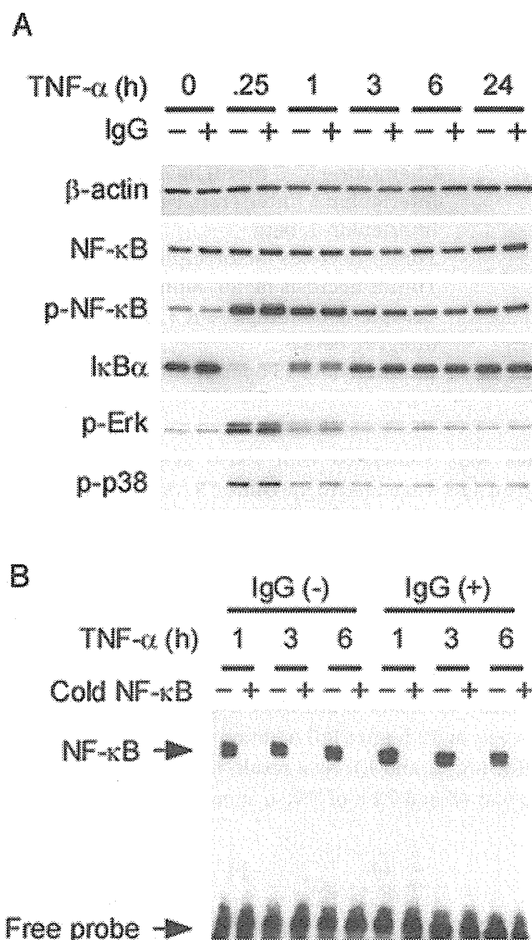

Figure 2. High-dose IgG did not affect TNF-α-mediated activation of NF-κB or MAPK. HCAECs were treated with 20 mg/mL IgG overnight and then stimulated with 10 ng/mL TNF-α for the indicated periods. (A) Whole-cell lysates prepared at the indicated time points after treatment were subjected to western blot analysis of expression of β-actin (as a loading control), NF-κB p65, phospho-NF-κB p65 (Ser536), IκBα, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) and phospho-p38 MAPK (Thr180/Tyr182). (B) Nuclear extracts from the indicated time points after treatment were subjected to EMSA assay to determine NF-κB activity. A 200-fold excess amount of unlabeled probe (cold NF-κB) was added to compete for specific binding of NF-κB-DNA. Data shown are representative of three experiments performed.

at 24 h were comparable to those at 24 h without IgG (Fig. 3B and D). These results indicate that IgG immediately exerts its inhibitory effects on cytokine production by HCAECs, even if added after TNF-α stimulation.

Involvement of C/EBPδ in the anti-inflammatory effects of high-dose IgG in HCAECs

To gain an insight into the molecular mechanisms of the effects of high-dose IgG, we reanalyzed the microarray data for HCAECs to find genes that were downregulated after IgG addition with kinetics similar to G-CSF mRNA. The genes that showed a similar expression profile to G-CSF mRNA, with a coefficient of correlation higher than 0.95, are presented in Supporting Information Fig. 4. Among them, we found that C/EBPδ, an NF-κB inducible transcription factor, was suppressed by IgG with the same kinetics as G-CSF mRNA. Previous studies reported that C/EBP binding sites located within the promoter regions of *G-CSF* and *IL-6* were critical for their transcriptional activation [20–22]. Therefore, as shown in Fig. 4A, B, and C, we examined the precise kinetics of C/EBPδ mRNA expression using the same experimental protocols as in Fig. 1A–F, Fig. 1 G–L, and Fig. 3, respectively. C/EBPδ mRNA was transiently induced by TNF-α, peaking at 2 h after stimulation and then returning to its basal level (Fig. 4A). It was later reinduced and continued to increase until at least 48 h after stimulation with TNF-α. High-dose IgG had no effect on the early transient induction of C/EBPδ mRNA, whereas it potently suppressed its reinduction, as seen in the results for IL-6 mRNA (Fig. 1C). More importantly, high-dose IgG did not suppress induction of C/EBPδ mRNA by IL-1β (Fig. 4B), as seen in such cytokine genes as G-CSF, IL-6 and IL-1β by IL-1β (Fig. 1G–L).

Western blot analysis of nuclear fractions from HCAECs showed that C/EBPδ protein gradually decreased starting at 3 h after IgG treatment compared with cells not exposed to IgG treatment (Fig. 4D). We further examined C/EBP binding activity to DNA by EMSA assay. TNF-α-induced C/EBP binding activity in the nuclear extracts of HCAECs was also reduced after IgG treatment and became undetectable after 24 h (Fig. 4E). Nuclear extracts prepared from HCAECs without IgG at 3, 6, and 12 h were pooled and used for supershift assay, which showed that nuclear C/EBP-binding protein after TNF-α stimulation consisted mainly of C/EBPδ, and partially C/EBPβ, but not C/EBPα (Fig. 4F).

$F(ab')_2$, but not the Fc fragment of IgG, had the same anti-inflammatory effects as intact IgG

We also compared the inhibitory activities of intact IgG and its $F(ab')_2$ and Fc fragments. HCAECs were pretreated overnight with the same molarity of IgG (5 mg/mL), Fc (1.67 mg/mL), or $F(ab')_2$ (3.33 mg/mL) and then stimulated with 10 ng/mL TNF-α for 48 h. The $F(ab')_2$ fragment suppressed TNF-α-induced G-CSF, IL-6, and IL-1β gene expression as effectively as intact IgG, with the same kinetics (Fig. 5). On the other hand, the Fc fragment of IgG only partially suppressed those gene expressions.

Discussion

In the present study, we examined the effects of high-dose IgG on cultured HCAECs, which are frequently injured in the pathogenesis of KD. We first scanned the microarray data and found that expression of some proinflammatory cytokines was selectively inhibited. Importantly, the most highly ranked genes, such as G-CSF, IL-1β, and IL-6 (Table 1), are known to be crucially associated with the pathogenesis of acute KD. In addition, these in vitro findings are entirely compatible with our recent in vivo evidence showing that

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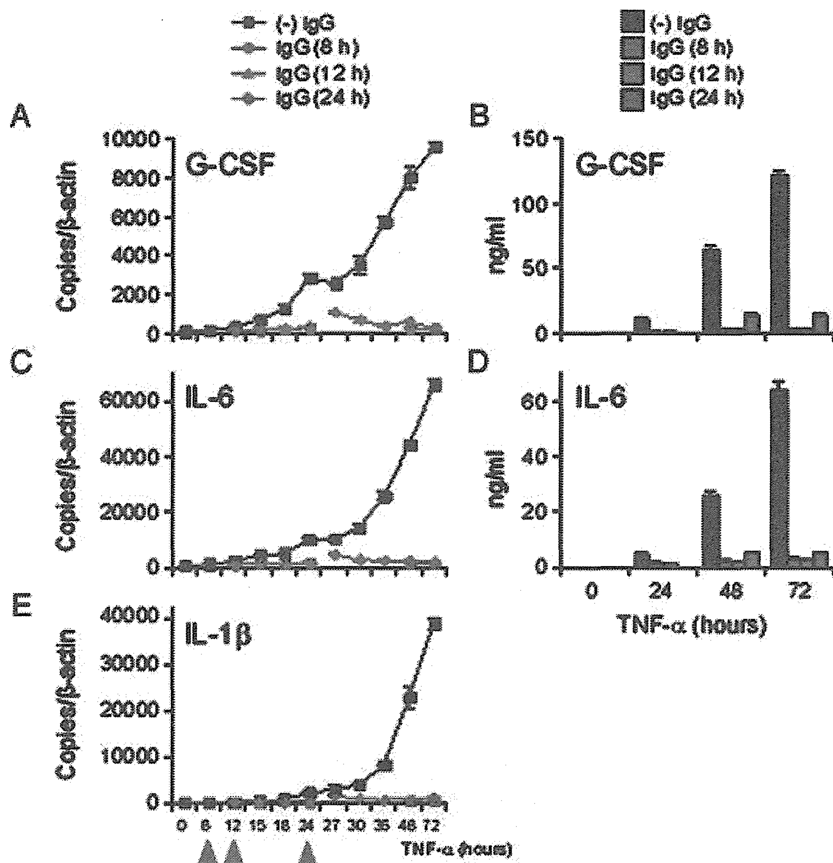

Figure 3. High-dose IgG rapidly inhibited cytokine production by HCAECs. HCAECs were stimulated with 10 ng/mL TNF-α at 0 h and then treated with 20 mg/mL IgG at 8 h (red), 12 h (green), and 24 h (yellow) after TNF-stimulation. The levels of mRNA for (A) G-CSF, (C) IL-6, and (E) IL-1β were examined by qPCR. Protein concentrations of (B) G-CSF and (D) IL-6 in the culture supernatants were measured by ELISA. Data are shown as the mean ± SD of triplicate samples and are representative of two experiments performed.

elevated serum G-CSF and IL-6 levels in pre-IVIG patients with KD were markedly decreased after IVIG therapy, especially in responsive patients [23]. Although it is not yet clear which type of cells are the major source of these proinflammatory cytokines in acute KD patients, tissue cells such as endothelial cells are likely candidates. Indeed, vascular endothelial cells are known to be major cellular sources of G-CSF under inflammatory conditions [24,25]. Furthermore, the agreement from both our in vitro and in vivo results suggests that some of IVIG's clinical effects on KD patients may be due to its direct suppressive effects on the coronary endothelium, which is the most important site of complications in KD.

On the contrary, high-dose IgG did not suppress induction of such cytokine genes as G-CSF, IL-6, and IL-1β after IL-1β stimulation (Fig. 1G–L). Currently, the most important clinical issue regarding IVIG therapy is that about 15% of KD patients do not respond. They develop CALs more frequently than responsive patients [26], and the cause of their nonresponsiveness remains unclear. However, previous studies by others suggested an association between IL-1β secretion and IVIG treatment in KD patients [27,28]. More recently, Fury et al. demonstrated increased transcript abundance for IL-1 pathway genes in IVIG-resistant patients with KD [29]. Weng et al. showed that IL-1β polymorphism was associated with initial IVIG treatment failure in Taiwanese children with KD [30]. Thus, higher IL-1β secretion and/or an IL-1β-mediated signaling pathway may be involved in the IVIG resistance in KD patients, and additional therapy by IL-1β blockade may help control the inflammation in the current IVIG-resistant patients with KD.

Previously, several papers reported on the effects of IVIG on human umbilical vein endothelial cells (HUVECs) [31,32]. Macmillan et al. demonstrated that IVIG completely inhibited neutrophil adhesion to HUVECs stimulated with IL-1α. On the contrary, they also showed that IVIG had no effect on adhesion when IL-1β or TNF-α were the activating cytokines [31]. These findings are compatible with our data that high-dose IgG had no effect on TNF-α-induced ICAM-1 expression in HCAECs (data not shown). On the other hand, Xu et al. [32] found that IVIG downregulated the expression induced by both TNF-α and IL-1β of mRNA coding for some proinflammatory molecules, including ICAM-1, IL-6, and IL-1β. The discrepancy among these different studies may be due to the difference in the IgG concentrations used: Macmillan et al. [31] and we used 15 mg/mL and 20 mg/mL, respectively, while Xu et al. [32] used 40 mg/mL. Importantly, our previous work [33] and that of others [34] demonstrated that the responses to cytokines and growth factors differ among various endothelial cell types depending on their origins. We believe that using HCAECs — which is a major lesion site in the pathogenesis of KD — rather than HUVECs, to examine the effects of IVIG is the most significant advantage of the present study.

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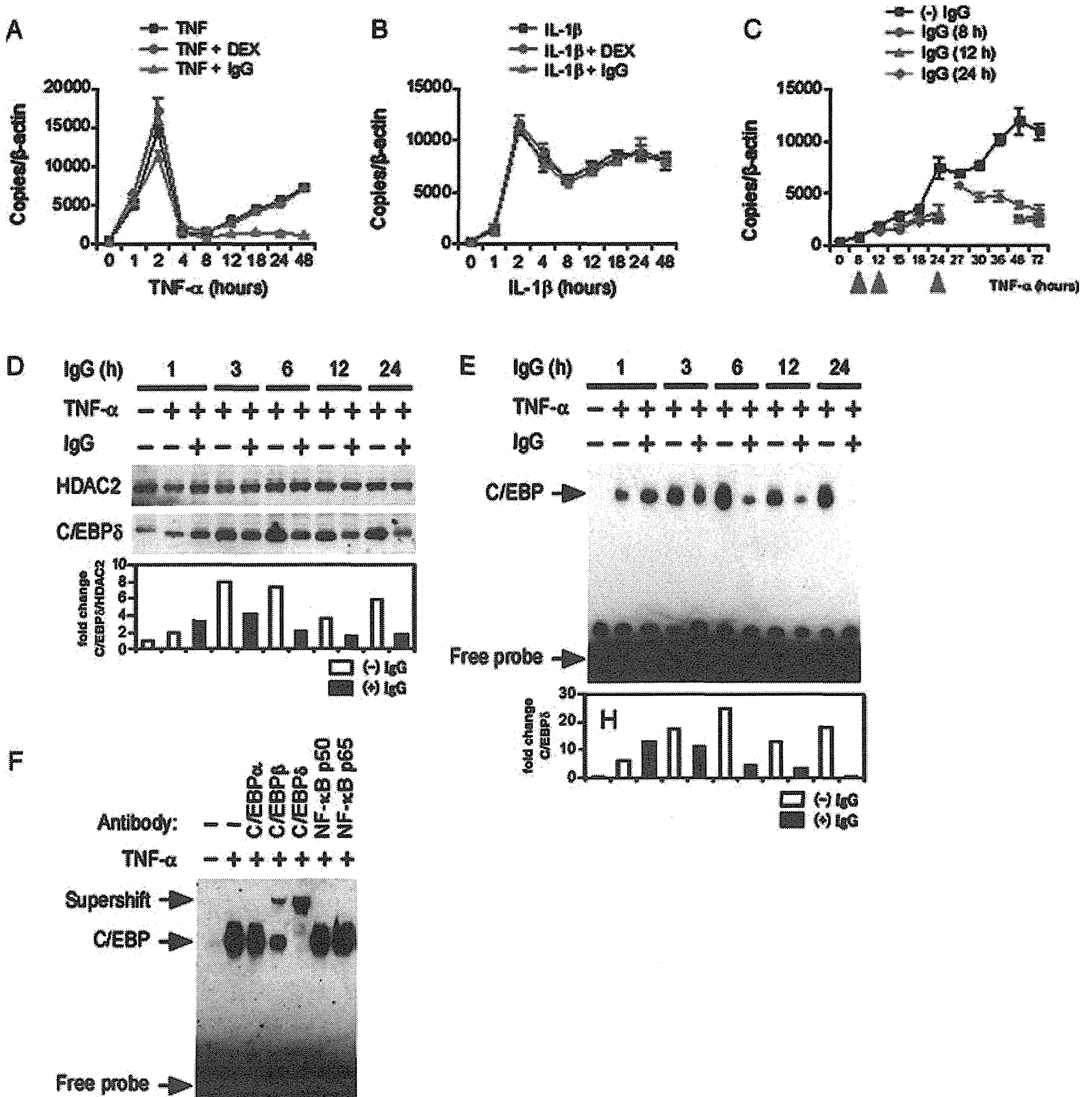

Figure 4. Effects of high-dose IgG on the expression and function of C/EBPδ in HCAECs. (A) HCAECs were treated with 10 ng/mL TNF-α in the presence and absence of 100 nM dexamethasone or 20 mg/mL IgG for the indicated time periods. (B) HCAECs were treated with 10 ng/mL IL-1β in the presence and absence of 100 nM dexamethasone or 20 mg/mL IgG for the indicated time periods. (C) HCAECs were stimulated with 10 ng/mL TNF-α at 0 h and then treated with 20 mg/mL IgG at 8 h (red), 12 h (green), and 24 h (yellow) after TNF-α-stimulation. (A–C) The levels of mRNA for C/EBPδ were examined by qPCR. Data are shown as the mean ± SD of triplicate samples and are representative of three experiments performed. (D–F) HCAECs were stimulated with 10 ng/mL TNF-α overnight and then treated with 20 mg/mL IgG for the indicated periods. (D) Five micrograms of nuclear extract prepared at each of the indicated time points after treatment were subjected to Western blot analysis of the expression of HDAC2 (as a loading control) and C/EBPδ. The fold change in nuclear C/EBPδ protein was determined using an Image J 1.43m and normalized to the respective HDAC2 level (lower graph). (E) Three micrograms of nuclear extract prepared at each of the indicated time points after treatment were subjected to EMSA assay to determine the C/EBP activity. Relative intensities were analyzed with an Image J 1.43 m (lower graph). (F) The nuclear extracts prepared from HCAECs without IgG at 3, 6, and 12 h were pooled and used for supershift assay using Abs for C/EBPα, C/EBPβ, C/EBPδ, NF-κB p50, or NF-κB p65. (D–F) Data shown are representative of two experiments performed.

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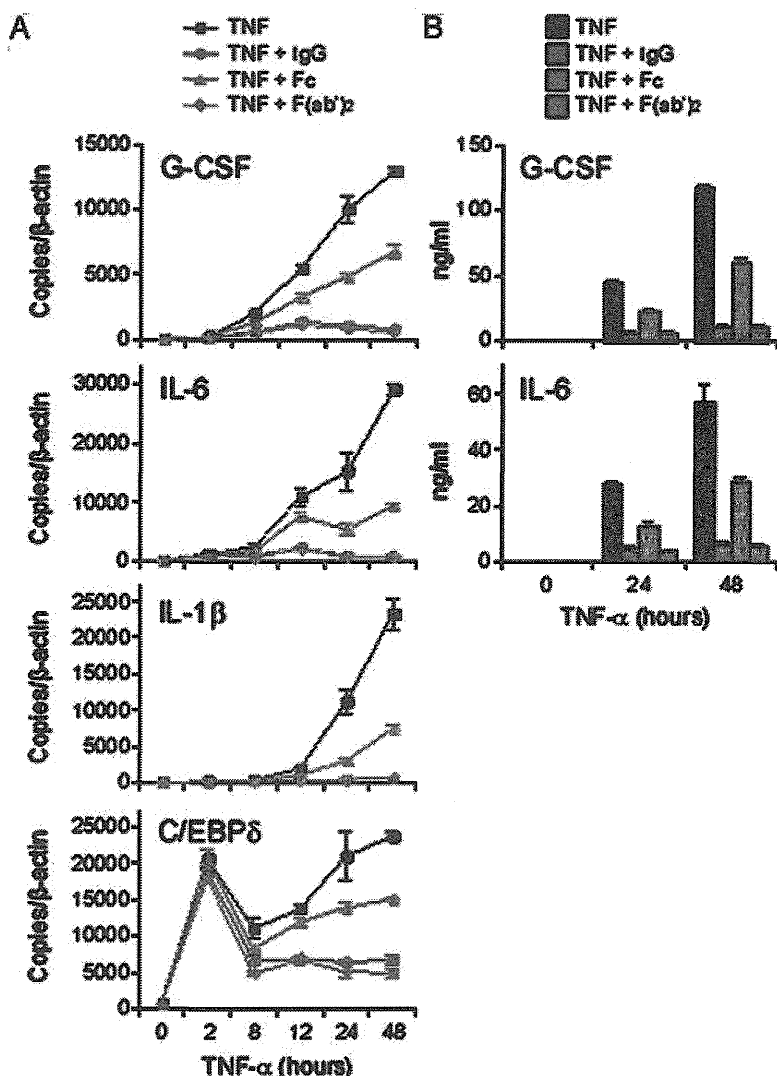

Figure 5. Effects of F(ab')$_2$ and Fc fragments of IgG on the TNF-α-induced cytokines and C/EBPδ in HCAECs. HCAECs were pretreated overnight with the same molarity of IgG (5 mg/mL), Fc (1.67 mg/mL), or F(ab')$_2$ (3.33 mg/mL) and then stimulated with 10 ng/mL TNF-α for the indicated periods. (A) The levels of mRNA for G-CSF, IL-6, IL-1β, and C/EBPδ were examined by qPCR. (B) Protein concentrations of G-CSF and IL-6 in the culture supernatants were measured by ELISA. Data are shown as the mean ± SD of triplicate samples and are representative of three experiments performed.

Compared to the potent anti-inflammatory effects of high-dose IgG, dexamethasone had a very limited effect on TNF-α-induced expression of these cytokines in HCAECs (Fig. 1). Previously, we demonstrated that dexamethasone treatment only weakly attenuated TNF-α-induced GRO-α and IL-8 production by human pulmonary microvascular endothelial cells [35]. In observations to date, corticosteroids have been much less effective on vascular endothelial cells than on other tissue cells such as epithelial cells [35–38]. These findings correspond to the clinical fact that while corticosteroids are the most commonly used anti-inflammatory drugs for treating various inflammatory diseases, their role in the treatment of KD remains controversial [39].

High-dose IgG exerted no influence on TNF-α-mediated early signaling events, including NF-κB activation (Fig. 2), suggesting that high-dose IgG's anti-inflammatory effects were not due to neutralization of exogenously added recombinant TNF-α. In contrast, Ichiyama et al. previously demonstrated that high-dose IgG partially inhibited TNF-α-induced activation of NF-κB in HCAECs [40]. We cannot fully explain this discrepancy between these different studies. However, one possibility is the difference in the TNF-α concentrations used: we used 10 ng/mL of TNF-α, while they used 2 ng/mL. Alternatively, the discrepancy may be due to the difference in culture conditions: we used EGM-2MV BulletKit supplemented with 5% FCS, while they used serum-free EGM-2 medium.

On the other hand, IgG completely suppressed induction of such cytokine genes as G-CSF, IL-6, and IL-1β later than 12 h after TNF-α stimulation (Fig. 1). These results suggest that a positive feedback mechanism, which accelerates expression of G-CSF, IL-6, and IL-1β mRNA in HCAECs, could be a specific target of IgG-regulated suppression. Importantly, we found that C/EBPδ, an NF-κB-inducible transcription factor, was also suppressed by IgG-treatment with the same kinetics as G-CSF (Fig. 4 and Supporting Information). Previous reports indicated that C/EBP binding sites in the promoters of *G-CSF* and *IL-6* are critical for their transcriptional activation [20–22]. Furthermore, Litvak et al.

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[41] recently demonstrated that C/EBPδ acted as an amplifier of LPS-induced NF-κB responses, resulting in substantially increased IL-6 production by murine macrophages. Our results are in agreement with their findings of a C/EBPδ-regulated positive-feedback mechanism, and we hypothesize as follows. TNF-α rapidly induces C/EBPδ and its target genes, such as IL-6 and G-CSF (initial phase). Subsequently, these cytokines further promote C/EBPδ expression, in an autocrine manner, resulting in an inflammation enhancing cycle (positive feedback amplification phase). We speculate that high-dose IgG specifically blocks the C/EBPδ-regulated inflammatory enhancing cycle in the positive feedback amplification phase, because our data indicated that high-dose IgG was able to specifically inhibit the late phase induction of the cytokine genes (Fig. 1A, C, and E) as well as C/EBPδ (Fig. 4A). On the contrary, high-dose IgG did not affect the IL-1β-induced expression of mRNA for either the cytokines (Fig. 1G–L) or C/EBPδ (Fig. 4B), suggesting that C/EBPδ plays a key role in the anti-inflammatory mechanisms of high-dose IgG in TNF-α-, but not IL-1β-, activated HCAECs. Clarifying the differences in the molecular pathways between TNF-α- and IL-1β-mediated activation of HCAECs will help us to further explore the mechanisms underlying IgG-regulated anti-inflammatory effects.

We also found that the inhibitory effects of intact IgG on HCAECs were mainly exerted via its $F(ab')_2$ fragment, and far less via its Fc fragment (Fig. 5). Although Fig. 2 suggests that high-dose IgG's anti-inflammatory effects were not due to neutralization of exogenously added recombinant TNF-α, the possibility remains that exogenous IgG exerts its potent anti-inflammatory effects via its $F(ab')_2$ fragment by neutralizing an autocrine factor or blocking an endogenous signaling molecule. This should be clarified in the near future.

In conclusion, we found that high-dose IgG specifically and completely inhibited accelerated expression of KD-related cytokines by HCAECs in the late response to TNF-α, but not to IL-1β, and it did not inhibit TNF-α-mediated early signaling events such as NF-κB activation. Our results also suggest that C/EBPδ, an NF-κB-inducible transcription factor, may play a key role in the anti-inflammatory mechanisms of high-dose IgG. Direct anti-inflammatory effects of high-dose IgG on coronary artery endothelial cells might contribute to the markedly reduced incidence of CALs brought about by IVIG therapy in KD patients. We anticipate that an improved understanding of the precise molecular mechanisms underlying the effects of high-dose IgG on coronary artery endothelial cells will contribute to development of new drug for KD that are more effective even for currently IVIG resistant patients.

Materials and methods

Reagents

Recombinant human TNF-α and IL-1β were purchased from PeproTech (Rocky Hill, NJ, USA). Dexamethasone was purchased from Sigma (St. Louis, MO, USA). All the IgG agents used in this study (intact IgG; Venoglobulin IH, Fc fragment, and F(ab')2 fragment) were provided by Benesis Corporation (Osaka, Japan).

Primary human endothelial cell culture and treatment

HCAECs were purchased from Lonza (Walkersville, MD, USA) and maintained exactly as recommended by the manufacturer by using an EGM-2MV BulletKit (Lonza). We obtained four different lots from individual donors, and all data in this study were reproducible among the different lots of HCAECs. HCAECs were stimulated with 10 ng/mL TNF-α overnight (about 18 h) prior to addition of 20 mg/mL of IgG to the culture. The IgG used in this study is clinically used to treat KD patients (Venoglobulin IH), and the final concentration of IgG (20 mg/mL) is approximately equivalent to the blood IgG level after IVIG therapy (2 g/kg). In some experiments, HCAECs were simultaneously treated with TNF-α and IgG, or were treated with 20 mg/mL of IgG overnight prior to stimulation with 10 ng/mL TNF-α. All the experiments described in this study were performed using second- or third-passage cells in 70–80% confluent monolayers unless otherwise noted.

RNA amplification and GeneChip expression analysis

Gene expression profiles were examined using the Human Genome U133 Plus 2.0 array (GeneChip; Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. Briefly, double-stranded cDNA was synthesized from 2 μg of total RNA using a One-Cycle cDNA Synthesis kit (Affymetrix). The cDNA was subjected to in vitro transcription using an IVT Labeling kit (Affymetrix) and hybridized with an HG-U133 Plus 2.0 array. The fluorescence intensity of each transcript was quantified using an Affymetrix GeneChip Scanner 3000 (Affymetrix), and the expression value was determined using GeneChip Operating Software (GCOS) (Affymetrix). The results of the microarray analysis can be found on our web site a (http://www.nch.go.jp/imal/GeneChip/HCAEC.htm).

The data were further analyzed with GeneSpring GX version 7.3 software (Agilent Technologies, Santa Clara, CA, USA). Each array was normalized (mean-centered) to the median intensity array. A one-way ANOVA analysis and unsupervised hierarchical clustering using Pearson's correlation were performed using GeneSpring GX version 7.3 software.

qPCR

Total RNA extraction, cDNA synthesis, and qPCR were performed as previously described [33]. Primer sets for five genes were synthesized at Fasmac (Kanagawa, Japan): G-CSF (sense, 5′-TGC TTA GAG CAA GTG AGG AAG ATC-3′; antisense, 5′-GCA CAC TCA CTC ACC AGC TTC T-3′), IL-6 (sense, 5′-CAA TAA CCA CCC CTG ACC

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CA-3′; antisense, 5′-GCG CAG AAT GAG ATG AGT TGT C-3′), IL-1β (sense, 5′-AAC TGA AAG CTC TCC ACC TCC AG-3′; antisense, 5′-CCC AAG GCC ACA GGT ATT TTG-3′), C/EBPδ (sense, 5′-GGT GCC CGC TGC AGT TT-3′; antisense, 5′-CTC GCA GTT TAG TGG TGG TAA GTC-3′), and β-actin (sense, 5′-CCC AGC CAT GTA CGT TGC TAT-3′; antisense, 5′-TCA CCG GAG TCC ATC ACG AT-3′). To determine the exact copy numbers of the target genes, quantified concentrations of the purified PCR products of G-CSF, IL-6, IL-1β, C/EBPδ, and β-actin were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of the total RNA samples were used for each qPCR. The mRNA expression levels were normalized to the β-actin level in each sample.

ELISA

The concentrations of the G-CSF and IL-6 proteins in cell-free supernatants were measured with specific ELISA kits (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions.

Nuclear and whole cell extracts

To prepare nuclear extracts from HCAECs, cells were seeded into 6-well plates at 1×10^5 cells/well and cultured until subconfluent (2 or 3 days). The cells were then treated with 20 mg/mL IgG overnight prior to stimulation with 10 ng/mL TNF-α (see Fig. 2B) or stimulated with 10 ng/mL TNF-α overnight prior to treatment with 20 mg/mL IgG (see Fig. 4D–F) for the indicated time periods. Nuclear fraction of HCAECs was extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL, USA) in accordance with the manufacturer's instructions. The protein concentration of each sample was determined with a BCA protein Assay Reagent Kit (Thermo Scientific). To prepare whole cell lysates of HCAECs, cells were seeded into 12-well plates at 5×10^4 cells/well and cultured until subconfluent (2 or 3 days). The cells were then treated simultaneously with 10 ng/mL TNF-α and 20 mg/mL IgG (see Fig. 1F) or with 10 ng/mL IL-1β and 20 mg/mL IgG (see Fig. 1L) or treated with 20 mg/mL IgG overnight prior to stimulation with 10 ng/mL TNF-α (see Fig. 2A) for the indicated time periods. Whole-cell lysates were extracted with 150 μL NuPAGE sample buffer (Invitrogen, Carlsbad, CA, USA) containing 5% 2-mercaptoethanol and lysed by sonication.

Western blotting

Equal amounts of whole cell lysates (see Fig. 1F and L, and Fig. 2A) or 5 μg of nuclear extracts (see Fig. 4D) were separated by SDS-PAGE (5–15% Ready Gels J; Bio-Rad, Hercules, CA, USA) gel electrophoresis and transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks, mini; Invitrogen). Immunoblotting was performed using the following Abs in accordance with the manufacturers' instructions: clone 2805, mouse mAb for human IL-1β (R&D Systems); clone AC-15, mouse mAb for β-actin (Sigma); clone C22B4, rabbit mAb for NF-κB p65; clone 93H1, rabbit mAb for phosphor-NF-κB p65 (Ser536); clone L35A5, mouse mAb for IκBα; clone D13.14.4E, rabbit mAb for phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204); rabbit polyclonal Ab for phospho-p38 MAPK (Thr180/Tyr182); rabbit polyclonal Ab for HDAC2 (Cell Signaling Technology, Danvers, MA, USA); and rabbit polyclonal Ab for C/EBPδ (M-17) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

EMSA and supershift EMSA

3′-biotin labeled oligonucleotides containing the consensus sequence for the NF-κB binding site, C/EBP binding site and their complementary fragments were synthesized at Fasmac. The oligonucleotides used in this study were as follows: NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; and C/EBP, 5′-TGC AGA TTG CGC AAT CTG CA-3′. They were annealed to generate double-stranded probes. EMSA and supershift EMSA assays were performed using a LightShift Chemiluminescent EMSA kit (Thermo Scientific). Briefly, 3 μg of nuclear extract was incubated with 20 fmol of biotinylated probes at room temperature for 20 min. For NF-κB-binding assay, a 200-fold excess amount of unlabeled probe (4 pmol) was added to compete for specific binding of NF-κB DNA. After the reaction, the DNA-protein complexes were subjected to 5% polyacrylamide gel electrophoresis (Bio-Rad) and transferred to a nylon membrane (Biodyne B, Thermo Scientific). The membrane was then immediately cross-linked for 15 min on a UV transilluminator equipped with 312 nm bulbs. Chemiluminescent detection was performed in accordance with the manufacturer's instructions. Relative intensities were analyzed using an Image J 1.43m. For supershift EMSA assay, the following Abs were added to the reaction mixture (2 μL/reaction): goat polyclonal Ab for C/EBPα (C-18); rabbit polyclonal Ab for C/EBPβ (C-19); rabbit polyclonal Ab for C/EBPδ (M-17); rabbit polyclonal Ab for NF-κB p50 (NLS) (Santa Cruz Biotechnology); and rabbit mAb for NF-κB p65; clone 93H1 (Cell Signaling Technology).

Acknowledgements:: We thank Naomi Wada of the Department of Allergy and Immunology, the National Research Institute for Child Health and Development, for her excellent technical assistance. This work was supported by a grant from the National Center for Child Health and Development (22A-1) and a Research Grant from the Japan Kawasaki Disease Research Center.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 Misra, N., Bayary, J., Dasgupta, S., Ephrem, A., Huyen, J. P., Delignat, S., Hassan, G. et al., Intravenous immunoglobulin and dendritic cells. *Clin. Rev. Allergy Immunol.* 2005. **29**: 201–205.

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2 **Ballow, M.**, The IgG molecule as a biological immune response modifier: mechanisms of action of intravenous immune serum globulin in autoimmune and inflammatory disorders. *J. Allergy Clin. Immunol.* 2011. **127**: 315–323.

3 **Basta, M., Van Goor, F., Luccioli, S., Billings, E. M., Vortmeyer, A. O., Baranyi, L., Szebeni, J. et al.**, F(ab)'2-mediated neutralization of C3a and C5a anaphylatoxins: a novel effector function of immunoglobulins. *Nat. Med.* 2003. **9**: 431–438.

4 **Kazatchkine, M. D. and Kaveri, S. V.**, Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. *N. Engl. J. Med.* 2001. **345**: 747–755.

5 **Samuelsson, A., Towers, T. L. and Ravetch, J. V.**, Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science* 2001. **291**: 484–486.

6 **Ravetch, J. V. and Bolland, S.**, IgG Fc receptors. *Annu. Rev. Immunol.* 2001. **19**: 275–290.

7 **Ephrem, A., Chamat, S., Miquel, C., Fisson, S., Mouthon, L., Caligiuri, G., Delignat, S. et al.**, Expansion of CD4+CD25+ regulatory T cells by intravenous immunoglobulin: a critical factor in controlling experimental autoimmune encephalomyelitis. *Blood* 2008. **111**: 715–722.

8 **Maddur, M. S., Vani, J., Hegde, P., Lacroix-Desmazes, S., Kaveri, S. V., Bayry, J.**, Inhibition of differentiation, amplification, and function of human TH17 cells by intravenous immunoglobulin. *J. Allergy Clin. Immunol.* 2011. **127**: 823–830.

9 **Kawasaki, T.**, Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes in children. *Arerugi* 1967. **16**: 178–222.

10 **Melish, M. E., Hicks, R. M., and Larson, E. J.**, Mucocutaneous lymph node syndrome in the United States. *Am. J. Dis. Child.* 1976. **130**: 599–607.

11 **Kawasaki, T., Kosaki, F., Okawa, S., Shigematsu, I., and Yanagawa, H.**, A new infantile acute febrile mucocutaneous lymph node syndrome (MLNS) prevailing in Japan. *Pediatrics* 1974. **54**: 271–276.

12 **Furusho, K., Kamiya, T., Nakano, H., Kiyosawa, N., Shinomiya, K., Hayashidera, T., Tamura, T. et al.**, High-dose intravenous gammaglobulin for Kawasaki disease. *Lancet* 1984. **2**: 1055–1058.

13 **Newburger, J. W., Takahashi, M., Burns, J. C., Beiser, A. S., Chung, K. J., Duffy, C. E., Glode, M. P. et al.**, The treatment of Kawasaki syndrome with intravenous gamma globulin. *N. Engl. J. Med.* 1986. **315**: 341–347.

14 **Newburger, J. W., Takahashi, M., Beiser, A. S., Burns, J. C., Bastian, J., Chung, K. J., Colan, S. D. et al.**, A single intravenous infusion of gamma globulin as compared with four infusions in the treatment of acute Kawasaki syndrome. *N. Engl. J. Med.* 1991. **324**: 1633–1639.

15 **Furukawa, S., Matsubara, T., Jujoh, K., Yone, K., Sugawara, T., Sasai, K., Kato, H. et al.**, Peripheral blood monocyte/macrophages and serum tumor necrosis factor in Kawasaki disease. *Clin. Immunol. Immunopathol.* 1988. **48**: 247–251.

16 **Matsubara, T., Ichiyama, T. and Furukawa, S.**, Immunological profile of peripheral blood lymphocytes and monocytes/macrophages in Kawasaki disease. *Clin. Exp. Immunol.* 2005. **141**: 381–387.

17 **Hui-Yuen, J. S., Duong, T. T. and Yeung, R. S.**, TNF-alpha is necessary for induction of coronary artery inflammation and aneurysm formation in an animal model of Kawasaki disease. *J. Immunol.* 2006. **176**: 6294–6301.

18 **Burns, J. C., Best, B. M., Mejias, A., Mahony, L., Fixler, D. E., Jafri, H. S., Melish, M. E. et al.**, Infliximab treatment of intravenous immunoglobulin-resistant Kawasaki disease. *J. Pediatr.* 2008. **153**: 833–838.

19 **Franchi, L., Eigenbrod, T., Munoz-Planillo, R. and Nunez, G.**, The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat. Immunol.* 2009. **10**: 241–247.

20 **Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T. et al.**, A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *Embo. J.* 1990. **9**: 1897–1906.

21 **Dunn, S. M., Coles, L. S., Lang, R. K., Gerondakis, S., Vadas, M. A. and Shannon, M. F.**, Requirement for nuclear factor (NF)-kappa B p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. *Blood* 1994. **83**: 2469–2479.

22 **Hu, H. M., Baer, M., Williams, S. C., Johnson, P. F. and Schwartz, R. C.**, Redundancy of C/EBP alpha, -beta, and -delta in supporting the lipopolysaccharide-induced transcription of IL-6 and monocyte chemoattractant protein-1. *J. Immunol.* 1998. **160**: 2334–2342.

23 **Abe, J., Ebata, R., Jibiki, T., Yasukawa, K., Saito, H. and Terai, M.**, Elevated granulocyte colony-stimulating factor levels predict treatment failure in patients with Kawasaki disease. *J. Allergy Clin. Immunol.* 2008. **122**: 1008–1013.

24 **Broudy, V. C., Kaushansky, K., Harlan, J. M. and Adamson, J. W.**, Interleukin 1 stimulates human endothelial cells to produce granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor. *J. Immunol.* 1987. **139**: 464–468.

25 **Zsebo, K. M., Yuschenkoff, V. N., Schiffer, S., Chang, D., McCall, E., Dinarello, C. A., Brown, M. A. et al.**, Vascular endothelial cells and granulopoiesis: interleukin-1 stimulates release of G-CSF and GM-CSF. *Blood* 1988. **71**: 99–103.

26 **Newburger, J. W., Takahashi, M., Gerber, M. A., Gewitz, M. H., Tani, L. Y., Burns, J. C., Shulman, S. T. et al.**, Diagnosis, treatment, and long-term management of Kawasaki disease: a statement for health professionals from the Committee on Rheumatic Fever, Endocarditis and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. *Circulation* 2004. **110**: 2747–2771.

27 **Leung, D. Y., Cotran, R. S., Kurt-Jones, E., Burns, J. C., Newburger, J. W. and Pober, J. S.**, Endothelial cell activation and high interleukin-1 secretion in the pathogenesis of acute Kawasaki disease. *Lancet* 1989. **2**: 1298–1302.

28 **Suzuki, H., Uemura, S., Tone, S., Iizuka, T., Koike, M., Hirayama, K. and Maeda, J.**, Effects of immunoglobulin and gamma-interferon on the production of tumour necrosis factor-alpha and interleukin-1 beta by peripheral blood monocytes in the acute phase of Kawasaki disease. *Eur. J. Pediatr.* 1996. **155**: 291–296.

29 **Fury, W., Tremoulet, A. H., Watson, V. E., Best, B. M., Shimizu, C., Hamilton, J., Kanegaye, J. T. et al.**, Transcript abundance patterns in Kawasaki disease patients with intravenous immunoglobulin resistance. *Hum. Immunol.* 2010. **71**: 865–873.

30 **Weng, K. P., Hsieh, K. S., Ho, T. Y., Huang, S. H., Lai, C. R., Chiu, Y. T., Huang, S. C. et al.**, IL-1B polymorphism in association with initial intravenous immunoglobulin treatment failure in Taiwanese children with Kawasaki disease. *Circ. J.* 2010. **74**: 544–551.

31 **Macmillan, H. F., Rowter, D., Lee, T. and Issekutz, A. C.**, Intravenous immunoglobulin G selectively inhibits IL-1alpha-induced neutrophil-endothelial cell adhesion. *Autoimmunity* 2010. **43**: 619–627.

32 **Xu, C., Poirier, B., Duong Van Huyen, J. P., Lucchiari, N., Michel, O., Chevalier, J. and Kaveri, S.**, Modulation of endothelial cell function by normal polyspecific human intravenous immunoglobulins: a possible mechanism of action in vascular diseases. *Am. J. Pathol.* 1998. **153**: 1257–1266.

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33 **Matsuda, A., Fukuda, S., Matsumoto, K. and Saito, H.,** Th1/Th2 cytokines reciprocally regulate in vitro pulmonary angiogenesis via CXC chemokine synthesis. *Am. J. Respir. Cell Mol. Biol.* 2008. **38**: 168–175.

34 **Shimizu, M., Pelisek, J. and Nikol, S.,** Vasculogenesis and angiogenesis depend on the developmental origin in the arterial tree. *Curr. Med. Chem.* 2002. **9**: 1619–1630.

35 **Matsuda, A., Orihara, K., Fukuda, S., Fujinaga, H., Matsumoto, K. and Saito, H.,** Corticosteroid enhances TNF-alpha-mediated leukocyte adhesion to pulmonary microvascular endothelial cells. *Allergy* 2008. **63**: 1610–1616.

36 **Orihara, K. and Matsuda, A.,** Pathophysiological roles of microvascular alterations in pulmonary inflammatory diseases: possible implications of tumor necrosis factor-alpha and CXC chemokines. *Int. J. Chron. Obstruct. Pulmon. Dis.* 2008. **3**: 619–627.

37 **Futamura, K., Orihara, K., Hashimoto, N., Morita, H., Fukuda, S., Sagara, H., Matsumoto, K. et al.,** beta2-adrenoceptor agonists enhance cytokine-induced release of thymic stromal lymphopoietin by lung tissue cells. *Int. Arch. Allergy Immunol.* 2010. **152**: 353–361.

38 **Yagami, A., Orihara, K., Morita, H., Futamura, K., Hashimoto, N., Matsumoto, K., Saito, H. et al.,** IL-33 mediates inflammatory responses in human lung tissue cells. *J. Immunol.* 2010. **185**: 5743–5750.

39 **Newburger, J. W., Sleeper, L. A., McCrindle, B. W., Minich, L. L., Gersony, W., Vetter, V. L., Atz, A. M. et al.,** Randomized trial of pulsed corticosteroid therapy for primary treatment of Kawasaki disease. *N. Engl. J. Med.* 2007. **356**: 663–675.

40 **Ichiyama, T., Ueno, Y., Isumi, H., Niimi, A., Matsubara, T. and Furukawa, S.,** An immunoglobulin agent (IVIG) inhibits NF-kappaB activation in cultured endothelial cells of coronary arteries in vitro. *Inflamm. Res.* 2004. **53**: 253–256.

41 **Litvak, V., Ramsey, S. A., Rust, A. G., Zak, D. E., Kennedy, K. A., Lampano, A. E., Nykter, M. et al.,** Function of C/EBP delta in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. *Nat. Immunol.* 2009. **10**: 437–443.

Abbreviations: **CAL**: coronary artery lesion · **HCAEC**: human coronary artery endothelial cell · **HUVEC**: human umbilical vein endothelial cell · **IVIG**: high-dose infusion of IgG · **KD**: Kawasaki disease

Full correspondence: Dr. Akio Matsuda, Department of Allergy and Immunology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, 157-8535, Tokyo, Japan.
Fax: +81-3-5494-7173
e-mail: amatsuda@nch.go.jp

Received: 17/1/2012
Revised: 6/4/2012
Accepted: 4/5/2012
Accepted article online: 14/5/2012

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2. Liu CH, Wu KH, Lin TY, Wei CC, Lin CY, Chen XX, et al. Wiskott-Aldrich syndrome with IgA nephropathy: a case report and literature review. Int Urol Nephrol 2012 [Epub ahead of print].
3. Matsukura H, Kanegane H, Miya K, Ohtsubo K, Higuchi A, Tanizawa T, et al. IgA nephropathy associated with X-linked thrombocytopenia. Am J Kidney Dis 2004; 43:e7-12.
4. Tomana M, Novak J, Julian BA, Matousovic K, Konecny K, Mestecky J. Circulating immune complexes in IgAN consist of IgA1 with galactose-deficient hinge region and antiglycan antibodies. J Clin Invest 1999;104:73-81.
5. Shimizu M, Nikolov NP, Ueno K, Ohta K, Siegel RM, Yachie A, et al. Development of IgA nephropathy-like glomerulonephritis associated with Wiskott–Aldrich syndrome protein deficiency. Clin Immunol 2012;142:160-6.
6. Lasseur C, Allen AC, Deminière C, Aparicio M, Feehally J, Combe C. Henoch-Schöenlein purpura with immunoglobulin A nephropathy and abnormal IgA in Wiskott-Aldrich syndrome carrier. Am J Kidney Dis 1997;29:285-7.
7. Moldoveanu Z, Wyatt RJ, Lee JY, Tomana M, Julian BA, Mestecky J, et al. Patients with IgA nephropathy have increased serum galactose-deficient IgA1 levels. Kidney Int 2007;71:1148-54.
8. Higgins EA, Siminovitch KA, Zhuang DL, Brockhausen I, Dennis JW. Aberrant O-linked oligosaccharide biosynthesis in lymphocytes and platelets from patients with the Wiskott-Aldrich syndrome. J Biol Chem 1991;266:6280-90.
9. Nguyen DD, Maillard MH, Cotta-de-Almeida V, Mizoguchiv E, Klein C, Fuss I, et al. Lymphocyte-dependent and Th2 cytokine-associated colitis in mice deficient in Wiskott-Aldrich syndrome protein. Gastroenterology 2007;133:1188-97.

Available online October 31, 2012.
http://dx.doi.org/10.1016/j.jaci.2012.08.040

Antigen-specific T-cell responses in patients with non–IgE-mediated gastrointestinal food allergy are predominantly skewed to T_H2

To the Editor:

IgE-mediated allergy is triggered by cross-linking of antigen-specific IgE antibodies on the cell surfaces of mast cells and basophils, followed by local accumulation and activation of inflammatory cells, including eosinophils and T_H2 cells. T_H2 cells produce such cytokines as IL-4, IL-5, and IL-13, which promote IgE production and eosinophilopoiesis and play central roles in the development of chronic allergic inflammation. On the other hand, non–IgE-mediated allergies, such as hypersensitivity pneumonitis, are considered mediated by cellular immunity, which has not been thought to involve antigen-specific T_H2 cells because IgE antibody would be detected if T_H2 cells were activated. Non–IgE-mediated gastrointestinal food allergies include food protein–induced enterocolitis syndrome (FPIES), food protein–induced proctocolitis, and food protein–induced enteropathy. The precise underlying mechanisms are almost unknown, except for a fundamental role of TNF-α,[1] presumably because this disease entity is relatively rare in incidence and is encountered during infancy in human subjects but not seen in experimental animals. Here, for the first time, we were able to detect antigen-specific T_H2 cell responses in infants with non–IgE-mediated gastrointestinal food allergies by analyzing 89 blood samples collected from all over Japan.

The antigen-specific lymphocyte stimulation test is a classic method for investigating antigen-specific T-cell proliferation and theoretically should be applicable to the study of gastrointestinal food allergies. However, a couple of previous studies demonstrated that the antigen-specific lymphocyte stimulation test was useful, whereas another study found no such usefulness.[2] We hypothesized that this controversy was due to contamination of the antigen preparations with LPS and tested this hypothesis. The limulus amebocyte lysate assay detected high concentrations of LPS in commercially available milk protein preparations, as previously reported (see Table E1 in this article's Online Repository at www.jacionline.org).[3] In addition, significant lymphoproliferative responses were found in the presence of as little as 10 pg/mL LPS (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org), and PBMCs from younger children showed more pronounced lymphoproliferation in response to LPS (see Fig E1, *B*). Therefore we attempted to remove contaminating LPS from milk protein preparations by passing them through a prepacked endotoxin affinity column. However, a high LPS concentration was detected even after that treatment (see Table E1), and therefore we obtained a special β-lactoglobulin preparation with very low contaminating LPS levels (kindly provided by Bean Stalk Snow, Tokyo, Japan). Further studies were performed by using these milk protein preparations, which contained LPS at a final concentration of less than 5 pg/mL.

TABLE I. Demographic characteristics of the patients

	IgE-mediated CMA		Gastrointestinal food allergies	
	No.		No.	
Age (mo)	12	38.0 (26.5-60.0)	65	2.0 (1.0-4.0)
Male/female sex	12	7/5	65	40/25
Day of onset	12	—	65	32.5 (7.0-115.5)
Symptoms at onset				
Vomiting	12	0% (0/12)	65	53.8% (35/65)
Bloody stool	12	0% (0/12)	65	47.7% (31/65)
Diarrhea	12	0% (0/12)	65	47.7% (31/65)
Failure to thrive	12	0% (0/12)	65	38.4% (22/65)
Lethargy	12	0% (0/12)	65	38.4% (22/65)
Fever	12	0% (0/12)	65	18.5% (12/65)
Eczema	12	100% (12/12)	65	7.7% (5/65)
Wheeze	12	33.3% (3/12)	65	0% (0/65)
Laboratory data				
Milk-specific IgE (IU/mL)	12	56.95 (11.74-90.8)	65	<0.34 (<0.34)
Peripheral blood eosinophils (%)		Not examined	53	7.7 (3.6-13.5)

Data are expressed as medians (interquartile ranges). The inclusion criteria were as follows: (1) gastrointestinal symptoms were present more than 2 hours after ingestion of milk and (2) 3 of Powell's criteria were fulfilled,[4] including (a) switch to therapeutic milk leading to resolution of symptoms, (b) differential diagnosis from other disorders, and (c) verified body weight gain. A definitive diagnosis based on the results of oral food challenge tests that were performed after complete resolution of the initial symptoms was achieved in 19 patients. Patients with gastrointestinal symptoms within 2 hours after ingestion of milk were excluded. On the basis of such symptoms as vomiting, diarrhea, and failure to thrive, the patient group (n = 65) consists of 34 patients with FPIES, 4 patients with food protein–induced enteropathy syndrome (enteropathy), and 27 patients with food protein–induced proctocolitis syndrome (proctocolitis). A definitive diagnosis based on the results of oral food challenge tests was achieved in 13 and 6 patients with FPIES and proctocolitis, respectively. None of the patients underwent endoscopic biopsy.

Next, to elucidate what types of antigen-specific immune responses are induced in patients with gastrointestinal food allergies, we cultured PBMCs from patients and control subjects in the presence and absence of LPS-depleted milk component proteins. The study enrolled 65 patients with gastrointestinal food allergies, 12 patients with IgE-mediated cow's milk allergy (CMA) who showed only nongastrointestinal symptoms on ingestion of milk, and 12 control subjects who showed absolutely no symptoms on ingestion of milk. Table I[4] summarizes the clinical symptoms, clinical diagnosis, and demographic data for the 2 patient groups. None of the patients with gastrointestinal food allergies had detectable levels of IgE against milk proteins in sera. We were unable to recruit infants with IgE-mediated CMA who were age matched with the infants with non–IgE-mediated

TABLE II. Antigen-specific lymphoproliferation and cytokine production profiles in patients with gastrointestinal food allergies, patients with IgE-mediated allergy, and control subjects

	Control subjects		IgE-mediated CMA		Gastrointestinal food allergies			
	No.		No.		No.		*P* value†	*P* value‡
Proliferation (SI)*	20	1.290 (0.830-1.738)	9	3.077 (2.484-3.492)	65	2.894 (2.004-7.147)	<.01	<.001
Cytokine (pg/mL)								
TNF-α	12	74.69 (58.44-144.8)	10	77.78 (58.04-141.4)	65	241.0 (89.21-729.6)	NS	<.05
IL-6	12	79.24 (36.36-193.8)	10	337.9 (57.43-1021)	65	1151 (157.0-4802)	NS	<.01
IL-1β	11	26.02 (6.880-46.47)	10	27.49 (6.548-65.04)	64	48.75 (11.7-136.1)	NS	NS
IL-2	12	4.15 (0.0-10.04)	10	12.31 (7.23-17.58)	58	16.32 (7.760-39.49)	NS	<.01
IL-3	12	0.0 (0.0-0.38)	10	0.40 (0.0-3.61)	62	4.22 (0.0-29.49)	NS	<.05
IL-4§	12	5.365 (2.895-6.358)	10	3.795 (2.033-7.788)	65	5.670 (2.775-12.06)	NS	NS
IL-5	12	2.080 (0.0-19.56)	10	46.59 (4.663-173.5)	65	63.66 (7.360-310.4)	NS	<.01
IL-10	12	9.285 (3.075-15.71)	10	56.17 (18.74-76.91)	65	57.92 (12.61-198.8)	NS	<.05
IL-13	12	21.61 (0.270-65.04)	10	82.56 (16.28-555.3)	65	291.7 (22.10-1417)	NS	<.01
IFN-γ	11	3.910 (0.0-67.06)	10	31.91 (3.635-102.0)	65	71.86 (5.49-303.4)	NS	NS
IL-17	12	0.0 (0.0-2.350)	10	7.635 (1.710-39.63)	65	7.150 (0.0-17.83)	NS	NS

PBMCs from each patient were stimulated separately with each of 5 different milk protein preparations, and the data show the highest concentration of each cytokine detected in response to the 5 different stimuli. Data are expressed as medians (interquartile ranges).

*The stimulation index *(SI)* was calculated as milk protein–specific tritiated thymidine uptake (cpm)/vehicle-induced tritiated thymidine uptake (cpm).

†Nonparametric test to compare control subjects and patients with IgE-mediated CMA.

‡Nonparametric test to compare control subjects and patients with gastrointestinal food allergies.

§According to the standard curve, the minimal detection limit was 5.88 pg/mL.

gastrointestinal food allergies. This study was approved by regional ethics committees, and written informed consent was obtained from the guardians of all patients and control subjects.

The details of the lymphoproliferation test and cytokine production assay are described in the Methods section in this article's Online Repository at www.jacionline.org. In brief, PBMCs from heparinized peripheral blood were suspended at a cell density of 1×10^6/mL in AIM-V medium (Gibco, Grand Island, NY) without serum. Lymphoproliferation was measured by using tritiated thymidine uptake during a 16-hour period after a 5-day stimulation with 100 μg/mL of each LPS-depleted milk protein preparation (α-lactalbumin, β-lactoglobulin, and α-, β- and κ-caseins). PBMCs were suspended at 1×10^6/mL in RPMI 1640 medium supplemented with 5% autologous plasma to investigate the antigen-specific cytokine production profiles. Culture supernatants were harvested at day 6 after stimulation with 100 μg/mL of each LPS-depleted milk protein preparation, and the cytokine production profiles were investigated by using the Luminex multiplex cytokine analysis kits (Millipore, Bedford, Mass) and ELISA (R&D Systems, Minneapolis, Minn).

In the first series of experiments, we investigated milk protein–specific lymphoproliferation in the control subjects, patients with IgE-mediated CMA, and patients with gastrointestinal food allergies. The lymphoproliferation level was similar in the patients with IgE-mediated CMA and those with gastrointestinal food allergies. Unlike in previous studies, however, the control subjects showed almost no proliferation (Table II). We presume that this was due to the extensive depletion of LPS contaminating the antigen preparations and the use of serum-free medium.

In the next experiments we investigated the cytokine production profiles in these subjects. TNF-α concentrations in the culture supernatants of milk protein–stimulated PBMCs from patients with gastrointestinal food allergies were significantly greater than those seen in patients with IgE-mediated CMA or control subjects. However, TNF-α levels in supernatants from patients with IgE-mediated CMA and control subjects were similar (Table II). Significantly higher concentrations of another proinflammatory cytokine, IL-6, were also seen only in the patients with gastrointestinal food allergies.

The concentrations of 3 T_H2 cytokines, IL-3, IL-5, and IL-13, in the supernatants of milk protein–stimulated PBMCs from patients with IgE-mediated CMA tended to be higher than those in the control subjects, but the differences did not reach statistical significance. In contrast, statistically significant and much higher concentrations of these T_H2 cytokines were found for the patients with gastrointestinal food allergies. Another T_H2 cytokine, IL-4, was undetectable in almost all subjects, and there were no differences among the 3 groups.

Concentrations of the T_H1 cytokine IFN-γ and the T_H17 cytokine IL-17 did not show statistically significant differences between any 2 groups.

The milk component that caused the most prominent tritiated thymidine uptake or the most prominent IL-2 or TNF-α production varied among the patients (see Fig E3 in this article's Online Repository at www.jacionline.org), suggesting that the lymphoproliferation and cytokine production observed in these assays were indeed antigen specific. In addition, the IL-5 concentration in the culture supernatant of cow's milk protein–stimulated PBMCs from patients with gastrointestinal food allergies correlated significantly with the peripheral blood eosinophil ratio at disease onset (see Fig E4 in this article's Online Repository at www.jacionline.org), suggesting that our *in vitro* assay reflects the *in vivo* conditions in these patients.

Collectively, T_H2 cytokines, including IL-3, IL-5, and IL-13, but not the T_H1 cytokine IFN-γ or the T_H17 cytokine IL-17 were significantly produced *in vitro* by milk protein–stimulated PBMCs from patients with gastrointestinal food allergies. The findings that tritiated thymidine uptake correlated significantly with IL-13 production (data not shown) along with the absence of milk-specific IgE antibody strongly suggest that the IL-13 detected in our assay was not produced by basophils in the PBMC fraction. IL-13 is a well-established mediator of intestinal

epithelial cell damage in patients with injuries and inflammatory diseases through activation of the tumor necrosis factor-like weak inducer of apoptosis-fibroblast growth factor-inducible molecule 14 (TWEAK-Fn14) axis.[5] Thus in addition to the previously known TNF-α, IL-13 might play a crucial role in the pathogenesis of gastrointestinal food allergies.

In conclusion, antigen-specific T-cell responses in patients with non–IgE-mediated gastrointestinal food allergy are predominantly skewed to T_H2. It remains unclear why antigen-specific IgE antibodies were not detected in these patients. Possible explanations are that neonatal B cells scarcely express IL-4/IL-13 receptors[6] or that production of IgE antibodies had just started but was still undetectable. This question warrants further study.

We express our sincere gratitude to all the members of the Japanese Research Group for Neonatal Infantile Allergic Disorders. We also thank all the doctors, nurses, and technicians, especially Ms Nao Aida from the Division of Allergy, Gastroenterology, Pathology, Surgery, Interdisciplinary Medicine and Neonatology of the National Center for Child Health and Development, for their hard work and valuable comments. We also thank Professor Mitsuaki Kimura (Department of Allergy and Clinical Immunology, Shizuoka Children's Hospital) for his valuable suggestions.

Hideaki Morita, MD, PhD[a,b]
Ichiro Nomura, MD, PhD[a,c]
Kanami Orihara, PhD[a]
Koichi Yoshida, MD[c,d]
Akira Akasawa, MD, PhD[c,d]
Hiroshi Tachimoto, MD, PhD[e]
Yoshikazu Ohtsuka, MD, PhD[f]
Yoshiyuki Namai, MD[g]
Masaki Futamura, MD, PhD[c]
Tetsuo Shoda, MD[a,c]
Akio Matsuda, PhD[a]
Norio Kamemura, MSc[h]
Hiroshi Kido, MD, PhD[h]
Takao Takahashi, MD, PhD[b]
Yukihiro Ohya, MD, PhD[c]
Hirohisa Saito, MD, PhD[a]
Kenji Matsumoto, MD, PhD[a]

From [a]the Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, Japan; [b]the Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan; [c]the Division of Allergy, National Center for Child Health and Development, Tokyo, Japan; [d]the Department of Allergy, Tokyo Metropolitan Children's Medical Center, Tokyo, Japan; [e]the Department of Pediatrics, Jikei University School of Medicine, Tokyo, Japan; [f]the Department of Pediatrics and Adolescence Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan; [g]the Department of Pediatrics, Ohta Nishinouchi Hospital, Koriyama, Japan; and [h]the Division of Enzyme Chemistry, Institute for Enzyme Research, University of Tokushima, Tokushima, Japan. E-mail: nomura-i@ncchd.go.jp, matsumoto-k@ncchd.go.jp.

Supported in part by Health and Labour Sciences Research Grants, Research on Intractable Diseases from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid for Clinical Research from the National Hospital Organization of Japan.
Disclosure of potential conflict of interest: I. Nomura has received support from the Ministry of Health, Labor, and Welfare. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Chung HL, Hwang JB, Park JJ, Kim SG. Expression of transforming growth factor beta1, transforming growth factor type I and II receptors, and TNF-alpha in the mucosa of the small intestine in infants with food protein-induced enterocolitis syndrome. J Allergy Clin Immunol 2002;109:150-4.
2. Giavi S, Megremis S, Papadopoulos NG. Lymphocyte stimulation test for the diagnosis of non-IgE-mediated cow's milk allergy: a step closer to a noninvasive diagnostic tool? Int Arch Allergy Immunol 2012;157:1-2.
3. Brix S, Bovetto L, Fritsché R, Barkholt V, Frøkiaer H. Immunostimulatory potential of beta-lactoglobulin preparations: effects caused by endotoxin contamination. J Allergy Clin Immunol 2003;112:1216-22.
4. Powell GK. Food protein-induced enterocolitis of infancy: differential diagnosis and management. Compr Ther 1986;12:28-37.
5. Kawashima R, Kawamura YI, Oshio T, Son A, Yamazaki M, Hagiwara T, et al. Interleukin-13 damages intestinal mucosa via TWEAK and Fn14 in mice—a pathway associated with ulcerative colitis. Gastroenterology 2011;141:2119-29.
6. Tian C, Kron GK, Dischert KM, Higginbotham JN, Crowe JE Jr. Low expression of the interleukin (IL)-4 receptor alpha chain and reduced signalling via the IL-4 receptor complex in human neonatal B cells. Immunology 2006;119:54-62.

Available online October 16, 2012.
http://dx.doi.org/10.1016/j.jaci.2012.09.005

Forkhead box protein 3 (*FOXP3*) hypermethylation is associated with diesel exhaust exposure and risk for childhood asthma

To the Editor:

Traffic-related air pollutants, such as diesel exhaust particles (DEP), significantly contribute to the pathogenesis of wheezing and asthma in early childhood.[1] These illnesses are characterized by chronic airway inflammation caused by a dysregulated immune system.[2] Attention has recently been directed toward regulatory T (Treg) cells because they are important in suppressing immune responses against nonspecific stimuli,[3] such as DEP. The suppressive phenotype of Treg cells is conferred by stable expression of forkhead box protein 3 (*FOXP3*).[3] Transcriptional silencing of *FOXP3* through hypermethylation of CpG islands in the promoter and intronic regions has been identified as a hallmark of committed Treg cells and human diseases, including asthma.[3,4] As such, Nadeau et al[4] reported increased *FOXP3* hypermethylation in blood DNA to be associated with diminished Treg cell function and increased asthma severity in children exposed to polycyclic aromatic hydrocarbons, a component of DEP. In this study we test the novel hypothesis that early (birth) and consistent exposure to high levels of traffic pollution alters *FOXP3* methylation status in DNA from saliva in a manner that correlates with DEP exposure, predicts wheezing/asthma in later life, or both. The oral cavity provides an important first line of defense against DEP exposure for children because mouth breathing is a common path of exposure.[5] Furthermore, other aerodigestive track tissues, such as buccal cells, have been successful in characterizing DNA methylation with respect to air pollutants and airway inflammation.[6,7] The ancillary goal is to establish a noninvasive, high-throughput, and quantitative assay for measuring risk of asthma linked to traffic-related air pollution.

TABLE I. Distribution of *FOXP3* percentage methylation in the sample population stratified by respiratory outcomes

Description	Mean	Minimum	Maximum	SD
Study sample	21.30	0.00	62.40	17.40
Wheezing phenotype				
Nonwheezers	17.00	0.00	55.10	16.20
Persistent wheezers*	35.84	18.10	58.35	15.20
Early transient wheezers*	24.16	0.32	61.30	17.90
Asthma status				
Nonasthmatic	19.50	0.00	62.40	16.90
Asthmatic†	32.70	1.10	58.40	17.90

*Significant difference between persistent wheezers ($P < .01$) and early transient wheezers ($P < .05$) compared with nonwheezers.
†Significant difference between asthmatic and nonasthmatic children ($P < .05$).

METHODS

Heparinized blood samples were stored at room temperature and transferred to the National Research Institute for Child Health and Development in Tokyo. The following procedures were performed no later than 24 hours after phlebotomy. PBMCs were obtained from peripheral blood by using Ficoll-Hypaque gradient sedimentation (Lymphocyte Separation Medium; ICN Biochemicals, Aurora, Ohio). The viability determined by using trypan blue dye exclusion (Sigma, St Louis, Mo) always exceeded 95%. PBMCs were suspended at a cell density of 1×10^6/mL in AIM-V medium (Gibco) without serum for lymphoproliferation, and in RPMI 1640 medium (GIBCO/Life Technologies, Gaithersburg, Md) in the presence of 5% autologous plasma for cytokine production assays.

Lymphoproliferation was measured based on tritiated thymidine (Amersham, Tokyo, Japan) uptake during a 16-hour period after 5 days of stimulation with 100 μg/mL of each LPS-depleted milk protein preparation (α-lactalbumin, Sigma; β-lactoglobulin, Bean Stalk Snow; and α-, β-, and κ-caseins, Sigma) at 37°C in a humidified 5% CO_2 atmosphere. Incorporated tritiated thymidine was counted with a liquid scintillation counter (TopCount NXT; PerkinElmer Life Sciences, Boston, Mass). The stimulation index was calculated as milk protein–specific tritiated thymidine uptake (cpm)/vehicle-induced tritiated thymidine uptake (cpm).

Culture supernatants were harvested at day 6, and the cytokine production profiles were investigated by using Luminex multiplex cytokine analysis kits (Millipore) and ELISA (R&D Systems).

The lymphoproliferation assays and cytokine production assays were performed in duplicates and triplicates, respectively.

There was a significant positive correlation between the IL-2 concentration in the PBMC culture supernatant and lymphoproliferation (stimulation index) after stimulation with κ-casein ($r = 0.269$, $P = .025$; see Fig E2). Similar tendencies were also found when PBMCs were stimulated with other milk protein preparations (data not shown).

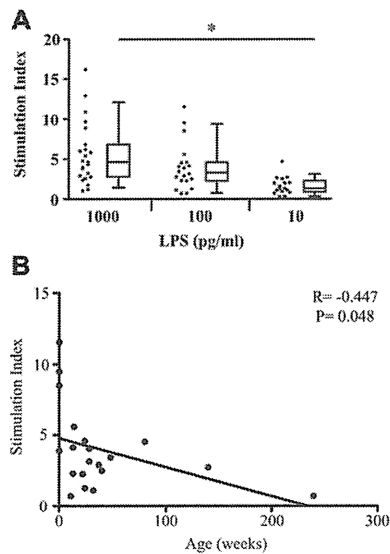

FIG E1. A, LPS at as little as 10 pg/mL can induce lymphoproliferation. PBMCs from young children (n = 60, 0-60 months of age) were stimulated with various concentrations of LPS (Sigma) for 5 days. Lymphoproliferation was measured by using tritiated thymidine uptake. The stimulation index was calculated as milk protein–specific tritiated thymidine uptake (cpm)/vehicle-induced tritiated thymidine uptake (cpm). $^*P < .05$. **B,** LPS-induced lymphoproliferation was inversely associated with age. PBMCs from young children (n = 21, 0-240 weeks of age) were stimulated with 100 pg/mL LPS (Sigma) for 5 days. Lymphoproliferation was measured by using tritiated thymidine uptake. The stimulation index was calculated as milk protein–specific tritiated thymidine uptake (cpm)/vehicle-induced tritiated thymidine uptake (cpm).

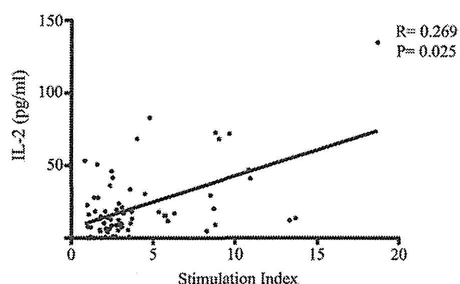

FIG E2. IL-2 concentrations in culture supernatant of cow's milk protein–stimulated PBMCs correlated significantly with antigen-specific lymphoproliferation. PBMCs from children with gastrointestinal food allergies were stimulated separately with 100 μg/mL of each of 5 LPS-depleted milk protein preparations in the absence of serum for the antigen-specific lymphoproliferation assay and in the presence of 5% autologous plasma for the IL-2 production assay. The stimulation index was calculated as milk protein–specific tritiated thymidine uptake (cpm)/vehicle-induced tritiated thymidine uptake (cpm), and the highest stimulation index shown among the 5 tested protein preparations was used as that patient's data in the plot. Even under slightly different culture conditions, antigen-specific lymphoproliferation and antigen-specific IL-2 production were significantly correlated ($r = 0.269$, $P = .025$).

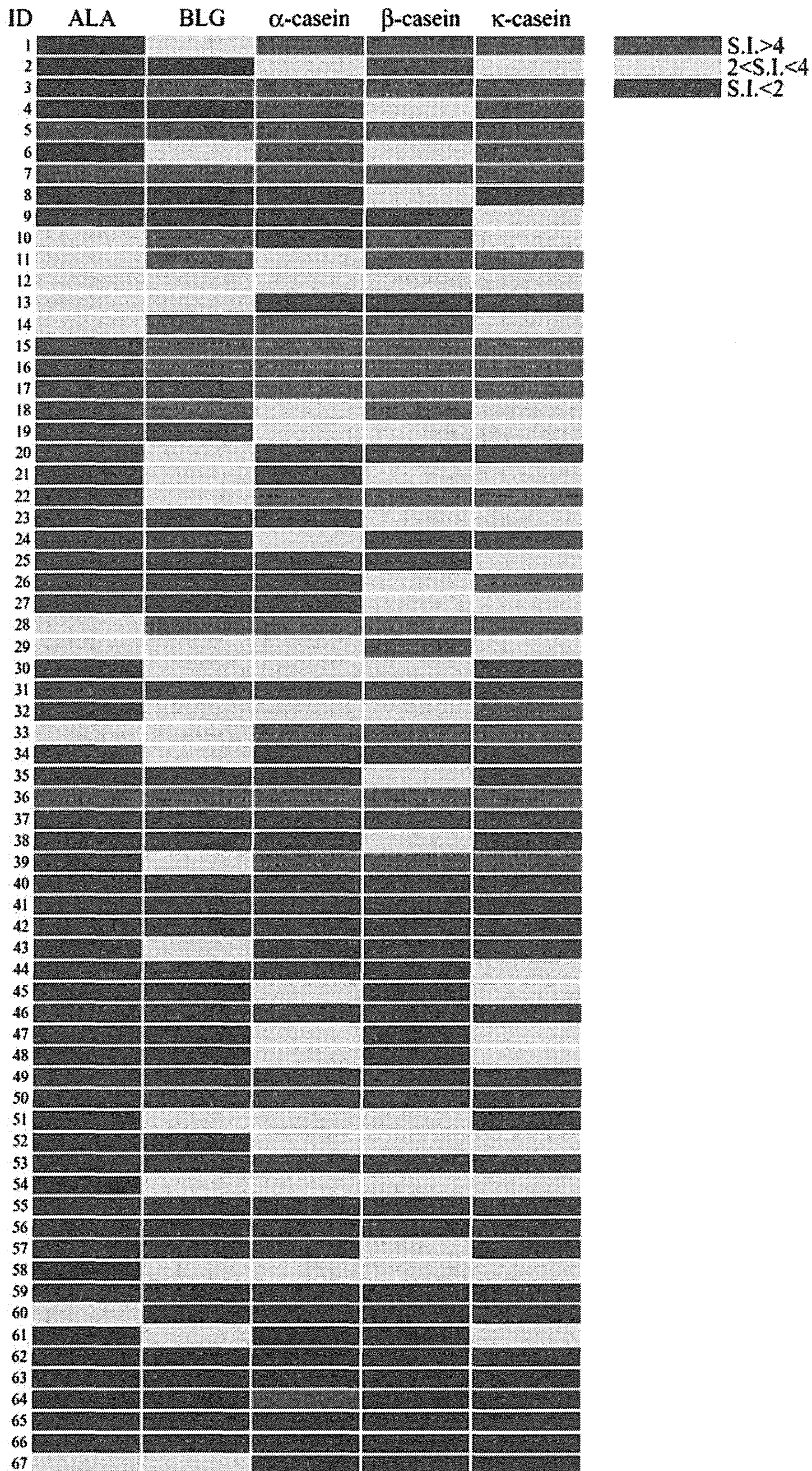

FIG E3. The milk protein component causing the most prominent tritiated thymidine uptake varied among the patients. PBMCs from children with gastrointestinal food allergies were stimulated separately with 100 μg/mL of each of 5 LPS-depleted milk protein preparations in the absence of serum. Lymphoproliferation was measured based on tritiated thymidine uptake. The stimulation index *(S.I.)* was calculated as milk protein–specific tritiated thymidine uptake (cpm)/vehicle-induced tritiated thymidine uptake (cpm). For

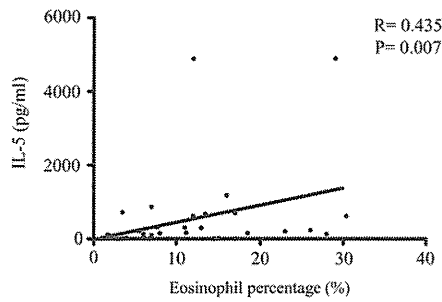

FIG E4. IL-5 concentration in the culture supernatant of cow's milk protein–stimulated PBMCs correlated significantly with the peripheral blood eosinophil percentage. PBMCs from children with gastrointestinal food allergies were stimulated separately with 100 μg/mL of each of 5 LPS-depleted cow's milk protein preparations in the presence of 5% autologous plasma for 6 days. Antigen-specific IL-5 production correlated significantly with the peripheral blood eosinophil percentage at disease onset (r = 0.435, P = .007).

each patient, the SI is shown for the PBMCs' response to each of the 5 milk protein preparations. Each *row* represents a single patient, and each *column* represents one of the 5 milk proteins. *ALA*, α-Lactalbumin; *BLG*, β-lactoglobulin; *blue*, SI < 2; *yellow*, 2.0 < SI < 4.0; *red*, SI > 4.

TABLE E1. Concentrations of LPS in commercially available milk protein preparations before and after treatment with a prepacked endotoxin affinity column

Cow's milk protein preparation	Before treatment (pg/mg)	After treatment (pg/mg)
α-Lactalbumin (Sigma L-6010)	184,200	14
β-Lactoglobulin (Sigma L-3908)	206,700	1,880
α-Casein (Sigma C-6780)	540	23
β-Casein (Sigma C-6905)	500	34
κ-Casein (Sigma C-0406)	400	41
LPS-depleted β-lactoglobulin (Bean Stalk Snow)	29	—

The indicated milk protein preparations were treated with a prepacked endotoxin affinity column (Detoxi-Gel; Pierce Chemical, Rockford, Ill) in accordance with the manufacturer's instructions. LPS concentrations were measured by using the limulus amebocyte lysate assay.