Superantigenic activity is responsible for induction of coronary arteritis in mice: an animal model of Kawasaki disease

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Abstract
Kawasaki disease (KD) is a multisystem vasculitis and the leading cause of acquired heart disease in children of the developed world. Lactobacillus casei cell wall extract-induced coronary arteritis in mice mirrors KD in children. Here, we report that responses to L. casei cell wall extract possess all the hallmarks of a superantigen-mediated response: marked proliferation of naive T cells, non-classical major histocompatibility restriction with a hierarchy in the efficiency of different class II molecules to present this superantigen, a requirement for antigen presentation, but not processing, and stimulation of T cells in a non-clonal, TCR Vb chain-dependent fashion. This superantigenic activity directly correlates with the ability to induce coronary arteritis in mice. Taken together, our findings demonstrate that superantigenic activity in L. casei cell wall extract is responsible for induction of coronary artery disease.

Introduction
Superantigens (SAg) are a group of proteins that differ in many ways from a typical protein or peptide antigen (1–4). They share the ability to stimulate a large proportion of T cells (up to 30% of the T cell repertoire compared to 1 in 10^4 to 1 in 10^6 T cells for conventional peptide antigens) by binding to a portion of the TCR Vβ chain in association with MHC class II molecules (5–8). SAg have been identified in a variety of organisms including bacteria and viruses (5,9). Staphylococcal enterotoxin B (SEB) is the best-studied prototypic bacterial SAg (1,10). Exposure to SEB leads to an initial proliferation and increase in the relative percentage of T cells expressing TCR Vβ8 (TCR Vβ skewing) (5,11,12). This initial stage of activation and proliferation is followed by a rapid disappearance of T cells expressing the reactive TCR Vβ chain (13–15). SEB also induces cytokines such as tumor necrosis factor (TNF)-α, IL-1, IL-2 and IFN-γ (16–19). T cell recognition of SAg requires MHC presentation, but not processing, and does not require degradation to peptides (20,21). Interestingly, presentation of SAg is not classically restricted to self MHC (7,9).

Kawasaki disease (KD) is the most common cause of vasculitis affecting children. KD is characterized by prolonged fever, non-purulent conjunctivitis, oral mucosal inflammation, cervical lymphadenopathy, induration and erythema of the hands and feet, and a diffuse polymorphous skin rash (22–24). Although the inflammatory response is found in medium and small vessels throughout the body, the most common site of end-organ damage is the coronary arteries. KD is now recognized as the leading cause of acquired heart disease in children in the developed world (25). Many gaps still exist in our knowledge of the etiology and pathogenesis of KD, making improvements in therapy difficult.

Although the etiology of KD is unknown, the epidemiologic and clinical features of the disease strongly suggest that an infectious agent is the cause or at least an inciting agent (26). It has been suggested that KD fits the spectrum between an infectious disease and a true autoimmune disease (27). Numerous infectious agents including Streptococcus pyogenes, Staphylococcus aureus, Propionibacterium acnes, Epstein–Barr virus and parvovirus have been identified in individual epidemics (28–33). Although still debated, current evidence suggests that there is an initial infectious trigger consistent with the presence of superantigenic activity (34).
The specific organism may not be as important as a common mechanism of stimulation of the developing immune system. The footprint of a SAg, i.e. TCR Vβ skewing, has been identified during some outbreaks of KD (35). TCR Vβ skewing has also been found in affected cardiac tissue, with extensive junctional region diversity within these T cell subpopulations (36). Following reports of the association of rug shampooing and KD (37,38), a variant of P. acnes (predominant bacteria found in dust mites in rugs) was recovered from lymph nodes and from cultures of a few patients (39,40). Interestingly, peptidoglycans from P. acnes have been shown to possess superantigenic activity (39–41). Other SAg-producing organisms, including streptococcal and staphylococcal bacteria, which secrete exotoxins B and C, and toxic shock syndrome toxin (TSST) respectively, have been isolated from patients with KD (42).

An animal model of coronary arteritis was developed by Lehman et al. (43). Strains of inbred mice responded to i.p. injections of Lactobacillus casei cell wall extract (LCWE) and developed coronary arteritis. The resultant vasculitis is similar to KD in children, demonstrating similar histologic changes, time course to coronary disease and response to therapeutic intervention with i.v. Ig (IVIG) (43–45). We have refined this animal model of LCWE-induced coronary arteritis by using genetically modified mice expressing human CD4 and human MHC class II DQ6 transgenes in a background lacking endogenous CD4 and CD8. These DQ6 transgenic (Tg) mice are phenotypically and functionally normal with respect to Tc, cell functions (46). They are unique in that they display human-like responsiveness to bacterial SAg and are super-sensitive to the pathogenic effects of bacterial SAg, and thus ideal for studies of SAg-mediated responses and assessing the pathogenesis of SAg-associated diseases.

In the present study, we dissected the immune response to LCWE. Our work confirms that LCWE contains superantigenic activity. Responses to LCWE possess all the characteristics of a SAg-mediated response: massive activation of naive T lymphocytes, requirement for MHC class II-expressing antigen-presenting cells (APC) and antigen presentation, but not processing, non-classical MHC restriction with a hierarchy of differing MHC class II ability for antigen presentation, and induction of TCR Vβ skewing. More importantly, presence of superantigenic activity correlated directly with induction of coronary arteritis. Further understanding of the mechanisms leading from immune activation to localized coronary artery damage will influence treatment and long-term morbidity/mortality in children with KD.

Methods

Mice

C57BL/6 and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice homozygous for the murine CD4 and CD8 mutations have previously been described (47). Briefly, using the method of targeted gene disruption and homologous recombination in embryonic stem cells, mouse strains carrying mutations in CD8α and CD4 genes (48,49) were bred together until homozygous for both mutations (mCD4 mCD8− double knockout). The human CD4 transgene (hCD4) is expressed under the control of the CD2 expression cassette (50), in a lymphocyte-specific, copy number-dependent, integration site-independent manner. These hCD4 transgenic mice were bred into the mCD4 mCD8− double-knockout mice. Concurrently, the human MHC class II DQ6 transgenic mice with co-injected DQ6 α and β genomic DNA fragments under control of their endogenous promoters (51) were bred into the mCD4 mCD8− background. The two strains of mutant mice were then bred together to obtain hCD4=DQ6+mCD4 mCD8− (DQ6 transgenic mice), which were backcrossed eight generations into C57BL/6 mice. Mice were genotyped by FACS of peripheral blood as previously described (46). All genetically modified animals had a H-2b background unless otherwise stated.

Preparation of LCWE

LCWE was prepared as previously described by Lehman et al. (52). Briefly, L. casei (ATCC 11578) was obtained from the ATCC (Rockville, MD). The bacteria were first cultured in Lactobacillus MRS broth (Difco, Detroit, MI) for 24 h at 37°C (on a shaker platform) and then harvested by centrifugation (10,000 g, 40 min) during the log phase of growth. After washing repeatedly with PBS (pH 7.2), bacteria were lysed by overnight incubation (at room temperature on a shaker platform) with 4% SDS (EM Science, Gibbstown, NJ) (10 volumes), and then washed thoroughly with PBS (10 times) to remove all cellular debris and SDS. Sequential incubations with 250 μg/ml RNase, DNase I and trypsin (Sigma, St Louis, MO) were performed to remove any adherent material from the cell walls. Each incubation was for 4 h at 37°C at twice the packing volume and was followed by two washes in PBS or four washes after the incubation with trypsin. Cell wall fragmentation was then achieved by sonication of the pellet (5 g packed wet weigh in 15 ml PBS) at 4°C in a continuous dry ice/ethanol bath for 2 h at a pulse setting of 5.0 (10 s pulse/5 s pause) (550 Sonic Dismembrator with a 1/2 in. tapped horn and tapered microtip; 1/8 in. diameter, tuned to vibrate at a fixed frequency of 20 KHz; Fisher Scientific, Nepean, Ontario, Canada). The sonicated preparation was then centrifuged for 1 h at 40,000 g at 4°C and the supernatant harvested to obtain the cell wall fragments for injection. The concentration of the preparation was based on a phenol/sulfuric acid colorimetric determination of the rhamnose content and expressed in mg/ml final concentration in PBS.

Flow cytometry analysis of TCR Vβ expression ex vivo

Mice (4–6 weeks old) were injected i.p. with 0.5 mg LCWE in 500 μl PBS on day 0. On days 3, 5 and 10, splenocytes were prepared into single-cell suspensions, and 1.0 × 106 cells were triple stained with appropriate dilutions of FITC-conjugated antibodies against TCR Vβ genes (mouse TCR Vβ screening panel; Pharmingen, San Diego, CA), biotinylated anti-mouse Thy-1.2 (53-2.1) (PharMingen) and either phycoerythrin (PE)-conjugated anti-human CD4 (SK3) (Becton Dickinson, Mountain View, CA) or PE-conjugated anti-mouse CD4 (Becton Dickinson) mAb. Biotinylated antibodies were visualized with Cy5–PE-conjugated streptavidin. Control samples were stained similarly but with isotype-matched mAb (PharMingen). Samples were analyzed using CellQuest software on a FACScan or FACSCalibur (Becton Dickinson).
Flow cytometry analysis of TCR Vβ expression in vitro

Splenocytes from 4- to 6-week-old mice were cultured in complete medium [IMDM (Gibco BRL, Burlington, Canada) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 50 μM 2-mecaptoethanol and 10 mM HEPES] or in complete medium containing either a mixture of 0.1 and 0.5 μg/ml of anti-CD3ε (145-2C11) and anti-CD28 (37.51) antibodies (PharMingen) respectively or 5–20 μg/ml LCWE (1 × 10⁶ cells/ml/well) in 24-well plates (Nunc, Roskilde, Denmark). After 3 days, cells were collected and triple stained for TCR Vβ as described above.

Preparation of APC

T lymphocytes were depleted from whole spleen by a standard protocol using Thy-1.2 antibody (AT-83) and rabbit complement (rabbit Low-Tox M complement; Cedarlane, Hornby, Ontario, Canada). Comparison of percentages of Thy-1.2-expressing cells before and after complement fixation by FACS analysis was employed to examine efficiency of T cell depletion. Thy-1.2 + cells were visualized using FITC-conjugated rat anti-mouse Ig (Becton Dickinson).

To obtain thioglycolate-induced peritoneal macrophages (Mac), 8- to 12-week-old mice were injected i.p. with 2 ml of 4% thioglycolate (Difco, Detroit, MI). At 3–5 days post-injection, peritoneal macrophages were harvested by a standard protocol. Purity of all preparations (>80% Mac-1+, <1% CD3+, <1% B220+) was examined by FACS of cell surface stains using FITC-conjugated anti-Mac-1 mAb (M1/70), biotinylated anti-CD3ε (145-2C11) or biotinylated anti-B220 (RA3-6B2) mAb followed by PE-conjugated streptavidin (PharMingen). Unless otherwise stated, T-depleted splenocytes (T-depl SPL) and macrophages were irradiated with 2000 and 5000 rad respectively prior to culturing with purified T cells.

Fixation of APC to prevent antigen processing was performed using paraformaldehyde (Sigma). T-depl SPL and macrophages were fixed at room temperature for 10–15 min in IMDM containing 1% PFA (4–5 × 10⁶ cells/ml) followed by a 20 min incubation with 100 mM lysine (Sigma) and washes in IMDM.

Proliferative assay

Splenocytes (0.5–1.0 × 10⁶) were cultured in complete medium alone or with various concentrations of LCWE. In specified experiments, T cells were purified by sorting for CD3+ cells from the axillary, inguinal and mesenteric lymph nodes. FACS analysis of surface staining of sorted cell preparations consistently indicated a purity of >98% CD3+. T cells were cultured at 1–1.5 × 10⁵ cells/well with or without 0.5–1 × 10⁶ APC in 96-well plates (Nunc). All cultures were set up in triplicates, incubated at 37°C in a humidiﬁed atmosphere containing 5% CO₂ and harvested at various times as indicated. They were pulsed with 1 μCi/well [3H]thymidine (Amersham Pharmacia Biotech, Baie d’Urfe, PQ, Canada) for the last 16 h of incubation.

Detection of TNF-α levels in culture supernatants

Purified T cells (5 × 10⁵) were co-cultured with 1.5 × 10⁶ irradiated (5000 rad) autologous thioglycolate-induced...
macrophages in 96-well plates (Nunc) with either 5±20 μg/ml LCWE or 2.5 μg/ml SEB (Toxin Technology, Sarasota, FL). Culture supernatants were collected at various time points and assayed for TNF-α levels by ELISA according to the manufacturer (Pierce Endogen, Brockville, Ontario, Canada).

Detection of TNF-α message by RT-PCR

DQ6 Tg mice (4±5 weeks old) were injected i.p. with 500 μl of either PBS alone or 500 μl PBS containing 0.5 μg of LCWE. Splenocytes were harvested at distinct time points during the following 72 h. RT-PCR was performed to detect TNF-α message levels using primers 5'AGCCACGTGAGC-AAACCAACA-3' and 5'TAACGAGACACTTCCCTAACCA-CA-3'. Levels of β-actin message in the same preparation were measured concurrently in the same PCR run using primers 5'GGAGAAGATCTGGACACCC-3' and 5'GG-TGCTTACACCTAGTCTGCC-3'. Relative levels of TNF-α message in each sample were expressed as the ratio of net intensity of the TNF-α band to that of the β-actin using Kodak 1D 2.02 software (Eastman Kodak, Rochester, NY).

Cardiac histology

DQ6 Tg mice (4±5 weeks old) were injected i.p. with 500 μl of PBS containing 0.5 mg LCWE or PBS alone and were sacrificed 28 days later. Cardiac tissues were removed and embedded in the embedding medium OCT (Tissue-Tek, Fort Washington, PA). They were then frozen immediately in liquid nitrogen, transferred and stored at −80°C. Serial sections (6 μm) were stained with hematoxylin & eosin for light microscopy.

Statistical analysis

Student’s t-test and Fisher’s exact test were used to determine statistical difference between experimental groups as appropriate.

Results

LCWE induces marked proliferation of naive T lymphocytes

To investigate whether LCWE can induce proliferation of naive T cells, we cultured splenocytes from C57BL/6 mice with various concentrations of LCWE ranging from 0 to 25 μg/ml and measured their proliferative response at day 3. LCWE was able to induce the proliferation of naive lymphocytes in a dose-dependent fashion, with maximal proliferation at 6.25 μg/ml (Fig. 1A). Splenocytes from lymphocyte-deficient mice (RAG-1−/−) failed to proliferate to LCWE, supporting the hypothesis that T cells were the responding cell population.

A hierarchy exists for presentation of LCWE by different MHC class II molecules

There is a hierarchy in the efficiency of different MHC class II isotypes to present SAg. CD4+ T cells respond more efficiently to presentation of SEB by human MHC class II molecules compared to their murine counterparts (53), with human HLA-DR > HLA-DQ > HLA-DP (54) and murine I-E > I-A (55,56), with the ranking being H-2k and H2d > H-2b > H-2q (5,7,57,58). To investigate whether the response to LCWE exhibits an MHC class II hierarchy, we compared the dose response of splenocytes from either wild-type C57BL/6 or MHC class II-deficient mice. (C) Cultures were set up as in (A) except that the APC were fixed with PFA. Proliferation was assayed 3 days later. Results are the mean values of Δc.p.m. of triplicate wells and are representative of two independent experiments.

Fig. 2. LCWE-induced proliferation requires presentation, but not processing, by MHC class II-expressing APC. (A) T cells (>98% CD3+) from DQ6 Tg mice were cultured with 20 μg/ml LCWE and APC which were either T-depl SPL (<1% CD3+) or Mac (> 80% Mac1+). T-depl SPL and Mac were used at 1.0 × 10⁶ and 0.5 × 10⁵ respectively, and were irradiated prior to use. (B) Purified T cells (1.5 × 10⁶) from C57BL/6 mice were cultured with various concentrations of LCWE and irradiated splenocytes from either wild-type C57BL/6 or MHC class II-deficient mice. (C) Cultures were set up as in (A) except that the APC were fixed with PFA. Proliferation was assayed 3 days later. Results are the mean values of Δc.p.m. of triplicate wells and are representative of two independent experiments.
at a concentration of 6.25 μg/ml LCWE or higher. In a separate experiment using a different preparation of LCWE, higher responses were also observed in cultures of splenocytes from DQ6 Tg mice in comparison to those from BALB/c mice (Fig. 1B). DQ6 splenocytes showed significantly higher proliferation than those from BALB/c mice.

Kinetics of LCWE-mediated T cell responses parallel that of known bacterial superantigens

To investigate the kinetics of LCWE-induced lymphocyte proliferation, we cultured splenocytes from either C57BL/6, BALB/c or DQ6 Tg mice with 5 μg/ml of LCWE and measured their proliferative response from days 1 to 10 (Fig. 1C). Consistent with the hierarchy of different class II MHC molecules to present SAg, splenocytes from DQ6 Tg animals consistently exhibited a more vigorous proliferative response in comparison to lymphocytes from all of the other strains. Similarly, splenocytes from BALB/c mice (I-E + H-2d) proliferated more dramatically than those from C57BL/6 mice (I-A + H-2b). However, splenocytes from all mouse strains tested responded to LCWE with similar kinetics. Proliferation was detectable by day 1, peaked between days 2 and 3, declined, and returned to baseline by day 5. The kinetics of the T cell response to LCWE corresponds directly to that elicited by the prototypic bacterial SAg, SEB.

LCWE-mediated responses require antigen presentation but not processing by APC

SAg stimulate T cells upon binding to the appropriate TCR Vβ chain and to MHC class II molecules expressed on APC (2). To determine whether the T cell response to LCWE requires the presence of APC, we cultured T cells from DQ6 Tg mice with LCWE in the absence of irradiated autologous APC (Fig. 2A). T-depleted SPL (<1% CD3+) or thioglycollate-induced peritoneal macrophages (>80% Mac-1+) were used as APC. When cultured alone, purified T cells failed to proliferate in response to LCWE. In contrast, marked proliferation in response to LCWE was detected when irradiated APC were added. Thymidine uptake detected in these cultures was exclusively due to T cells since cultures containing only irradiated APC did not exhibit any significant proliferation. APC from class II-deficient mice failed to support the proliferation of T cells in response to LCWE (Fig. 2B), consistent with reports that SAg are presented to T cells in the context of MHC class II molecules.

One unique property of SAg is that they do not require antigen processing (5,21,55). To address the question of whether LCWE requires antigen processing, we cultured purified T cells from DQ6 Tg mice with LCWE in the presence or absence of PFA-fixed T-depleted SPL or PFA-fixed thioglycollate-induced macrophages (Fig. 2C). Consistent with earlier data, purified T cells alone did not respond to LCWE. Interestingly, LCWE-induced T cell proliferation was fully restored when PFA-fixed T-depleted SPL or PFA-fixed macrophages were added. Proliferation was comparable to that in cultures using fully functional APC (Fig. 2A and C). Taken together, these data demonstrate that LCWE-induced T cell proliferation requires presentation by MHC class II+ APC, but not processing by the same cells.

T cell response to LCWE is non-classically MHC class II restricted

The response to various SAg has been shown to be non-classically MHC class II restricted (7,9). To investigate the MHC class II restriction of LCWE-induced responses, we cultured T cells from DQ6 Tg mice with LCWE in the presence of either irradiated autologous APC or non-autologous APC which were T-depleted SPL of BALB/c origin (H-2d) (Fig. 3). Mixed lymphocyte reaction in cultures of T cells from DQ6 Tg mice and non-autologous APC was detectable on day 3 with maximal proliferation on day 6 (Fig. 3A). As seen before, T cells from DQ6 Tg mice proliferated vigorously in response to LCWE when cultured with autologous APC (Fig. 3B). Interestingly, DQ6 T cells also responded to LCWE when

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**Fig. 3.** T cell response to LCWE is non-classically MHC restricted. (A) T cells (1.0 × 10^6) from DQ6 Tg mice were cultured with 0.5 × 10^6 irradiated, non-autologous T-depleted SPL from BALB/c mice in medium (mixed lymphocyte reaction cultures). The proliferative response was measured both at 3 and 6 days. Results are the mean values of triplicate wells and are expressed as c.p.m. (B) T cells (1.0 × 10^6) from DQ6 Tg mice were cultured with 0.5 × 10^6 irradiated T-depleted SPL from either DQ6 Tg or BALB/c mice in medium containing 6.5 μg/ml LCWE. In some cultures as indicated, purified T cells from BALB/c mice were cultured with either autologous SPL or SPL from DQ6 Tg mice. Proliferation was assayed after 3 days. Results are the mean values of triplicate wells and are expressed as Δc.p.m. In all cases, the T cell population was >98% CD3+.
cultured with non-autologous APC from BALB/c animals. The levels of proliferation in these cultures were comparable to that of BALB/c T cells cultured with autologous APC and LCWE, and were significantly higher than the basal mixed lymphocyte reaction response measured concurrently (Fig. 3A). Consistent with other known bacterial SAg (7,9), these data demonstrate non-classical MHC class II restriction of LCWE-mediated T cell responses.

**TNF-α production elicited by LCWE parallels that of SEB**

The prototypic bacterial SAg, SEB, induces a characteristic biphasic TNF-α response, which is a T cell-dependent process, with elimination of the second macrophage peak if the T cell component is absent (17,53). To assess LCWE-induced TNF-α production, we cultured T cells from DQ6 Tg animals and irradiated syngenic macrophages with either varying concentrations of LCWE or SEB, and measured TNF-α levels in the supernatants at multiple time points (Fig. 4A and B). TNF-α production in response to LCWE was biphasic and paralleled the response to SEB. In both cases, it was detectable by 30 min post-stimulation, peaked at 1.5 h and declined to basal levels by 10 h. A second TNF-α peak occurred at 18 h, with a decline to baseline by 24 h. This response was dose dependent and, importantly, all LCWE doses tested induced similar kinetics of TNF-α production. LCWE produces the classic footprint of TCR Vβ skewing

The hallmark of SAg stimulation is a skewing of the TCR Vβ repertoire. To investigate whether LCWE preferentially stimulates T cells that express specific TCR Vβ chains, we cultured splenocytes from C57BL/6 (I-A) and DQ6 Tg mice with either LCWE or anti-CD3 plus anti-CD28 antibodies and assayed for surface TCR Vβ expression by FACS on day 3. The data presented in Fig. 5(A and B) are representative of four to six experiments, and show percentages of LCWE-stimulated and anti-CD3 + anti-CD28 antibody-stimulated, CD4+ T cell blast

**Fig. 4.** LCWE-induced TNF-α production mirrors that induced by SEB. Purified T cells (5 x 10^5) from DQ6 Tg mice were cultured with 1.5 x 10^6 autologous, irradiated thioglycolate-induced macrophages and either 5, 10 or 20 μg/ml LCWE, or 2.5 μg/ml SEB. Culture supernatants were collected during a period of 48 h and TNF-α levels were assayed by ELISA. Results are the mean values of duplicate wells and are representative of three independent experiments.

**Fig. 5.** TCR Vβ skewing in response to LCWE. Splenocytes from (A) C57BL/6 or (B) DQ6 Tg mice were cultured with either LCWE or with anti-CD3e + anti-CD28 antibodies. Three days later, cells were collected and stained with FITC-conjugated antibodies against various murine TCR Vβ chains, and either PE-conjugated anti-mouse CD4 or PE-conjugated anti-human CD4 antibody and Cy5–PE-conjugated anti-mouse Thy-1.2 antibody. Percentages of CD4+ cell blasts (based on side scatter and forward cell scatter) expressing specific TCR Vβ families were assayed by FACS analysis. LCWE-stimulated cultures showing significant TCR Vβ expansion in comparison to anti-CD3 and CD28 antibody-stimulated cultures are marked with asterisks (P < 0.05). Each data set is representative of four to six experiments.
subpopulations expressing TCR V\(_b\) families. In both mouse strains, in LCWE-stimulated cultures, the percentages of T cells expressing TCR V\(_b\)2, 4 and 6 demonstrated a significant increase in comparison to pan-TCR stimulation with anti-CD3 + anti-CD28 antibodies or with phorbol myristate acetate + ionomycin (P < 0.05) (data not shown). No other TCR V\(_b\) families assayed responded this way. Lymphocytes from DQ6 Tg animals stimulated with LCWE exhibited the highest increase in absolute numbers of CD4\(^+\) cells expressing TCR V\(_b\)2, 4 and 6, and those from C57BL/6 exhibited the lowest.

Fig. 6. LCWE-induced expansion is followed by deletion of CD4\(^+\) cells expressing reactive TCR V\(_b\) chains. DQ6 Tg mice were injected i.p. with 500 \(\mu\)g LCWE on day 0. On day 3, 5 and 10 post-injection, CD4\(^+\) splenocytes expressing TCR V\(_b\) were assayed by FACS analysis. Shaded histograms represent samples stained with isotype-matched antibodies. All isotype-matched control samples showed similar results. Values indicate percentages of CD4\(^+\) T cells expressing TCR V\(_b\)6. Data are representative of five independent experiments.

Fig. 7. Presence of superantigenic activity in LCWE correlates with induction of coronary arteritis. (A) Splenocytes from DQ6 Tg mice were cultured with 25 \(\mu\)g/ml of three different preparations of LCWE: LCWE A, LCWE B and LCWE C. Proliferation was assayed on days 1–10. Results are the mean values of \(\Delta\)c.p.m. of triplicate wells and are representative of three independent experiments. (B) DQ6 Tg mice (4–5 weeks old) were injected i.p. with 500 \(\mu\)l PBS only, or 500 \(\mu\)l PBS containing 0.5 mg LCWE preparation A or preparation B. Mice were sacrificed at set time points for 72 h following the injection and RT-PCR was performed to detect TNF-\(\alpha\) RNA message from splenocytes. Relative levels of TNF-\(\alpha\) message are expressed as the ratio of net intensity of the TNF-\(\alpha\)-PCR band to that of \(\beta\)-actin in the same samples. Results are the mean values of two to four animals (\(n = 2\) for PBS, \(n = 2\) for LCWE A and \(n = 4\) for LCWE B). On day 28 post-injection, serial sections of coronary artery from mice injected with either (C) PBS, (D) LCWE B or (E) LCWE A were prepared and stained with hematoxylin & eosin. Order of magnification was \(\times 40\) in all panels.
Superantigenic activity leads to coronary vasculitis

This correlates with the hierarchy in the efficiency of different MHC class II molecules to present SAg as discussed previously (7,54–56). Similar findings were observed in the BALB/c mice and with different preparations of LCWE, demonstrating the same superantigenic activity in different preparations (data not shown).

**LCWE-induced T cell proliferation is followed by deletion in vivo**

One hallmark of SAg stimulation is the deletion of T cells expressing reactive TCR Vb families following their activation and proliferation in vivo (13, 19,59,60). To address the question of T cell fate following exposure to LCWE, we injected DQ6 Tg mice with LCWE on day 0, and examined the percentages of splenic CD4 T cells expressing TCR Vb 2, 4 and 6 on days 3, 5 and 10 post-injection. Figure 6 presents data obtained for TCR Vb 6, but similar patterns were observed with TCR Vb 2 and 4. Consistent with earlier observations, the CD4 TCR Vb 6-reactive population was over-represented by day 3 post-injection (6.9%). However, by day 5, this population was reduced to 1.73% and by day 10 to <1%. Non-reactive TCR Vb family-expressing T lymphocytes, such as TCR Vb12+ T cells, neither proliferated nor were deleted. Thus, the classical kinetics of in vivo T cell fate following superantigen stimulation was observed following LCWE injection.

**Superantigenic activity in LCWE correlates with induction of coronary arteritis in mice**

To investigate the relationship between superantigenic activity and coronary arteritis, we compared different preparations of LCWE for superantigenic activity in vitro and examined the coronary arteries for end-organ damage in LCWE-injected mice. In vitro proliferative responses directly correlated with in vivo T cell activation as evidenced by increased total splenocyte numbers and absolute numbers of T cells expressing reactive TCR Vb families (unpublished data). Proliferative responses also correlated with both in vitro and in vivo production of TNF-α. In Fig. 7, LCWE preparation A induced a robust in vitro T cell proliferation as well as in vivo TNF-α production (Fig. 7A and B). Conversely, LCWE preparation B induced minimal proliferation and minimal TNF-α production. More importantly, LCWE preparation A was able to induce an inflammatory response at the coronary artery and LCWE preparation B was not (Fig. 7C and E). We consistently found that only LCWE preparations able to stimulate a vigorous proliferative response and/or TNF-α production were able to induce high incidence of inflammation at the vessel wall. Table 1 summarizes data collected from different LCWE preparations. Our data from 27 LCWE preparations consistently demonstrated that the ability to induce coronary arteritis correlates directly with the presence of superantigenic activity, as measured by T cell proliferative response and cytokine production. The incidence of inflammation at the coronary artery in mice injected with LCWE preparations with SAg activity was significantly greater than that in mice injected with LCWE possessing no SAg activity or injected with PBS alone ($P < 0.001$).

### Table 1. Incidence of coronary arteritis in mice injected with LCWE preparations with or without SAg activity

<table>
<thead>
<tr>
<th>Agent injected</th>
<th>Incidence of coronary arteritis (no. mice affected/no. mice injected)</th>
</tr>
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<tbody>
<tr>
<td>LCWE with SAg activity</td>
<td>71/78</td>
</tr>
<tr>
<td>LCWE with no SAg activity</td>
<td>0/11</td>
</tr>
<tr>
<td>PBS</td>
<td>0/15</td>
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*Experimental mice (4–5 weeks old) were injected i.p. with 500 μl PBS alone, or PBS containing 0.5 mg LCWE from preparations with or without SAg activity, as measured by the ability to induce strong proliferative responses and/or TNF-α production of naive splenocytes. On day 28 post-injection, cardiac tissues were examined for inflammation at the coronary artery. Values were obtained from testing a total of 27 LCWE preparations with SAg activity and three preparations without.

**Discussion**

Bacterial SAg exert their effects over a broad range of clinical settings and remain a major health problem. KD has been added to the growing list of diseases in which SAg are suspected to be of etiologic importance. The nature of the inflammatory response and the processes involved in vascular damage and aneurysm formation are not clearly understood. Cardiac tissue is not readily available from children with KD and even obtaining serial blood samples from children is difficult ethically. Thus, an accurate animal model is required to address the pathogenic mechanisms leading from inflammation to vascular damage and aneurysm formation in KD. LCWE-induced coronary arteritis in mice is currently the best animal model for KD, having similar age dependence, time course, pathology and response to IVIG (43–45). In our study, we have, for the first time, shown that LCWE acts as a SAg whose activity correlates directly with development of disease.

SAg can be broadly divided into two groups—foreign and self-SAg (5). Foreign SAg comprise a family of soluble proteins synthesized by a number of bacteria, of which the best studied is SEB. Self-SAg are common in mice and are products of mammary tumor retroviruses (61). Regardless of the type of SAg, SAg all share unique features that distinguish them from conventional peptide antigens. Firstly, SAg can cause dramatic proliferation of naive, unprimed T lymphocytes in a TCR Vb-specific way. SAg presentation, but not processing, by MHC class II-expressing APC is required. There is a hierarchy in the efficiency of different MHC class II isotypes to present SAg: human HLA-DR > HLA-DQ > HLA-DP, which are better than murine class II, with murine I-E > I-A and H-2k, H-2d > H-2b > H-2q (7,54,62). Our study demonstrated that LCWE possesses all such unique features of SAg. LCWE induced a marked proliferative response in naive lymphocytes. Our observations that purified T cells alone, APC alone or splenocytes from RAG-1−/− mice failed to respond to LCWE support the concept that the immune response to LCWE is T cell dependent and requires presentation by APC, thus excluding the possibility that LCWE possesses a non-specific mitogen, endotoxin or B cell SAg (63,64). When tested for the presence of lipopolysaccharide (E-Toxate kit; Sigma), all
preparations of LCWE tested negative for lipopolysaccharide with the highest detectable concentration of <25 pg/ml. As in the case of T cell responses to SEB (5), the T cell response to LCWE did not require antigen processing since inhibition of antigen processing by PFA fixation of the APC did not influence the response. Presence of MHC II molecules was the requisite feature of APC to support T cell response to LCWE, as MHC class II-deficient APC were not able to present LCWE and elicit a T cell response.

Consistent with observations from other bacterial SAg (14,54,55,58), T cells from human MHC class II (DQ6) Tg mice reacted most vigorously to LCWE and I-E+ mice (BALB/c) LCWE and I-E+ mice (C57BL/6). The DQ6 Tg mice express human CD4 and human HLA-DQ6, but not murine CD4 or CD8, and are phenotypically and functionally normal with respect to Tn cell functions, but have no CD8 cytotoxic functions (46). T cells from these Tg mice are much more sensitive to SEB than wild-type mice and respond to similar concentrations of SEB to which human cells react. In vivo, they succumb to normally sublethal amounts of SEB. This SAg supersensitivity was also reflected in LCWE-induced coronary artery disease, with a more intense immune response both systemically and at the vessel wall found in the DQ6 Tg mice. Superantigenic activity in LCWE was also evidenced by the kinetics of the T cell proliferative response which mirrored that of the prototypic SAg, SEB.

Consistent with other known SAg, the response to LCWE was not classically MHC class II restricted. T cells from DQ6 Tg mice responded to LCWE in the presence of either syngenic or non-syngenic APC from BALB/c (H-2d, I-Ed) mice. After taking the mixed lymphocyte reaction into account, the proliferation of T cells from DQ6 Tg mice in response to LCWE presented by DQ6 APC was always higher than that in response to LCWE presented by H-2d APC. This supports the earlier finding that human MHC class II molecules are more effective at presenting bacterial SAg than murine MHC class II molecules (55,56).

T cell effector functions in response to LCWE including cytokine production are also reminiscent of a bacterial SAg. Maximal TNF-α production in response to LCWE occurred within the first hour of stimulation, followed by a second peak of production at 18 h. This biphasic response is classic for SAg and is consistent with earlier reports that TNF-α mRNA expression after SEB stimulation in BALB/c mice was biphasic, with an early (30 min) post-injection peak attributed to T cells and a subsequent peak identified as a T cell-dependent macrophage-mediated event (17,53). The observations that the kinetics of both LCWE-induced proliferation and cytokine production mirror that induced by SEB further support the presence of superantigenic activity in LCWE.

Recognition of SAg is mainly dependent on the TCR Vb chain. Since the relative number of Vb genes is limited, many T cells within an individual will bear a particular Vb element and, therefore, a given SAg is capable of interacting with or activating a large fraction of T cells expressing the reactive TCR Vb chain (5–30%) resulting in deviation of the TCR Vb profile. SAg-induced responses are characterized by an initial activation and proliferation of T cells expressing specific TCR Vb families, followed by the deletion of these reactive T cells (5,15). The consistent skewing of the TCR Vb response to LCWE is the strong evidence of superantigenic activity in LCWE. In contrast to pan-stimulation by anti-CD3 and anti-CD28 antibodies, which activates all TCR Vb families, T cells expressing TCR Vb2, 4 and 6 families were over-represented after stimulation with LCWE. These reactive TCR Vb families remained the same in all mouse strains tested, as well as with different preparations of LCWE. TCR Vb chain bias in response to LCWE also follows an MHC class II hierarchy. DQ6 Tg animals exhibited the greatest increase in absolute numbers of reactive T cell families. Our data also demonstrated the characteristic fate of in vivo SAg-activated T cells with proliferation and expansion of reactive TCR Vb families followed by deletion. The absence of these cells by day 7 following LCWE stimulation parallels the decline in proliferative response to baseline seen in vitro.

The same TCR Vb2, 4 and 6 families were consistently over-represented in all mouse strains tested in response to different preparations of LCWE. Our observations demonstrate that the same superantigenic activity is present in different preparations and thus argue against the possibility that SAg activity of LCWE was due to contamination with other bacterial SAg. In addition, LCWE appears to mediate responses in T cells expressing a unique combination of TCR Vb families which are not mediated by any single known bacterial SAg.

Different LCWE preparations showed marked variation in the ability to induce coronary arthritis. This is reflected in variation in the magnitude of responses induced by various LCWE preparations. It is the potency of responses elicited by different LCWE preparations that varied. Some preparations were able to initiate a strong response at lower doses. Others required higher concentrations. Some did not possess superantigenic activity. However, it should be noted that the patterns of response mediated by those exhibiting superantigenicity are the same: same kinetics of proliferation, TNF-α production, and TCR Vh family expansion and deletion. The characteristic polysaccharide of L. casei is rhamnosyl-(1→3)N-acetyl-galactosamine and rhamnose is the specific antigen determinant of L. casei and other Lactobacilli bacteria. Currently, LCWE is quantitated and expressed as carbohydrate (i.e. rhamnose) concentration. The carbohydrate content, however, may not be the best measure of the ability to generate an immune response leading to inflammatory reaction at the coronary vessel wall.

It is important to note that the ability of different LCWE preparations to induce T cell proliferation and TCR Vh skewing correlated with the ability of the same preparation to induce TNF-α production. The presence of mononuclear infiltrate and invasion of murine cardiac tissue have been demonstrated to lead to coronary arteritis and heart lesions that pathohistologically mimic those of KD (43,44,67). In all these studies, the presence of the cellular infiltrates at the coronary artery was the earliest indication of coronary arteritis which progressed to aneurysm formation. In our study, we showed that only LCWE preparations that were able to induce TCR Vh production and TNF-α production both in vitro and in vivo were able to produce inflammation at the coronary artery. Perivascular inflammation leads to parvasculitis, and subsequently dilation and aneurysm formation in our animal model, identical to KD in children. Thus, the superantigenic activity of LCWE is directly related to its ability to induce coronary artery disease.
Superantigenic activity leads to coronary vasculitis

In summary, our data shows that a novel SAg is present in LCWE. This SAg has all the hallmarks of a classic bacterial SAg: (i) marked T cell proliferation in naïve cells, (ii) non-classical MHC restriction with a hierarchy of differing class II MHC ability to present it, (iii) the requirement for antigen presentation, but not processing, (iv) the characteristic footprint of TCR Vβ skewing and (v) the disappearance of reactive T cells following initial proliferation which typifies the fate of SAg-reactive T cells in vivo. More importantly, superantigenic activity correlated directly with development of coronary arteritis. It has been suggested that an infectious agent is the cause or at least an inciting agent in KD, and that the disease fits in the spectrum between an infectious disease and a true autoimmune disease (26,27). LCWE may act as an inciting agent that activates and expands autoreactive T cell subpopulations which lead to coronary arteritis. KD is the most common multisystem vasculitis of childhood. Improved understanding of the mechanisms leading from immune activation to localized coronary artery disease will influence treatment and long-term morbidity/mortality in children with KD.

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>DQ6Tg mice</td>
<td>mice expressing human CD4 and DQ6 transgenes in an endogenous mouse CD4- and CD8-deficient background</td>
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<tr>
<td>KD</td>
<td>Kawasaki disease</td>
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<td>IVIG</td>
<td>i.v. Ig</td>
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<td>LCWE</td>
<td>Lactobacillus casei cell wall extract</td>
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<td>Mac</td>
<td>thioglycolate-induced peritoneal macrophages</td>
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<tr>
<td>PE</td>
<td>phycoerythrin</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>SAg</td>
<td>superantigen</td>
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<tr>
<td>SEB</td>
<td>staphylococcal enterotoxin B</td>
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<tr>
<td>T-depl SPL</td>
<td>T-depleted splenocytes</td>
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<td>TSST</td>
<td>toxic shock syndrome toxin</td>
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References

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