed mice \((p < 0.05)\) (Fig. 3). The average increase over time from baseline in the thickness of posterior paws was 0.75 mm for the OVA-fed, 0.41 mm for the \emph{S. gordonii}-fed, 0.23 mm for the \emph{S. sanguis}-fed, and 0.21 mm for type II collagen-fed mice.

Early feeding of CB11 (days 1–5 postpartum) reduced the initial rate \((p < 0.001)\) and final severity of arthritis \((p < 0.05)\) when compared with untreated mice (data not shown). In contrast to late feeding \((days 6–10)\), early feeding with \emph{S. sanguis} reduced neither the initial rate \((p = 0.073)\) nor the final severity \((p > 0.05)\) (data not shown).

\textit{T cell cross-reactivity between type II collagen and \emph{S. sanguis}}

Immunization at day 56 with chick type II collagen (Fig. 4A) or a 19-mer peptide of chick type II collagen (Fig. 4B) primed T cells to proliferate in vitro when stimulated with heat-killed PAAP\(^+\) \emph{S. sanguis} 133–79 (ANOVA, \(p < 0.05\), respectively). The primed T cells proliferated significantly more in response to PAAP\(^+\) \emph{S. sanguis} than PAAP\(^+\) \emph{S. gordonii} M5 \((p < 0.05)\), which was similar to background. After priming with type II collagen, T cells proliferated more in response to \emph{S. sanguis} than homologous type II collagen \((p < 0.05)\) (Fig. 4A), while T cells primed with the 19 mer proliferated similarly in response to \emph{S. sanguis} or the homologous 19 mer (Fig. 4B). Type II collagen- and 19-mer-primed T cells proliferated significantly above background in response to type II collagen preparations \((p < 0.05)\).

Primed by immunization with live \emph{S. sanguis} 133–79, T cells proliferated significantly only in response to homologous heat-killed cells (Fig. 4C). \emph{S. sanguis}-primed T cells did not respond to type II collagen or \emph{S. gordonii}. In contrast, \emph{S. gordonii}-primed T cells responded to both \emph{S. gordonii} and \emph{S. sanguis} (Fig. 4D). OVA-primed T cells proliferated significantly above background in response only to the homologous OVA Ag \((p < 0.05)\) (Fig. 4E).

\textbf{FIGURE 3.} Perinatal feeding of \emph{S. sanguis} reduces paw swelling. Six-day-old, untreated, and unimmunized mice \((n = 9)\) were fed OVA \((n = 8)\) at 20 \(\mu\)g/g body weight or 3 \(\times 10^8\) live \emph{S. sanguis} cells \((n = 13)\) for 5 consecutive days. Immunized mice received 130 \(\mu\)g type II collagen in CFA at 56 days of age. The paw swelling in all the mice was assessed with a constant tension caliper (15 g force). The graph represents the changes in paw thickness before and after immunization. Each point represents the group average of summed changes from baseline of the four paws \(\pm\) SEM.

\emph{S. sanguis} infection exacerbates autoimmune arthritis

Mice primed by immunization at day 56 with type II collagen in CFA and boosted with \emph{S. sanguis} develop more arthritis than mice immunized with type II collagen in CFA alone (ANOVA, \(p < 0.05\)) (Fig. 5). In animals primed with \emph{S. sanguis} and boosted with type II collagen, the onset of arthritis is delayed, but the rate of onset is accelerated, when compared with type II collagen alone (one-way ANOVA, \(p < 0.05\)). At the time of type II collagen boosts, viridans streptococci were identified in purulent exudates, indicating of persistent \emph{S. sanguis} infection. The final severity of arthritis in mice primed with \emph{S. sanguis} and boosted with type II collagen was similar to mice immunized with type II collagen in CFA and boosted with \emph{S. sanguis}.

\textbf{Discussion}

The data show that the PAAP\(^+\) \emph{S. sanguis} strain 133–79 modulates transmucosally the systemic response against type II collagen. DBA/II mice fed perinatally with PAAP\(^+\) \emph{S. sanguis} developed significantly less arthritis after immunization later with type II collagen than unfed or OVA-fed mice. When fed from 6–10 days after birth, \emph{S. sanguis} appeared to selectively inhibit the systemic cell-mediated response because the Ab response was not affected (data not shown).

PAAP\(^+\) \emph{S. sanguis} expresses a T cell epitope that is homologous to the tolerogenic epitope on type II collagen. Peripheral lymph node T cells primed against a 19-mer peptide of type II collagen or against

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Inoculum & 6 Days Postpartum & No. Males/Females & No. Arthritic & Percent Arthritic Limbs & Time of Arthritis Onset & AAI at Term \((\pm SEM)\) \\
\hline
\textbf{Oral} & & & & & & \\
OVA & 8 & 2/6 & 7 & 2/5 & 62.5 & 31 \pm 3 & 7.3 \pm 1.9 \\
\emph{S. gordonii} M5 & 10 & 6/4 & 9 & 6/3 & 70.0 & 44 \pm 10 & 7.4 \pm 1.4 \\
\emph{S. sanguis} 133–79 & 13 & 6/7 & 9 & 4/5 & 46.1 & 43 \pm 6 & 4.3 \pm 1.2 \\
\textbf{Native type II collagen} & & & & & & \\
Native (unimmunized) & 9 & 4/5 & 6 & 4/2 & 44.4 & 35 \pm 3.5 & 4.1 \pm 1.6 \\
\hline
\textbf{i.p.} & & & & & & \\
Native type II collagen & 8 & 3/5 & 4 & 2/2 & 18.7 & 74 \pm 18 & 1.4 \pm 0.9 \\
\hline
\end{tabular}
\caption{Reduction of arthritis after neonatal oral and i.p. tolerization at day 6 postpartum followed by immunization with type II collagen}
\end{table}

\(a\) At 138 days after primary immunization (194 days old).

\(b\) Mean days after immunization \(\pm\) SEM for mice that became arthritic.
Type II collagen-primed T cells do not respond to PAAP induction is significant. Infection with S. sanguis boosted 20 days later with type II collagen. Even though the initial result also. In vivo, the exacerbation of arthritis is consistent with this finding. S. sanguis is responsible for the proliferation of T cells. Conclusively, S. sanguis do not prime T cells that respond to type II collagen. The exacerbation of arthritis in vivo is consistent with this result also. In S. sanguis-infected mice, arthritis appears only after boosting 20 days later with type II collagen. Even though the initial infection with S. sanguis does not initiate arthritis, the rate of arthritis induction is significantly increased after type II collagen immunization. Type II collagen-primed T cells do not respond to PAAP \( S. \) gordonii, suggesting that only the “collagen-like” platelet aggregation-associated protein of S. sanguis is responsible for the proliferation of memory type II collagen-specific T cells.

Our findings are consistent with the assumption that the PAAP mimics the type II collagen peptide interacting with I-A\(^d\) and TCR molecules. The peptide (residues 260–270) IAGFKGEGQPKP of type II collagen (32) fits the I-A\(^d\) groove of DBA/1 mice during Ag presentation (35). Single substitutions of aa 245–270 showed that residues 260–270 are the most important for T cell activation (31, 36). Apparently, the PAAP collagen-like motif, PGEGQPKP, with a nearby isoleucine residue (15) is sufficiently homologous to the critical residues 260–270 of type II collagen to affect the type II collagen-specific T cell response.

In humans, the fit of the type II collagen peptide AEGKKGEGQPKP to the HLA-DR4 molecule has been modeled by analogy to the crystallinography data of HLA-DR4 fitted with type II collagen peptide YQMRADQAAAGGLR (1168–1180) (35). Although the HLA-DR4/1180 peptide complex is only a hypothetical computer-generated model, the analogy to the HLA-DR4/1168–1180 peptide is so close and the mode of peptide binding so conserved that HLA-DR4 (DRB1*0401) individuals harboring PAAP \( S. \) sanguis may be either at risk or protected against autoimmune recognition.

The T cell cross-reactivity data are consistent with a T cell-mediated antiarthritic effect induced by the oral route. The effects elicited by S. sanguis during the perinatal period can be measured indirectly into adulthood. The antiarthritic effects in our study may have been generated by the immunosuppressive cytokines TGF-\( \beta \) and IL-10. CD4\(^+\) and CD8\(^+\) T cells producing TGF-\( \beta \) in the Peyer’s patches do so in an Ag-specific manner (37). Adoptive transfer experiments with Peyer’s patch T cells from S. sanguis-fed mice will test this hypothesis (M. Costalonga, et al., manuscript in preparation). Unlike clonal deletion or anergy induced by i.p. injections of proteins (20, 38), mucosal administration of low Ag doses to neonatal pups elicits suppressive T cell clones (37, 39). Suppressive T cells in the Peyer’s patches are defined as Th3 cells and in the spleen as T regulatory cells (CD25\(^+\)CD45RB\(^{hi}\)CD4\(^+\)) (40). These T cells prevent Th1-mediated hypersensitivity reactions (reviewed in Ref. 41) against exogenous Ags and commensal microorganisms (40) and promote IgA isotype switching (reviewed in Ref. 42). Our data support the hypothesis that commensal microorganisms induce Ag-specific suppressive mechanisms.

Despite the persistence of a large number of bacteria in the intestinal lumen, immunosuppressive cytokines are released in the lamina propria of the intestinal walls (43). Commensal microorganisms may be indirectly responsible for the release of inhibitory cytokines because oral delivery of Lactobacillus casei strain Shirota reduced the severity of type II collagen-induced arthritis in adult DBA/1 mice (8) or diabetes in nonobese diabetic mice (44). The mechanism of transmucosal inhibition of the local and systemic cell-mediated immune responses by commensal bacteria remains unclear (45), and the bacterial Ags involved have not been previously reported.

Our data suggest PGEQGPKP of PAAP to be a candidate self-mimicking epitope. Both closely related species S. sanguis and S. gordonii are encased in a peptidoglycan wall of similar structure and chemistry (46). A major difference is expression of PAAP. Although peptidoglycans have been associated with inhibition of experimental arthritis (47), feeding PAAP \( S. \) sanguis reduced arthritis more than S. gordonii. This finding also argues against a T-independent tolerogenic effect mediated by peptidoglycans (47) or bacterial heat shock proteins (48), and suggests an Ag-specific effect mediated by the PAAP\(^+\) epitope. To confirm the Ag-specific effects of PAAP, genetic approaches would negate any unexpected effects of the similar, but nonidentical backgrounds of S. sanguis and S. gordonii. For example, an S. sanguis PAAP\(^+\)-isogenic mutant and S. gordonii engineered to express PAAP could be compared with their respective wild types.
Our results suggest the following model in which the specificities and abundance of memory T cells are far more restricted than the population of primed T cells (Fig. 6). The PAAP\(^+\) *S. sanguis* was expected to stimulate T cells primed with type II collagen-related Ags, and to prime T cells that would react with type II collagen. Surprisingly, T cells primed with *S. sanguis* failed to proliferate in vitro when stimulated with type II collagen or with the 19 mer. Hence, reciprocal cross-reactivity between *S. sanguis* and type II collagen epitopes is not apparent. After priming with either Ag (Fig. 6A), the TCR heterogeneity of emerging memory T cells is reduced such that T cells that respond to low concentrations of peptide become memory cells (49). In contrast to the diverse population of primed T cells, memory cells show a less diverse repertoire. Therefore, even though the specificities generated during priming overlap, the populations of memory cells generated by the peptide IA2FGEQGPK of type II collagen and the PAAP epitope on *S. sanguis* are completely distinct. Type II collagen-specific memory cells respond also to PAAP\(^+\) *S. sanguis*. The specificities of memory type II collagen-specific cells are therefore broad (Fig. 6A, ↔) and overlap the specificities generated by *S. sanguis* during priming (Fig. 6A, filled arrow). This mechanism is consistent with *S. sanguis* infection exacerbating type II collagen-induced arthritis. In contrast, *S. sanguis*-specific memory cells do not respond to type II collagen because the spectrum of specificities is smaller (Fig. 6, ↔) and does not overlap with the specificities generated after priming with type II collagen (Fig. 6A, hatched arrow). In vivo peripheral tolerance is indeed maintained, because *S. sanguis* 133–79 in CFA is unable to induce autoimmune arthritis when injected s.c. into DBA/1 mice (data not shown). Nonetheless, the persistence of *S. sanguis* infection accelerates the onset of arthritis after type II collagen immunization. Nineteen-mer and type II collagen-primed T cells recognized type II collagen. Both primed and memory T cells appear to show overlapping specificities, suggesting reciprocal cross-reactivity (Fig. 6B, open arrows). T cells primed with the control Ag OVA did not recognize any of the Ags tested (Fig. 6C). Conversely, T cells primed with other Ags failed to recognize OVA (Fig. 4, A–D), confirming that primed and memory T cells have distinct, nonoverlapping specificities. Collectively, our data are consistent with the hypothesis that an individual retains T cells with a low, but not negligible, autoreactive potential to maintain a diverse T cell repertoire.

Occasionally, autoreactive potential may be exploited by virulent pathogens expressing self-mimicking epitopes. For example, post-streptococcal carditis is caused by streptococcal M protein (50), and ankylosing spondylitis is associated with *Klebsiella pneumoniae* infections (51). *K. pneumoniae* pullulanase shares homologies with types I, III, and IV collagen. Ankylosing spondylitis patients have elevated IgG and IgA Abs against *K. pneumoniae*, pullulanase, and type I and IV collagens (51), indicating T cell activation and isotype switching. In these two instances, it appears that the virulent pathogen activates naive T cells and initiates the autoimmune response. Despite the epitope mimicry, colonization with the avirulent, commensal *S. sanguis* may not be sufficient to initiate an autoimmune response. In humans, oral colonization with *S. sanguis* does not appear to stimulate an autoimmune response to self proteins in the connective tissue. In vitro, *S. sanguis* does not activate type II collagen-specific naive T cells, but reactivates only memory T cells and exacerbates type II collagen-induced arthritis in vivo. Type II collagen-specific memory T cells, however, can be found in the joints of rheumatoid arthritis patients (17). Harbored in the dental plaque of humans (12, 52), *S. sanguis* could induce a systemic challenge through ulceration of the epithelial barrier at the gingival sulcus (13, 14). The systemic challenge through the gingiva would be expected to exacerbate progression, but not initiate autorecognition in patients harboring type II collagen-specific memory cells as in rheumatoid arthritis (17). Consistent with this concept, rheumatoid arthritis patients appear to have higher prevalence of advanced adult chronic periodontitis (53), suggesting that autoreactive T cells are reactivated locally.

In summary, this study shows that a commensal microorganism (*S. sanguis* strain 133–79) expressing a type II collagen-mimicking epitope exerts transmucosal inhibitory effects on type II collagen-induced arthritis. The effect appears to be Ag specific, because *S. sanguis* is recognized by type II collagen-primed T cells. The transmucosal antiarthritic effects are initiated after 6 days of age and are detected after type II collagen immunization at 56 days. Consequently, a host-mimicking microorganism such as *S. sanguis* may be protective if colonization and transmucosal interactions occur early in life, when primarily naive type II collagen-specific T cells are present. Alternatively, if repeated systemic bacterial challenge occurs in the presence of type II collagen-primed or memory T cells as in rheumatoid arthritis, the anti-type II collagen
response may increase with exacerbation of the rheumatoid condition. The prevalence of PAAP /H11001
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