Genotyping

DNA was extracted from whole blood cells by standard procedures. For each of the polymorphisms studied, the forward and reverse primers used for polymerase chain reaction (PCR) amplification, including those previously described (Akahoshi et al. 2003; Golovleva et al. 1996; Liu et al. 1995; Miterski et al. 1999), are listed in Table 1. Genomic DNA (20 ng) was amplified by PCR in a total reaction volume of 10 µl containing 25 pmol each primer, 0.2 nmol each dNTP, 1.5 mM $MgCl_2$, and 0.5 U *Taq* polymerase. The cycling conditions for PCR in all reactions consisted of an initial 5-min denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 55–65°C for 30 s, and 72°C for 30 s, and finally a 7-min extension at 72°C. Following PCR, the reaction products were digested overnight with the appropriate enzyme (Table 1) at the correct temperature for optimal enzyme activity according to the manufacturer's instructions and analyzed by agarose gel electrophoresis.

For the *IFNA10* gene, we typed polymorphism T→A at nucleotide position 60 in which cysteine (Cys) at position 20 is replaced by a stop codon (nonsense mutation). We amplified DNA samples from 94 healthy individuals with two primers: 5'-AAAGCTCAGCTACAAATCCATCAG-3' and 5'-ATCAAACTCCTCCTGGGGGAT-3', the former mismatched primer (underlined nucleotide) introducing a restriction site for *Bgl*II in polymorphic alleles with the A substitution. We carried out PCRs with 10 ng genomic DNA, 12.5 pmol each primer, 0.2 nmol each dNTP, 1.5 mM $MgCl_2$, and 0.5 U *Taq* polymerase. DNA was denatured at 95°C for 2 min, and then PCR was performed for 37 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final 7-min extension at 72°C. The reaction products were digested overnight with endonuclease *Bgl*II at 37°C, and each allele was detected after separation on polyacrylamide gels.

Measurement of IFN and IL-12p70 production in human whole blood

Healthy blood volunteers were classified according to their *IFNA* genotypes as wild-type (1/1), heterozygous (1/2), or homozygous (2/2). IFN production induced by HVJ in vitro was measured as described in detail elsewhere (Uno et al. 1996); 2 ml heparinized blood from healthy individuals was cultured with 500 hemagglutinin U/ml Sendai virus (HVJ) within 5 h after blood was drawn. Supernatants were harvested after incubation (37°C for 20 h) of the blood-virus mixture. In the bioassay for IFN, IFN activity in the supernatants was assayed by suppression of the viral cytopathic effects in FL cells (derived from human amnion). Duplicate samples of standard reference IFN were serially diluted with Eagles' MEM supplemented with 5% fetal calf serum (FCS) in the wells of flat-bottomed 96-well microtiter plates (Falcon 3072) and irradiated with UV light (1000 J/m²) for HVJ inactivation. FL cells (5×10^4/well) were added following incubation at 37°C for 18 h. Sindbis virus (10^5 PFU/50 µl per well) suspended in Eagles' MEM supplemented with 1% FCS was added to the FL cells after removal of the supernatant. Sindbis virus and FL cells were cultured for another 40 h. Neutral red dye (0.5%) was added to the cells 1 h before harvesting, and the amount of dye incorporated into the cells was measured in a spectrophotometer (Titertek Multiskan MCC/340) at 540 nm following extraction of dye with 0.1 M sodium phosphate and 50% ethanol solution. The results were standardized to NIBSC international reference IFN-α leukocyte 69/19. The main subtype of IFN produced from whole blood-HVJ mixtures has previously been identified as IFN-α (Uno et al. 1996).

Subsequently, the amounts of IL-12p70 in the culture supernatants were determined by a specific enzyme-linked immunosorbent assay (ELISA) by using a Human IL-12 Ready-SET-Go! Kit (eBioscience, San Diego, Calif.). ELISA was conducted according to the manufacturer's recommended procedure.

Statistical analysis

We determined *P*-values for the differences in allele frequencies of each candidate gene polymorphism between cases (sarcoidosis or tuberculosis) and controls by using a 2×2 contingency χ^2 test with one degree of freedom (df) or Fisher exact test. Odds ratios (ORs) were calculated with 95% confident intervals (95% CI). For multiple comparisons, the *P*-values were corrected by Bonferroni method, where P_c was the corrected value. Analysis of the linkage disequilibrium (LD) coefficient between *IFNA* polymorphisms was performed ($D'=D/D_{max}$) with the ARLEQUIN 2.0 software package as described elsewhere (http://anthro.unige.ch/arlequin). The association between the in vitro production of IFN-α and of IL-12 and individual genotypes was analysed by the Mann-Whitney *U* test. A *P*-value of less than 0.05 was judged statistically significant.

Results

Association analysis between Th1 response-related genes and sarcoidosis

We ascertained the genotype of 10 previously identified polymorphisms of Th1-related genes (*IFNA17*, *IFNB*, *IFNG*, *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *IL12RB2*, *ETA-1*, and *NRAMP1*) for 102 Japanese patients with sarcoidosis, 114 patients with tuberculosis, and 115 healthy controls by restriction-fragment-length polymorphism analysis. We first conducted statistical calculations for each single polymorphism locus by χ^2 tests with 1 df and an overall

Table 2 Results of case-control association studies

Gene	Polymorphism	Sarcoidosis (*n*=102) vs controls (*n*=115)		Tuberculosis (*n*=114) vs. controls (*n*=115)	
		Odds ratio[a] (95%CI)	*P*[b]	Odds ratio[a] (95%CI)	*P*[b]
IFNA17	551 T/G	3.27 (1.44–7.46)	0.004	0.63 (0.33–1.20)	0.155
IFNB	153 C/T	0.96 (0.55–1.67)	0.873	0.66 (0.39–1.14)	0.137
IFNG	1348 T/A	1.02 (0.49–2.12)	0.952	1.24 (0.62–2.48)	0.550
IFNGR1	167 T/C	0.60 (0.33–1.09)	0.093	0.67 (0.34–1.28)	0.213
IFNGR2	839 G/A	1.50 (0.74–3.04)	0.256	0.80 (0.43–1.51)	0.498
IL12B	1146 C/A	1.47 (0.79–2.70)	0.221	0.50 (0.27–0.94)	0.030
IL12RB1	641 A/G	0.52 (0.29–0.94)	0.028	1.18 (0.63–2.19)	0.610
IL12RB2	365 C/T	1.49 (0.83–2.54)	0.197	0.86 (0.50–1.49)	0.593
ETA-1 (*SPP1*)	2514 C/T	1.20 (0.57–2.53)	0.632	1.14 (0.66–1.96)	0.643
NRAMP1 (*SLC11A1*)	1703 G/A	1.95 (0.97–3.91)	0.058	0.59 (0.29–1.20)	0.144

[a]Odds ratios are for the comparison between wild-type homozygous and variant homozygous genotype (*CI* confidence interval)
[b]2×2 Table

Table 3 *IFNA17* genotype and allele frequencies in patients with sarcoidosis and in healthy controls in the Japanese population (*CI* confidence interval)

IFNA17 Polymorphism	Sarcoidosis patients (n=102)	Healthy controls (n=115)	Odds ratio (95%CI)	P
Genotype				
T/T	11 (10.8%)	30 (26.1%)	1.00	
T/G	49 (48.0%)	50 (43.5%)	2.67 (1.21–5.92)	0.014
G/G	42 (41.2%)	35 (30.4%)	3.27 (1.44–7.46)	0.004
Allele				
T	71 (34.8%)	110 (47.8%)	1.00	
G	133 (65.2%)	120 (52.2%)	1.72 (1.17–2.53)	0.006
T/T+T/G	60 (58.8%)	80 (69.6%)	1.00	
G/G	42 (41.2%)	35 (30.4%)	1.60 (0.91–2.80)	0.099
T/T	11 (10.8%)	30 (26.1%)	1.00	
T/G+ G/G	91 (89.2%)	85 (73.9%)	2.92 (1.38–6.19)	0.004

comparison (2 df) between patients and controls. The results of the case-control studies for each polymorphism are listed in Table 2 (ORs are for the comparison between the homozygous wild-type and the homozygous variant genotype). For each of the markers investigated, there was no evidence of departure from Hardy-Weinberg equilibrium in either cases or controls. In patients with sarcoidosis, among these polymorphisms, polymorphisms in the *IFNA17* and *IL12RB1* genes were shown to have a significance in the genotypic frequency distribution (OR 3.27 [95% CI: 1.44–7.46], P=0.004, and OR 0.52 [95% CI: 0.29–0.94], P=0.028, respectively; Table 2). In contrast, in patients with tuberculosis, the *IL12B* polymorphism was found to be of significance for its genotype distribution (OR 0.50 [95% CI: 0.27–0.94], P=0.03; Table 2). However, only the result for an *IFNA17* polymorphism (551T→G) in sarcoidosis cases remained significant after Bonferroni correction for multiple testing (P_c=0.04). No significant associations with the other polymorphisms including *IFNB* were observed. Moreover, there was no statistical difference in genotype frequencies for each polymorphism between patients with sarcoidosis and tuberculosis after adjustment for age and sex. Table 3 shows the distribution of *IFNA17* genotypes in healthy Japanese individuals and patients with sarcoidosis. The frequency of the 551G allele was reported to be high in the Asian Mongoloid population, e.g., Chinese (approximately 50%), compared with Swedes (11%) or Finns (24%; Golovleva et al. 1997b). There was also a significant association (P=0.013) when using a χ^2 test with 2 df for the comparison of overall genotype frequencies.

Confirmation of two major haplotypes of the *IFNA* gene

The *IFNA17* polymorphism, viz., a T→G transversion occurring at nucleotide position 551, changes amino acid position 184 encoding isoleucine to arginine (I184R) and alters an *Ssp*I restriction site. The 551T allele has the *Ssp*I site, whereas the 551G allele lacks this site. Golovleva and colleagues (1996) have reported another functionally relevant polymorphism within the *IFNA10* gene, i.e., a T→A transversion at nucleotide position 60 (60T→A) creating a stop codon (C20X), which is associated with the I184R polymorphism of *IFNA17*. To determine the relationship between these two functionally relevant polymorphisms in Japanese populations, we further analyzed the genotype frequencies of the *IFNA10* gene variant, 60T→A. The haplotype frequencies for *IFNA10* [60T/A] and *IFNA17* [551T/G], which are 45 kb apart, were then estimated in 94 control subjects. The genotype distribution of *IFNA10* was also in Hardy-Weinberg equilibrium. Haplotype frequencies of [60T]-[551T], [60T]-[551G], [60A]-[551T], and [60A]-[551G] were 0.4149, 0.0053, 0.0160, and 0.5638, respectively. Thus, as reported previously (Golovleva et al. 1996), we confirmed the strong LD (D'=0.98) between alleles *IFNA10* [60T] and *IFNA17* [551T] and between alleles *IFNA10* [60A] and *IFNA17* [551G], two major haplotypes in Japanese populations. We designated the wild-type *IFNA10* [60T]-*IFNA17* [551T] allele as allele 1 and the polymorphic *IFNA10* [60A]-*IFNA17* [551G] allele as allele 2. The three *IFNA* genotypes were designated 1/1, 1/2, and 2/2. These results indicate that the haplotype in the *IFNA* gene, i.e., allele 2, is associated with increased susceptibility to sarcoidosis.

Association between IFNA genotype and in vitro IFN-α and IL-12p70 production

To assess whether this set of polymorphisms (haplotype) is associated with variations in protein production, in vitro IFN-α production induced by HVJ in cultures of whole blood cells from healthy individuals was measured and compared with genotype. As a result, we found that cells from 2/2 individuals produced significantly more IFN-α than did cells from 1/1 (P=0.028) or 1/2 (P=0.020) individuals (2/2 vs 1/1+1/2, P=0.010; Fig. 1A). Taken together, the high IFN-α-producing haplotype (allele 2) is associated with increased risk of sarcoidosis. Interestingly, when HVJ-induced in vitro IL-12 production was measured, a similar tendency to the results of IFN-α production was observed, i.e., like IFN-α, the 2/2 genotype is associated with a higher production of IL-12 and the 1/1 genotype with a lower production of IL-12 in whole blood cell cultures (2/2 vs 1/1, P=0.024; Fig. 1B).

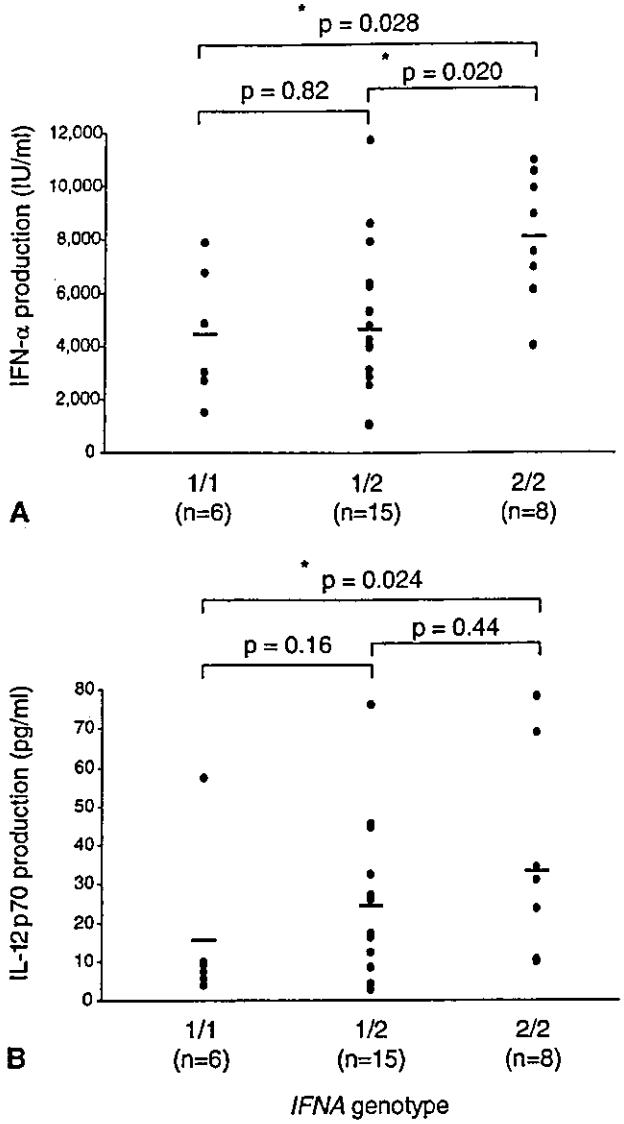

Fig. 1A, B Cytokine production in the three different allelic groups. **A** HVJ-induced IFN-α for whole blood cell cultures from six homozygotes for *IFNA* allele 1 (1/1), 15 heterozygotes (1/2), and eight homozygotes for *IFNA* allele 2 (2/2). The median IFN-α concentrations±SD for 1/1, 1/2, and 2/2 are 4472±2481 IU/ml, 4651±2917 IU/ml, and 8144±2428 IU/ml, respectively. *P* (2/2 vs 1/1)=0.028, *P* (2/2 vs 1/2)=0.020, *P* (2/2 vs others)=0.010, *P* (1/1 vs 1/2)=0.82. **B** HVJ-induced IL-12p70. The median IL-12p70 concentrations±SD for 1/1, 1/2, and 2/2 are 15.5±20.5 pg/ml, 24.3±19.5 pg/ml, and 33.4±26.7 pg/ml. *P* (2/2 vs 1/1)=0.024, *P* (2/2 vs 1/2)=0.44, *P* (1/1 vs 1/2)=0.16. One representative set of results from two separate experiments is shown (*bars* median, $^*P<0.05$)

Discussion

Many studies have supported the notion that sarcoidosis occurs after environmental exposure of a genetically susceptible population (ATS/ERS/WASOG Committee 1999; Newman et al. 1997) and have confirmed that the disease is characterized by highly polarized Th1 cytokine profiles with dominant expression of IFN-γ and IFN-γ-promoting cytokines, IL-12 and IL-18 (Moller et al. 1996; Greene et al. 2000). To identify a key genetic factor in the pathogenesis of sarcoidosis, we have investigated a range of cytokine-related genes involved in the type 1 immune response in patients with sarcoidosis and control subjects in an association-based study. We have found a significant association with the *IFNA17* and *IL12RB1* genes, which have not previously been studied in sarcoidosis. After correction for multiple testing, a significant difference was observed only in the distribution of *IFNA17* genotypes in sarcoidosis patients (P_c=0.04), but not in patients with tuberculosis, compared with controls. Then, we confirmed the presence of a strong LD between the *IFNA10* and *IFNA17* polymorphisms in the Japanese population and observed that one of the two major *IFNA* haplotypes, viz., allele 2: *IFNA10* [60A]-*IFNA17* [551G], was significantly associated with an increased risk of sarcoidosis. Moreover, in healthy controls, these genotypes correlated with total IFN-α production in vitro induced by HVJ infection in whole blood cell cultures, and our results showed that the 2/2 genotype resulted in a higher production of IFN-α and of IL-12 in vitro than did the 1/1 or 1/2 genotypes.

Type I IFNs (IFN-α and IFN-β) are produced in response to viral infection by many cell types and exert antiviral, antiproliferative, and multiple immunomodulatory activities (De Maeyer and De Maeyer-Guignard 1998). In addition to many pseudogenes, the human IFNs are encoded by a family of at least 15 different genes, including 13 IFN-α genes and a single IFN-β gene, and all known intronless type I IFN genes are clustered on the short arm of chromosome 9 spanning 400 kb (De Maeyer and De Maeyer-Guignard 1998; Roberts et al. 1998). Although numerous IFN-α sequence variants, including those in tumor cell lines, have been described, polymorphisms in populations have been reported in only a few loci of the *IFNA* genes. A previous association study and LD analysis revealed significant correlation between the microsatellite *IFNA* $(GT)_n$ allele and the risk of multiple sclerosis (Epplen et al. 1997) and correlations among alleles of the *IFNA* microsatellite, *IFNA10*, and *IFNA17* (Miterski et al. 1999). Although it remains to be determined whether the *IFNA* haplotype has a similar effect in other forms of immune disease, the *IFNA* haplotype appears to be widespread, suggesting that it contributes to the pathogenesis of a variety of autoimmunity.

We have also investigated the interaction between IFN-α production and *IFNA* genotype. Our findings indicate that *IFNA* allele 2, which is over-represented in patients with sarcoidosis, is associated with increased in vitro IFN-α production. IFN-α is especially highly produced by plasmacytoid dendritic cells (DCs), also called natural interferon-producing cells (IPCs), during a number of viral infections (Colonna et al. 2002). In humans, IPC-secreted type I IFNs are important in inducing Th1 polarization of T helper cells (Brinkmann et al. 1993; Farrar and Murphy 2000). Accordingly, increased IFN-α synthesis against an unknown agent from IPC, resulting from the set of poly-

morphisms within the *IFNA* gene, could result in inappropriate cellular immune responses with granuloma formation. In addition, taking into account the anti-cancer ability of IFN-α, our result is consistent with the previous finding of a significant increase in allele 1, the putative low IFN-α-producing allele, among patients with nasopharyngeal carcinoma compared with controls in southern China (Golovleva et al. 1997a). Here, we report a novel candidate gene, *IFNA*, in sarcoidosis. This is the first report demonstrating the functional consequences of the gene variations associated with sarcoidosis.

Although the mechanism by which these polymorphisms affect IFN-α production remains to be defined, the nearby sequence changes in the non-coding regulatory regions (an as yet unidentified locus of *IFNA*) may influence the production of IFN-α. Thus, our data raise the possibility that allele 2 is in LD with another polymorphism, which may be related to gene transcription or expression, in the *IFNA* gene. Interestingly, the results of in vitro IL-12p70 production between the genotype groups also show a similar pattern to those of IFN-α production. Although the precise mechanism of virus-induced IL-12 production is unclear, IFN-α priming of human monocytes has been shown to increase bioactive IL-12p70 secretion (Hermann et al. 1998). These observations suggest that IFN-α produced by IPC acts in an autocrine or paracrine manner to up-regulate DC production of IL-12.

The importance of IFN-α in sarcoidosis development is supported by several observations. First, there is an increasing number of reported cases of sarcoidosis induced by IFN-α therapy (Cogrel et al. 2002; Raanani and Ben-Bassat 2002). Furthermore, in some cases, the sarcoid lesions improved following dose reduction or cessation of the therapy. Second, the most common adverse effects of IFN-α therapy, such as early flu-like symptoms or later persistent fatigue, malaise, weight loss, and depression, resemble the systemic symptoms of most patients with sarcoidosis (ATS/ERS/WASOG Committee 1999; Newman et al. 1997). In addition, sarcoidosis is also associated with a high prevalence of depressive symptoms (Chang et al. 2001). Third, there is evidence that IFN-α/β induces the activation of alveolar macrophages from normal subjects to the level seen in patients with sarcoidosis (Fels et al. 1987). Fourth, the finding that T cells in bronchoalveolar lavage fluid in sarcoid lungs express a functional IL-12 receptor (IL-12R) composed of both the β1 and β2 subunits (Rogge et al. 1999) and the enhancing effect of IFN-α on the expression of the IL-12R β2 chain after antigen triggering (Rogge et al. 1997) also support a role of IFN-α in sarcoid pathogenesis.

In contrast, in patients with tuberculosis, we have found no significant association with *IFNA* gene. To date, cell-mediated or Th1 immunity has been shown to play a crucial role in defenses against intracellular pathogens such as *Mycobacterium* and *Salmonellae* (Akahoshi et al. 2003; Casanova and Abel 2002). Taken together, our results indicate that the activated IFN-α (type I IFN) pathway and a defective IFN-γ (type II IFN) pathway in individuals are associated with the genetic susceptibility of two distinct granulomatous lung diseases, i.e., sarcoidosis and tuberculosis, respectively. Further study in larger or ethnically distinct populations of patients will be necessary to confirm the effect of the *IFNA* polymorphisms.

In summary, this study has identified a significant association between *IFNA* genotype and risk of sarcoidosis in Japanese. The *IFNA* allele 2, which is associated with high IFN-α production, is over-represented in patients with sarcoidosis. Thus, the genetically determined, high endogenous production of IFN-α (and subsequent strong Th1 polarization) in these individuals could predispose toward development of sarcoidosis.

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Reciprocal Regulation of Thymus and Activation-Regulated Chemokine/Macrophage-Derived Chemokine Production by Interleukin (IL)-4/IL-13 and Interferon-γ in HaCaT Keratinocytes Is Mediated by Alternations in E-cadherin Distribution

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Keratinocytes produce many cytochemokines that are involved in the pathogenesis of skin disorders. In particular, the CC chemokines thymus and activation-regulated chemokine (TARC)/macrophage-derived chemokine (MDC) play an important role in the infiltration of Th2 cells. This study was undertaken to examine the regulatory effects of interleukin (IL)-4, IL-13, and interferon (IFN)-γ on TARC/MDC production in the human keratinocyte cell line HaCaT. HaCaT cells spontaneously secrete TARC and MDC. The production of TARC/MDC was downregulated by IL-4/IL-13, whereas it was upregulated by IFN-γ. To explore these regulatory mechanisms, we investigated the capacity of cytokines to regulate expression of several adhesion molecules that may affect TARC/MDC production. Of the adhesion molecules examined, the constitutive surface expression of E-cadherin was downregulated by IL-4/IL-13, but was upregulated by IFN-γ. Moreover, disruption of the homophilic adherence of E-cadherin by anti-E-cadherin antibody or calcium chelation abolished the production of TARC/MDC. We further examined the distribution of the adherens junction complex composed of E-cadherin, α-catenin, β-catenin, and γ-catenin. IL-4/IL-13 decreased the levels of membrane staining for adherens junction proteins, whereas IFN-γ increased membrane staining. Taken together, these results suggest that IL-4/IL-13 and IFN-γ induce alternations in the distribution of adherens junctions in a different fashion and thereby contribute to the reciprocal regulation of TARC/MDC production.

Key words: TARC/E-cadherin/IL-4/IFN-g/Keratinocyte.
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Keratinocytes produce a vast repertoire of cytokines, including interleukins, growth factors, colony-stimulating factors, and chemokines. The pattern of cytochemokines secreted locally plays a critical role in modulating the nature of tissue inflammation (Uchi *et al*, 2000). Thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) are ligands for the CC chemokine receptor 4 (CCR4), which is expressed selectively on Th2 cells. Ligation of TARC/MDC and CCR4 plays an important role in the migration of Th2 cells into inflamed tissues (Imai *et al*, 1999; Yoshie *et al*, 2001). Very recently, serum levels of TARC and MDC were shown to correlate well with disease severity in Th2-prone skin disorders, such as atopic dermatitis and mycosis fungoides (Kakinuma *et al*, 2001, 2002, 2003). Both chemokines are produced from various cell types, such as dendritic cells, T cells, fibroblasts, and epithelial cells (Alferink *et al*, 2003; Vulcano *et al*, 2001; Hirata *et al*, 2003; Fukuda *et al*, 2003; Albanesi *et al*, 2001).

Immunohistologic staining has revealed that keratinocytes express TARC and MDC in the lesional, but not in the nonlesional, skin of atopic dermatitis *in vivo* (Vestergaard *et al*, 2000; Kakinuma *et al*, 2001; Horikawa *et al*, 2002). In addition, the expression of TARC was demonstrated in the lesional skin of atopic model mice NC/Nga (Vestergaard *et al*, 1999). Moreover, the expression of TARC was detected in the lesional skin of mycosis fungoides (Kakinuma *et al*, 2003). In sharp contrast to these *in vivo* reports, cultured normal human keratinocytes were reported to be capable of secreting only MDC, but not TARC, whereas they expressed mRNA for both cytokines (Albanesi *et al*, 2001; Horikawa *et al*, 2002; Tsuda *et al*, 2003). On the other hand, a spontaneously immortalized, nontumorigenic human HaCaT keratinocyte cell line has been described to synthesize and secrete TARC upon stimulation with interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Vestergaard *et al*, 2000; Tsuda *et al*, 2003). The production of TARC by keratinocytes was also confirmed by culturing a murine keratinocyte cell line (PAM212) in the presence of IFN-γ,

Abbreviations: BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; MDC, macrophage-derived chemokine; p-, phospho-; PBS, phosphate buffered saline; TARC, thymus and activation-regulated chemokine; TNF, tumor necrosis factor.

TNF-α, or interleukin (IL)-1β (Vestergaard *et al*, 1999). Although the results, thus far, remain controversial, HaCaT keratinocytes may mimic the lesional keratinocytes of atopic dermatitis and mycosis fungoides.

To understand the regulatory mechanisms for TARC and MDC production in keratinocytes, we investigated the effects of IL-4, IL-13, and IFN-γ on TARC and MDC production by HaCaT keratinocytes. We found that IL-4/IL-13 and IFN-γ induce different alternations in the distribution of adherens junctions and, thereby, contribute to the reciprocal regulation of TARC and MDC production.

Results

HaCaT cells, but not normal human keratinocytes, secreted TARC and MDC spontaneously We examined the production of TARC and MDC from normal human keratinocytes and HaCaT keratinocytes. As previously documented (Albanesi *et al*, 2001; Horikawa *et al*, 2002; Tsuda *et al*, 2003), both normal human keratinocytes and HaCaT keratinocytes expressed mRNA for TARC and MDC (Fig 1*A*). We examined whether normal keratinocytes and HaCaT keratinocytes express the IL-4/IL-13 and IFN-γ receptor components. As shown in Fig 1*A*, mRNA for IL-4Rα, IL-13Rα1, and IL-13Rα2 was expressed, whereas mRNA for IL-2Rγ was undetectable. For IFN-γR1 expression, we examined using flow cytometric analysis. As shown in Fig 1*B*, IFN-γR1 was expressed on the surface of both normal keratinocytes and HaCaT keratinocytes. Only HaCaT keratinocytes produced substantial amounts of TARC and MDC (Fig 1*C*). Because the spontaneous secretion of TARC and MDC by HaCaT keratinocytes achieved a plateau after 48 h of incubation, the following experiments were designed with 48 h of incubation.

The spontaneous production of TARC/MDC mRNA and protein was suppressed by IL-4/IL-13, but enhanced by IFN-γ We addressed the effects of IL-4/IL-13 or IFN-γ on TARC/MDC production. HaCaT cells were cultured with or without 0.1 to 10 ng per ml each of IL-4, IL-13, or IFN-γ for 48 h. As shown in Fig 2, both IL-4 and IL-13 downregulated the spontaneous production of TARC/MDC in a concentration-dependent manner. In contrast, IFN-γ augmented the spontaneous production of TARC/MDC. We evaluated whether the regulation of TARC/MDC production by IL-4/IL-13 or IFN-γ accompanied the regulation of TARC/MDC mRNA expression using RT-PCR. As shown in Fig 3*A*, in HaCaT keratinocytes, TARC and MDC mRNA expression was suppressed as early as 1 h after the addition of either IL-4 or IL-13 and gradually decreased thereafter. In contrast, TARC and MDC mRNA expression was enhanced significantly for 24 h by IFN-γ. Like HaCaT cells, TARC/MDC mRNA expression was suppressed by IL-4 and IL-13, but enhanced by IFN-γ for 24 h of incubation in normal human keratinocytes (Fig 3*B*).

E-cadherin expression was downregulated by IL-4 or IL-13, but was upregulated by IFN-γ To explore the regulatory mechanisms involved, we investigated the capacity of IL-4/IL-13 or IFN-γ to induce or regulate the expression of several adhesion molecules that may affect TARC and MDC production. After 48 h of incubation with the medium alone or with 10 ng per mL IL-4, IL-13, or IFN-γ, the expression of adhesion molecules was determined by flow cytometry. As indicated in Fig 4, of the adhesion molecules examined, the constitutive expression of E-cadherin and CD54 was downregulated by IL-4, whereas this expression was upregulated by IFN-γ in HaCaT keratinocytes. IL-13 presented similar results as that observed for IL-4 (data not shown). Nevertheless, neither CD11b nor CD18, which are ligands for CD54, was induced, even after cytokine stimulation. In normal human keratinocytes, IFN-γ also upregulated the E-cadherin expression and IL-4/IL-13 downregulated its expression (Table II).

Disruption of homophilic adhesion of E-cadherins abolished the spontaneous production of TARC and MDC To determine whether the homophilic interaction between E-cadherins might be involved in the regulation of TARC/MDC production, the production of TARC and MDC was assessed after using an anti-E-cadherin antibody that blocks binding activity. The anti-E-cadherin antibody (1–30 μg/ml) dramatically reduced TARC/MDC production in a concentration-dependent manner (Fig 5). To confirm the participation of E-cadherin in TARC/MDC production, we examined the effect of Ca^{2+} depletion on TARC/MDC production. As indicated in the top part of Fig 6, the constitutive expression of E-cadherin was suppressed by Ca^{2+}-free medium treatment and was more potently suppressed by EGTA plus Ca^{2+}-free medium treatment. In accordance with the levels of E-cadherin expression, TARC/MDC production was inhibited (bottom part of Fig 6). These data suggest that the levels of E-cadherin expression may contribute to the regulation of TARC/MDC production by HaCaT cells. Thus, we focused our experiments on the adherens junction complex composed of E-cadherin, α-catenin, β-catenin, and γ-catenin.

IL-4/IL-13 and IFN-γ induced alternations in the distribution of adherens junction proteins We examined whether IL-4/IL-13 or IFN-γ induced any change on the amount of the each adherens junction protein. As shown in Fig 7, no significant changes in the amount of each adherens junction protein were detected in IL-4/IL-13- or IFN-γ-treated cells. These data indicate that the level of E-cadherin expression by IL-4/IL-13 or IFN-γ is not regulated by changes in the amount of adherens junction proteins. We then examined the distribution of the adherens junction complex by confocal immunofluorescent microscopy. As shown in Fig 8, in nontreated cells, staining for E-cadherin, α-catenin, β-catenin, and γ-catenin was localized to the cell membrane. Nevertheless, membrane staining for E-cadherin and catenins was reduced after IL-4/IL-13 stimulation, whereas the immunoreactivity for surface E-cadherin was increased after IFN-γ stimulation (Fig 9). Taken together, these results may indicate that IL-4 and IL-13 promote the internalization of adherens junction complex, leading to a downregulation of TARC/MDC production. In contrast, IFN-γ inhibits the internalization of adherens junction complex, thus upregulating TARC and MDC production.

We also investigated whether the activation of ERK1/2, p38 MAPK, and JNK was triggered by IL-4, IL-13, and

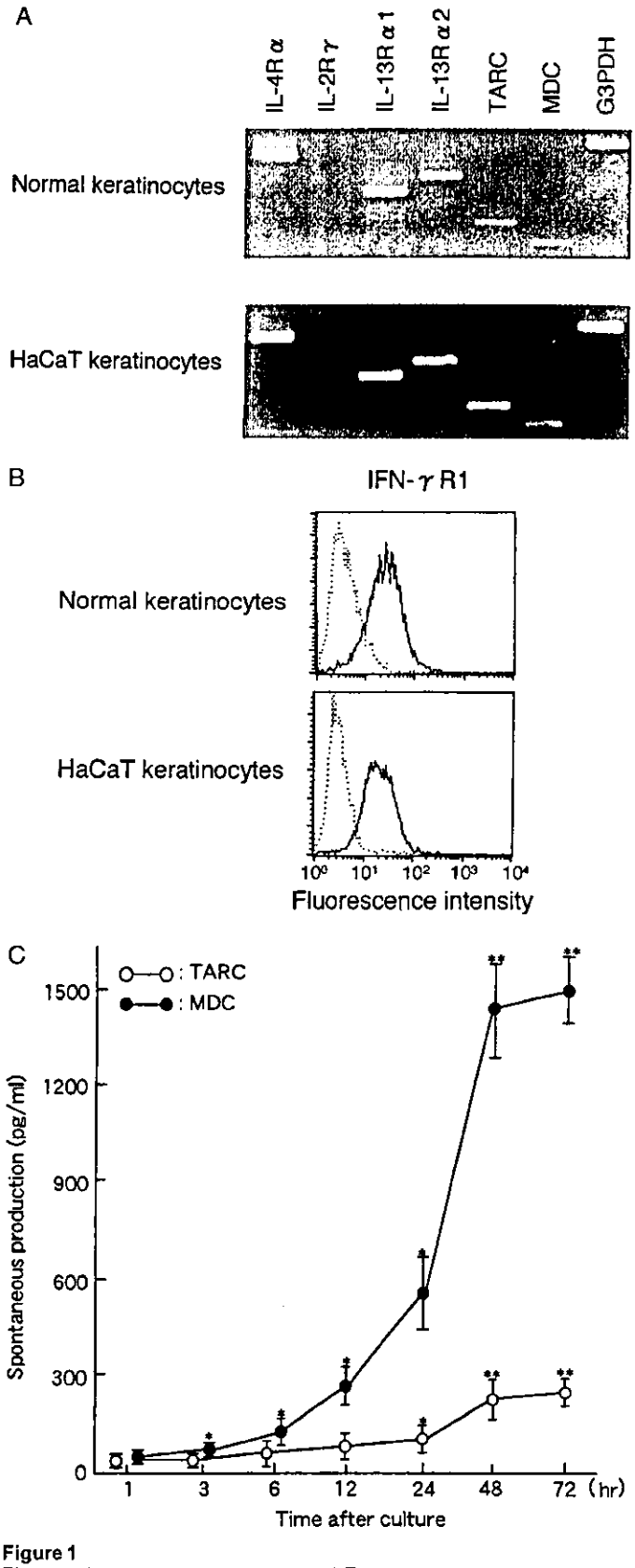

Figure 1
Phenotypic and functional profiles. (*A*) The expression of mRNA for IL-4/IL-13 receptor components and TARC/MDC was analyzed by RT-PCR. (*B*) The surface expression of IFN-γR1 was analyzed by flow cytometry. (*C*) A time-course study of spontaneous TARC/MDC production in HaCaT cells. All values are the mean ± SEM of triplicate experiments. $^{*}p<0.05$; $^{**}p<0.005$.

IFN-γ. The activation of p38 MAPK or JNK by any cytokine was not detected (data not shown). In contrast, ERK1/2 was spontaneously activated, but was not regulated by cytokines (Fig 10).

Discussion

TARC and MDC are very potent chemokines for CCR4 + Th2 cells (Kawasaki *et al*, 2001; Yoshie *et al*, 2001). These chemokines are produced from various cell types such as immunocompetent dendritic cells, T cells, and endothelial cells (Alferink *et al*, 2003; Vulcano *et al*, 2001; Hirata *et al*, 2003; Fukuda *et al*, 2003; Albanesi *et al*, 2001). Because keratinocytes are one of the candidates capable of producing TARC and MDC, the regulatory mechanisms involved in their secretion are of interest. In this study, we compared TARC and MDC secretion from normal human keratinocytes and HaCaT keratinocytes. Consistent with previous findings, HaCaT keratinocytes, but not normal human keratinocytes, secreted TARC and MDC spontaneously. The upregulation of the ERK1/2 signaling pathway in the HaCaT cells may explain this difference. This hypothesis is supported by the fact that an ERK1/2 inhibitor inhibited TARC production by HaCaT keratinocytes (our unpublished data). Further study is necessary to elucidate this point.

HaCaT keratinocytes constitutively express IL-4Rα, IL-13Rα1, and IFN-γR1 on their surface, similar to normal human keratinocytes (David *et al*, 2001; Nickoloff *et al*, 1987; Akaiwa *et al*, 2001). Therefore, we investigated the effect of IFN-γ (Th1 cytokine) and IL-4/IL-13 (Th2 cytokine) on TARC and MDC production. Interestingly, IFN-γ significantly augmented and IL-4/IL-13 inhibited TARC and MDC production in a concentration-dependent manner. Many investigators have reported the regulatory effects of cytokines on TARC/MDC production. PAM 212 cells, a murine keratinocyte cell line, produced TARC after stimulation with either TNF-α or IFN-γ (Vestergaard *et al*, 1999). IL-10 greatly augmented the TNF-α + IFN-γ-induced TARC production by HaCaT cells (Vestergaard *et al*, 2001). In bronchial epithelial cells, Sekiya *et al* (2000) reported that A549 and BEAS-2B cells produce TARC protein after dual stimulation with TNF-α + IL-4. Moreover, IFN-γ synergistically upregulated the TNF-α + IL-4-induced TARC production. Terada *et al* (2001) reported that combined stimulation with IL-4/IL-13 and TNF-α induced TARC expression in nasal epithelial cells. Similarly, human corneal fibroblasts have been shown to produce TARC only in the presence of both IL-4 and TNF-α (Kumagai *et al*, 2000). In monocytes, IL-4/IL-13 induces MDC production, whereas IFN-γ inhibits it (Andrew *et al*, 1998; Bonecchi *et al*, 1998). In HaCaT keratinocytes, the production of TARC and MDC are reciprocally regulated by Th1 and Th2 cytokines.

E-cadherin is a potent cell adhesion molecule and is indispensable in the maintenance of the structural and functional rigidity of the epithelium. The blockage of cadherins by antibodies induced tissue destruction and the dissociation of the cultured tissue (Vestweber and Kelmer, 1985). Also, misregulated E-cadherin expression or

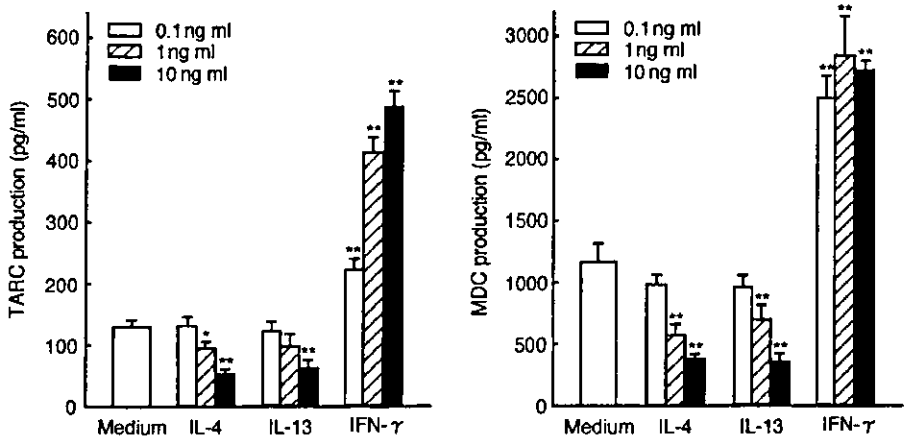

Figure 2
Effects of IL-4/IL-13 and IFN-γ on TARC/MDC production. Cells were incubated with medium alone or with IL-4, IL-13, or IFN-γ for 48 h. After incubation, the concentrations of TARC/MDC in the supernatants were quantified by ELISA procedures. Results represent the mean ± SEM of triplicate experiments. *$p<0.05$ and **$p<0.005$ indicate a statistically significant difference versus unstimulated control cells.

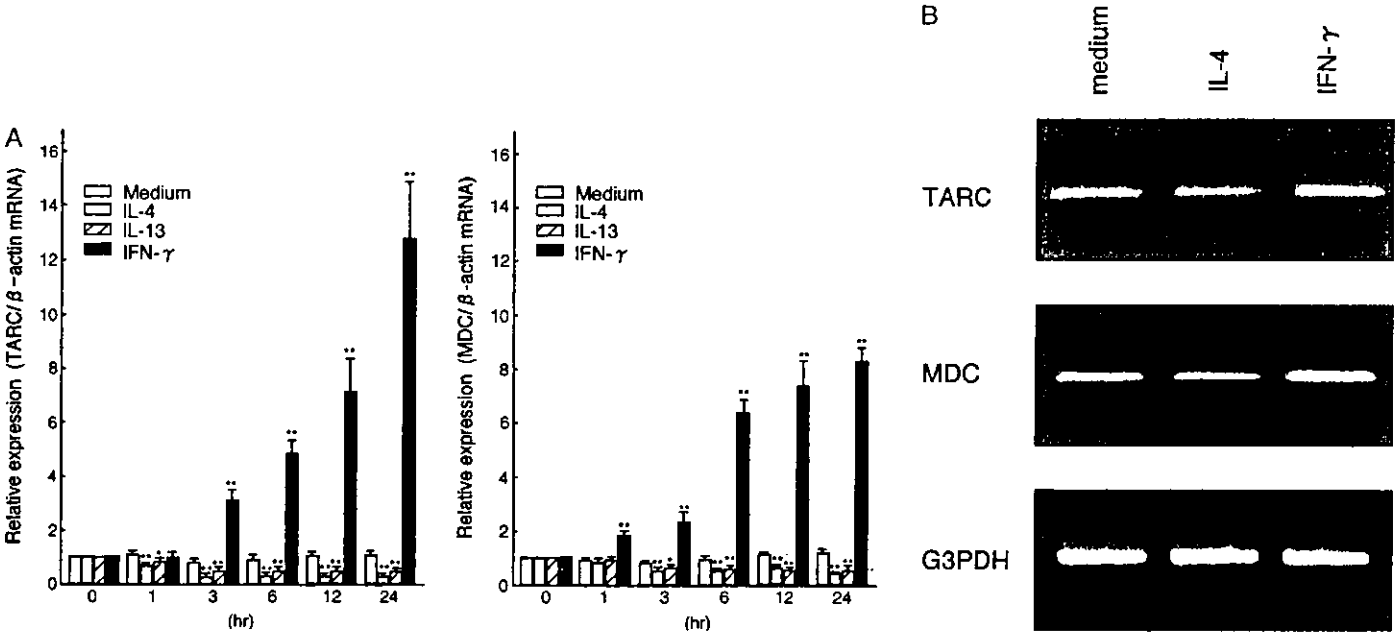

Figure 3
Effects of IL-4/IL-13 and IFN-γ on TARC/MDC mRNA expression. (*A*) HaCaT cells were incubated with medium alone or with 10 ng per mL each of IL-4, IL-13, or IFN-γ for the indicated times. The ratio of TARC/MDC mRNA to β-actin mRNA was determined by quanti-tative real-time RT-PCR. Results represent the mean ± SEM of triplicate experiments. *$p<0.05$; **$p<0.005$. (*B*) Normal human keratinocytes were incubated with medium alone or with 10 ng per mL each of IL-4 or IFN-γ for 24 h. mRNA for TARC/MDC was analyzed by RT-PCR. Like IL-4, IL-13 gave similar results (data not shown).

function can alter the pattern of epithelial cell growth, and differentiation (Gumbiner, 1996).

Several investigators have reported the regulation of E-cadherin expression by IL-4, IL-13, and IFN-γ (Fenyves *et al*, 1993; Kanai *et al*, 2000). In bovine microvascular endothelial cells, IFN-γ upregulated E-cadherin expression (Fenyves *et al*, 1993). In a human colon cancer cell line (colo205), IL-4 and IL-13 inhibit cell–cell adhesion by downregulating E-cadherin (Kanai *et al*, 2000). In this study, we demonstrated that the surface expression of E-cadherin in keratinocytes is suppressed by IL-4/IL-13, but is enhanced by IFN-γ. E-cadherin-mediated cell–cell adhesion is disrupted by Th2 cytokines, but enhanced by the Th1 cytokine. We demonstrated that the integrity of surface E-cadherin was closely linked to the production of TARC and MDC. Disruption of the homophilic adhesion of E-cadherins by anti-E-cadherin antibody blockade or by extracellular calcium chelating abolished the spontaneous production of TARC and MDC. This strongly suggests that E-cadherin is involved in the regulation of TARC/MDC production. In keratinocytes, E-cadherin forms an adherens junction complex with β-catenin or γ-catenin and α-catenin. β-Catenin or γ-catenin binds to the cytoplasmic tail of E-cadherin and to α-catenin. α-Catenin in turn binds to the actin cytoskeleton. This assembly is important for cell–cell adhesion (Kondo *et al*, 1998; Kobayashi *et al*, 2000; Pece and Gutkind, 2000; Hu *et al*, 2001). Interestingly, IL-4/IL-13 enhanced the internalization of E-cadherin/catenins complex into the cytoplasm and disrupted surface E-cadherin expression, resulting in the downregulation of TARC/MDC production. Conversely, IFN-γ augmented the surface membrane distribution of E-cadherin/catenins, resulting in the upregulation of TARC/MDC production.

Hu *et al* (2001) reported that in peroxovanadate-treated human keratinocytes, the distribution of adherens junction proteins is altered with the tyrosine phosphorylation of

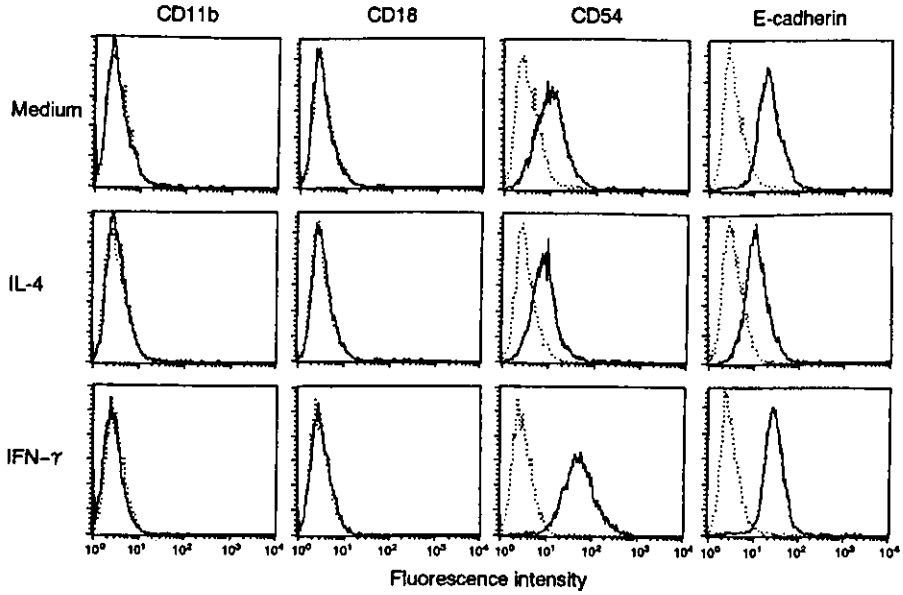

Figure 4
Effects of IL-4 and IFN-γ on CD11b, CD18, CD54, and E-cadherin expression. Cells were cultured with medium alone or with 10 ng per mL each of IL-4 or IFN-γ for 48 h. Expression of these adhesion molecules was analyzed by flow cytometry. Representative data are shown (n = 3). Like IL-4, IL-13 gave similar results (data not shown).

Table I. Sequences of primers used for PCR

Amplified RNA	Primer sequence	Size of amplified product (bp)
IL-4Rα	5′-GCTATGTCAGCATCACCAAGA-3′	830
	5′-TTCTACTTCCTCCAGGTGTCT-3′	
IL-2Rγ	5′-TTTCTTCCTGACCACTATGCC-3′	479
	5′-CAGTCCAGCTGTGGTCCCAG-3′	
IL-13Rα1	5′-GGAGCCAGCTCAATTTGTAG-3′	510
	5′-CACACGGGAAGTTAAAGGCA-3′	
IL-13Rα2	5′-ATGGCTTTCGTTTGCTTGGCTA-3′	620
	5′-TAGTCTGATGCCTCCAAATAGG-3′	
TARC	5′-ATGGCCCCACTGAAGATGCT-3′	351
	5′-TGAACACCAACGGTGGAGGT-3′	
MDC	5′-GCTCGCCTACAGACTGCACTC-CTG-3′	268
	5′-GCTTATTGAGAATCATCTTCACC-CAGG-3′	
G3PDH	5′-TGAAGGTCGGAGTCAACGGATTT-GGT-3′	983
	5′-CATCTGGGCCATGAGGTCCACC-AC-3′	

Table II. Effects of IL-4, IL-13, and IFN-γ on E-cadherin expression in normal human keratinocytes[a]

	Mean fluorescence intensity	Significance versus medium
Medium	7.01 ± 0.63	
IL-4	5.69 ± 0.61	$p < 0.2$
IL-13	5.34 ± 0.83	$p < 0.2$
IFN-γ	8.90 ± 0.87	$p < 0.2$

[a]Cells were cultured with medium alone or with 10 ng per mL each of IL-4, IL-13, or IFN-γ for 48 h. Expression of E-cadherin was analyzed by flow cytometry. Results represent the mean fluorescence intensity ± SEM of triplicate experiments.

β-catenin and γ-catenin. Thus, we examined the tyrosine phosphorylation of adherens junction proteins in the presence of cytokines. No tyrosine phosphorylation was induced by any cytokine stimulation (data not shown). Nelms *et al* (1999) described that in the A431 keratinocyte cell line, IL-4 not only activated the JAK/STAT pathway, but also regulated the activity of various members of the MAPK family, which is in contrast to what has been described in most hematopoietic cells. Therefore, we investigated whether the activation of ERK1/2, p38 MAPK, and JNK were modulated by IL-4, IL-13, and IFN-γ. Our results show that the activation of p38 MAPK, JNK, or ERK1/2 was not modified by any cytokine.

The function of the keratinocyte is known to be regulated via intracellular signaling pathways triggered by growth factors (Hashimoto, 2000). We explored the role of some growth factors in the reciprocal regulation of TARC/MDC production by IL-4/IL-13 and IFN-γ. The growth factors examined, PDGF-AB, IL-6, amphiregulin, EGF, basic FGF, and PDGF-BB, had no effect on the regulation of TARC/MDC production by HaCaT cells (data not shown).

In conclusion, our results suggest that in HaCaT keratinocytes, IL-4/IL-13 and IFN-γ induce differing alterations in the distribution of adherens junctions and, thereby, contribute to the reciprocal regulation of TARC and MDC production. Although the molecular mechanisms triggered by these cytokines remain unclear, our findings that TARC and MDC production are regulated in parallel with E-cadherin expression in keratinocytes may provide a new clue for understanding the orchestrated regulation of TARC/MDC production.

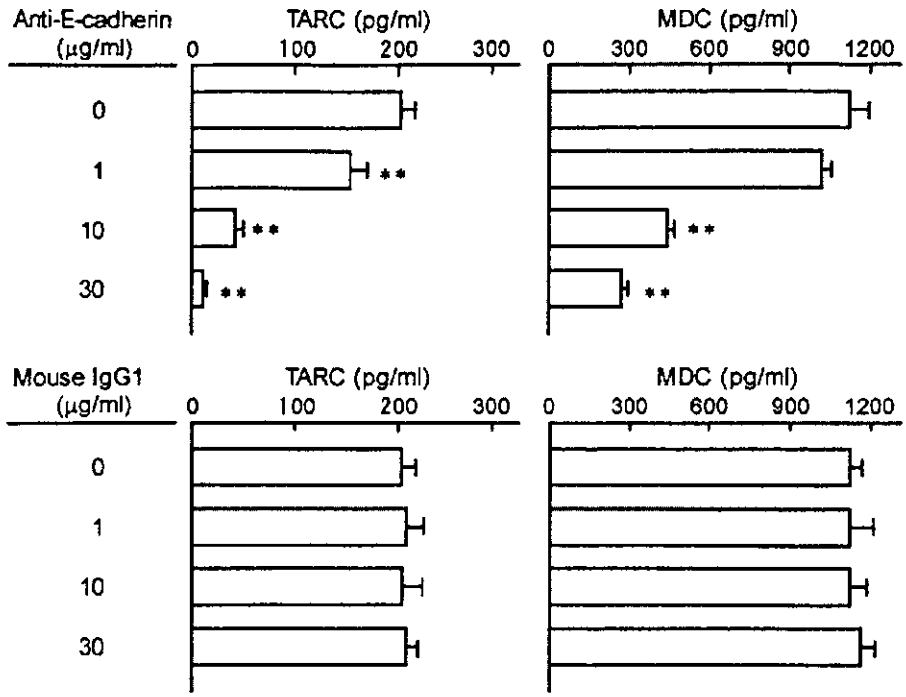

Figure 5
Inhibitory effect of anti-E-cadherin antibody on the spontaneous production of TARC/MDC. Cells were incubated with or without the anti-E-cadherin antibody or control antibody for 48 h. Levels of TARC/MDC in the supernatants were quantified by ELISAs. Results represent the mean ± SEM of triplicate experiments. *$p<0.05$; **$p<0.005$.

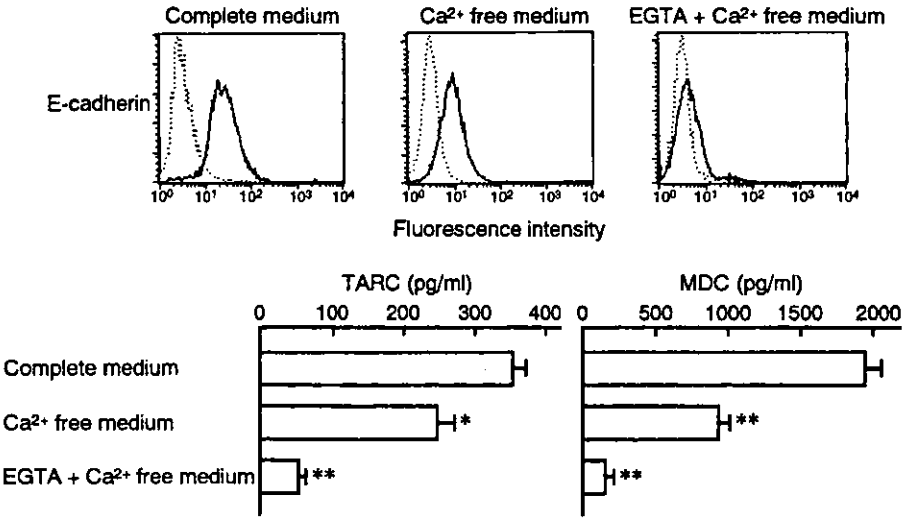

Figure 6
Effects of Ca^{2+} depletion on E-cadherin expression and TARC/MDC production. Cells were cultured with ordinary Ca^{2+}-containing medium for 48 h, with Ca^{2+}-free medium for 48 h, or with Ca^{2+}-free medium for 47 h followed by EGTA (4 mM) treatment for 1 h. After incubation, E-cadherin expression and TARC/MDC production were determined using flow cytometry and ELISA procedures, respectively. Representative data (in top panels) and mean ± SEM (in bottom panels) are shown (n = 3). *$p<0.05$; **$p<0.005$.

Materials and Methods

Reagents Recombinant human IL-4, IL-13, and IFN-γ were purchased from Genzyme (Cambridge, MA). ELISA kits for TARC and MDC were obtained from R&D Systems (Minneapolis, MN). All reagents for quantitative real-time PCR were from Roche Molecular Biochemicals (Germany). For flow cytometry staining, polyclonal anti-IFN-γR1 antibody was obtained from R&D systems (Minneapolis, MN). Fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-CD11b antibody was purchased from Beckman Coulter (Fullerton, CA). FITC-conjugated monoclonal anti-CD18 antibody was purchased from Dako (Denmark). FITC-conjugated monoclonal anti-CD54 antibody was purchased from Serotec (UK). Monoclonal anti-E-cadherin antibody (Immunotech, France) was used for flow cytometry as well as for blocking experiments. Phycoerythrin-conjugated $F(ab')_2$ fragments of goat anti-mouse immunoglobulin antibody was from Biosource (Camarillo, CA). Streptavidin-phycoerythrin was purchased from Becton Dickinson (San Jose, CA). For immunoblotting and immunofluorescent staining, monoclonal anti-α-catenin, monoclonal anti-β-catenin, horseradish peroxidase-conjugated goat anti-mouse IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Zymed (South San Francisco, CA). Monoclonal anti-E-cadherin and anti-γ-catenin antibodies were from Transduction Laboratories (Lexington, KY). Polyclonal anti-phospho (p)-ERK1/2 (Thr202, Tyr204), anti-p-p38 MAPK (Thr180, Tyr182), anti-p-JNK (Thr183, Tyr185), anti-ERK1/2, anti-p38 MAPK, and anti-JNK antibodies were obtained from Cell Signaling Technology (Beverly, MA). FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse immunoglobulin antibody was from Biosource (Camarillo, CA).

Cell culture The HaCaT keratinocyte cell line was provided by N. E. Fusenig, DKFZ Heidelberg (Boukamp *et al*, 1988). HaCaT cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (Gibco-BRL), 100 U per mL penicillin, 100 μg per mL streptomycin (Gibco-BRL) at 37°C. In specific experiments described below, cells were serum-starved for 24 h before

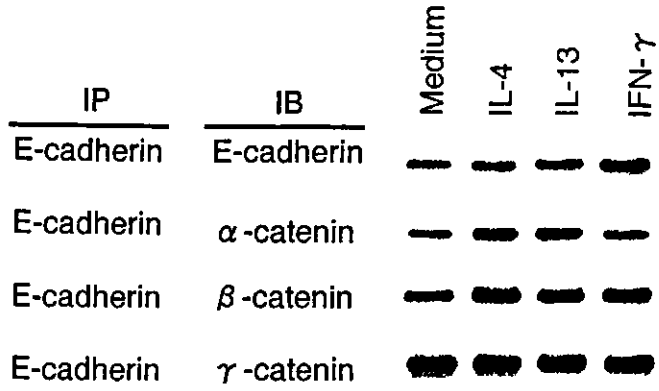

Figure 7
Analysis of adherens junction complexes composed of E-cadherin, α-catenin, β-catenin, and γ-catenin. Cells were stimulated with or without 10 ng per mL each of IL-4, IL-13, or IFN-γ for 48 h. The lysates were immunoprecipitated with anti-E-cadherin antibody and blotted with monoclonal antibodies to E-cadherin, α-catenin, β-catenin, or γ-catenin.

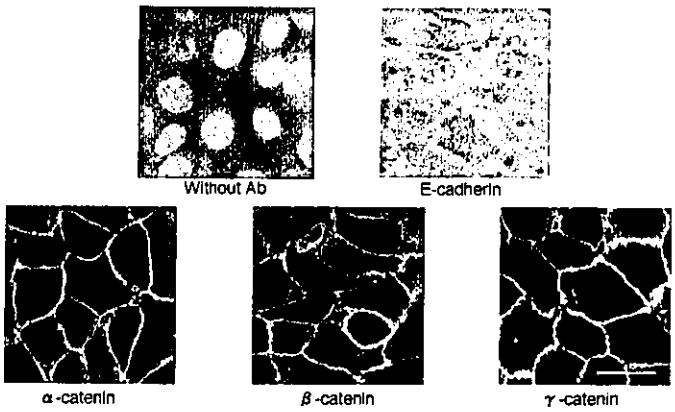

Figure 8
Distribution of adherens junction proteins. After fixation and permeabilization, cells were stained with monoclonal antibodies to E-cadherin, α-catenin, β-catenin, or γ-catenin and detected with FITC-conjugated anti-mouse IgG. Stained cells were analyzed by confocal laser microscopy. *Bar,* 10 μm.

treatment with or without IL-4, IL-13, or IFN-γ. Normal human keratinocytes from neonatal foreskin (Kurabou, Osaka, Japan) were grown initially in keratinocyte basal medium (HuMedia-KB2, Kurabou) containing 0.15 mM calcium supplemented with 0.1 ng per mL epidermal growth factor, 0.4% bovine pituitary extract, 50 μg per mL gentamycin, and 50 ng per mL amphotericin B.

ELISA Concentrations of TARC and MDC in culture supernatants were measured by commercially available ELISA kits according to the manufacturer's instructions. These ELISA procedures enabled detection of TARC concentrations of greater than 15.6 pg per mL and MDC concentrations of greater than 62.5 pg per mL.

RT-PCR Total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden Germany). RT was performed using oligo$(dT)_{12\text{-}18}$ primer (Invitrogen Corp., Carlsbad, CA) and a Superscript II RNase reverse transcriptase kit (Invitrogen Corp.) according to the manufacturer's protocol. To analyze the expression of IL-4/IL-13 receptor components and TARC/MDC in unstimulated HaCaT cells, semiquantitative PCR was performed on a thermal cycler (TaKaRa, Tokyo, Japan) using a Pyrobest DNA polymerase kit (TaKaRa, Tokyo, Japan). The sequences of the PCR primers are shown in Table I. G3PDH was used as a reference gene. The thermal cycling conditions were set to 98°C for 5 min, followed by 28 cycles of 98°C for 10 s, 62°C for 30 s, 72°C for 1 min, and 72°C for 4 min. The amplified products were subjected to electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. We investigated whether 10 ng per mL each of IL-4, IL-13, or IFN-γ had any effect on the expression of TARC/MDC mRNA. Quantitative real-time PCR was performed on a LightCycler (Roche Molecular Biochemicals, Germany) in LightCycler capillaries using the LightCycler FastStart DNA master SYBR green I kit (Roche Molecular Biochemicals) and LightCycler primer sets for TARC (CCL17), MDC (CCL22), and β-actin (Roche Molecular Biochemicals) were used. Thermal cycler conditions were set to 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, 68°C for 10 s, and 72°C for 16 s. To control for the specificity of the amplification products, a melting curve analysis was performed. No amplification of unspecific products was observed. The copy number was calculated from a standard curve.

Flow cytometry The surface expression of IFN-γR1 or adhesion molecules was determined by flow cytometry after 0 or 48 h of incubation with or without 10 ng per mL each of IL-4, IL-13, or IFN-γ. A total of 5×10^5 cells were suspended in 50 μL of phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.05% NaN_3 (FACS buffer). To analyze IFN-γR1 expression, the cells were sequentially incubated with biotinylated anti-IFN-γR1 antibody for 30 min at 4°C and streptavidin-phycoerythrin for 30 min at 4°C. Expression of CD11b, CD18, and CD54 was assessed by staining with the appropriate antibodies labeled with FITC for 30 min at 4°C. To analyze E-cadherin expression, the cells were first incubated with monoclonal anti-E-cadherin antibody for 30 min at 4°C and detected with phycoerythrin-conjugated goat anti-mouse immunoglobulins. In control samples, staining was performed with isotype-matched control antibodies. After being washed and fixed with 0.5% paraformaldehyde, the cells were

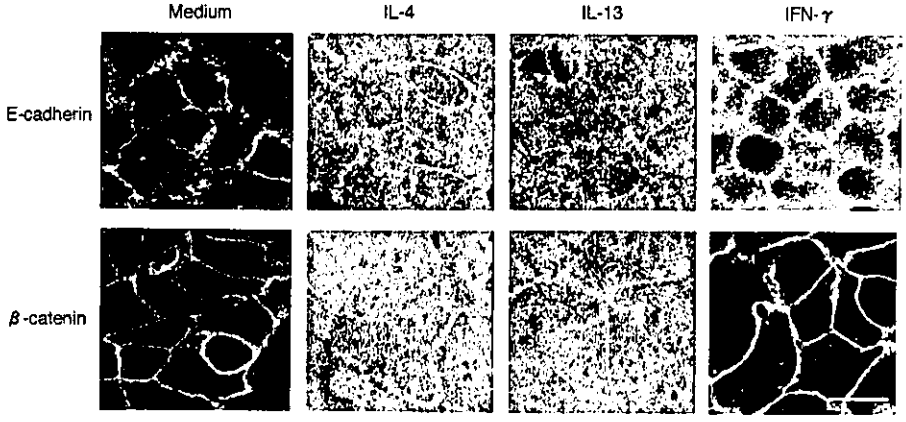

Figure 9
Effects of IL-4/IL-13 and IFN-γ on the distribution of E-cadherin and β-catenin. Cells were treated with medium alone or with 10 ng per mL each of IL-4, IL-13, or IFN-γ for 48 h. After fixation and permeabilization, immunostaining, and microscopic analyses were performed. Similar results were obtained for α-catenin and γ-catenin (data not shown). *Bar,* 10 μm.

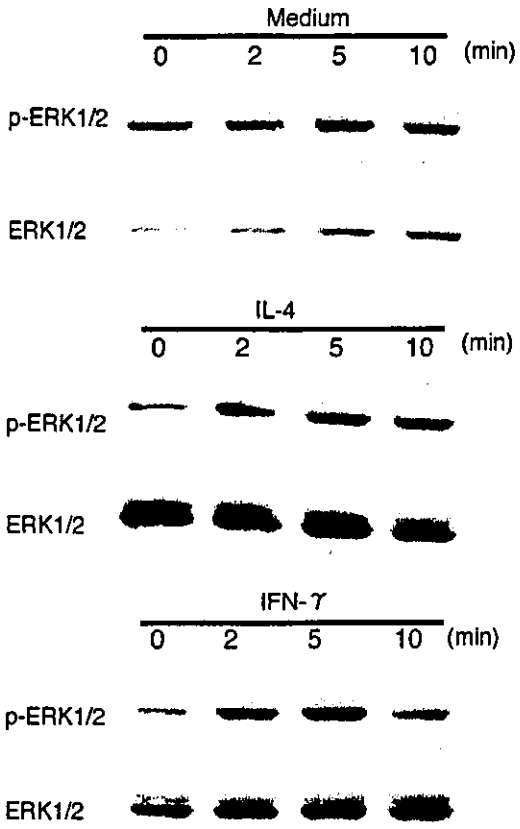

Figure 10
Effects of IL-4 and IFN-γ on ERK1/2 activation. Cells were treated with medium alone or with 10 ng per mL each of IL-4 or IFN-γ for the indicated times. The lysates were blotted with anti-p-ERK1/2 antibody. To evaluate the total amount of ERK present on the blot, the blot was stripped and reprobed with anti-ERK antibody. Like IL-4, IL-13 gave similar results (data not shown).

analyzed by FACSCalibur using CELLQUEST software (Becton Dickinson). Forward- and side-scatter gates were set to identify living cells and 10,000 events were acquired.

Effect of anti-E-cadherin antibody on the spontaneous production of TARC/MDC To assess the involvement of E-cadherin in the regulation of TARC/MDC production, an anti-E-cadherin antibody (1, 10, and 30 μg/ml) and isotype-matched control antibody were added to the cultures for 48 h. After incubation, the supernatant was collected for ELISA procedures for TARC/MDC. In addition, because E-cadherin is a calcium-dependent adhesion molecule, we examined the effect of Ca^{2+} depletion on TARC/MDC production. Cells were cultured with ordinary Ca^{2+}-containing medium (1.8 mM) for 48 h, with Ca^{2+}-free medium for 48 h, or with Ca^{2+}-free medium for 47 h followed by EGTA (4 mM) treatment for 1 h. After incubation, E-cadherin expression and TARC/MDC production were determined using flow cytometry and ELISAs, respectively.

Immunoprecipitation and western blotting The effect of cytokines on the amount of adherens junction complex composed of E-cadherin, α-catenin, β-catenin, and γ-catenin was analyzed by western blotting. After stimulation with or without 10 ng per mL each of IL-4, IL-13, or IFN-γ for 48 h, the cells were lysed on ice with lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM β-glycerol phosphate, 1 mM phenylmethylsufonyl fluoride, 10 μg per mL aprotinin, and 10 μg per mL leupeptin. Protein concentration in extracts was determined by the Bradford assay (Bio-Rad, Hercules, CA). The lysates were precleared with protein A-Sepharose beads (Pharmacia, Sweden) for 1 h and incubated with anti-E-cadherin antibody (3 μg/ml) for 1 h at 4°C. Immunocomplexes were precipitated overnight with protein A-Sepharose beads at 4°C, washed with lysis buffer, eluted by boiling in SDS-PAGE sample buffer containing 2-mercaptoethanol (Wako, Japan), resolved on SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were then blocked with 2% BSA in PBS for 1 h and analyzed by western blotting with sequential probing of the same membrane using monoclonal antibodies to E-cadherin, α-catenin, β-catenin, and γ-catenin diluted to the manufacturer's recommendation in antibody dilution buffer (2% BSA, 0.1% Tween 20 in PBS) (1:250 E-cadherin, 1:1000 α-catenin, 1:1000 β-catenin, 1:2000 γ-catenin) for 1 h at room temperature. Blots were then washed in PBS with 0.1% Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:5000 in antibody dilution buffer. After being washed in PBS with 0.1% Tween 20, the blots were visualized by SuperSignal West pico chemiluminescent substrate (Pierce, Rockford, IL). When necessary, blots were stripped with Restore western blot stripping buffer (Pierce) for 15 min at 50°C, washed in PBS with 0.1% Tween 20, and sequentially reprobed with the appropriate antibodies.

The effects of cytokines on ERK1/2, p38 MAPK, and JNK activation were also examined. Cells were treated with or without 10 ng per mL each of IL-4, IL-13, or IFN-γ for 0 to 10 min. The lysates were blotted with polyclonal antibodies to p-ERK1/2, p-p38 MAPK, or p-JNK. To evaluate the total amount of ERK1/2, p38 MAPK, or JNK present on the blot, the same blot was stripped and reprobed with appropriate antibodies, respectively.

Confocal immunofluorescent microscopy For immunofluorescent staining, HaCaT cells were grown on ethanol-sterilized glass coverslips. Cells were incubated with or without 10 ng per mL each of IL-4, IL-13, or IFN-γ for 48 h before fixation in 3.7% paraformaldehyde in PBS for 5 min at room temperature, permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature, and blocked with 2% BSA in PBS for 15 min at room temperature. Fixed cells were incubated overnight at 4°C in a humidity chamber with each primary antibody (E-cadherin, α-catenin, β-catenin, and γ-catenin) diluted 1:100 in antibody dilution buffer (2% BSA, 0.1% Tween 20 in PBS). After incubation, the coverslips of fixed cells were washed in PBS at 4°C, blocked with 2% BSA in PBS at 4°C for 20 min, incubated for 60 min at 4°C in the dark with FITC-conjugated anti-mouse immunoglobulins diluted 1:100 in antibody dilution buffer, washed in PBS at 4°C, and then mounted with FluoroGuard antifade reagent (Bio-Rad). Stained cells were analyzed by confocal laser microscopy at 600× magnification (Bio-Rad).

Statistical analysis Statistical analyses were performed using an unpaired Student's *t* test.

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Differential effect of IL-4 and IL-13 on the expression of recombination-activating genes in mature B cells from human peripheral blood

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Abstract

We examined the expression of recombination-activating genes (RAG-1 and RAG-2) and activation-induced cytidine deaminase (AID) by mature human blood B cells stimulated with anti-CD40 in the presence of IL-4 or IL-13. IL-4 was an effective cofactor for RAG-1 and RAG-2 expression, whereas IL-13 was not. In addition, IL-4-dependent RAG expression combined with AID and IgE expression allowed predominant expression of newly rearranged λ light chains on IgE^+ cells generated from κ^+ cells. Although the magnitudes of IL-4- and IL-13-dependent AID and IgE expression were related to expression levels of binding subunits of the IL-4 and IL-13 receptors, IL-13 was ineffective for light chain replacement in the induced IgE^+ cells due to the failure in RAG expression. Our studies using mature blood B cells indicate that IL-4-responsive cells, unlike IL-13-responsive cells, undergo λ gene rearrangement leading to replacement in parallel with RAG expression and suggest that this replacement may contribute to the regulation of affinity maturation of IgE antibodies.

Keywords: Mature B cells; IL-4; IL-13; Recombination-activating genes; Light chain rearrangement; Activation-induced cytidine deaminase; IgE isotype switching

1. Introduction

IL-4 and IL-13, which are produced by activated Th2 cells, mast cells, and basophils, are related cytokines that have similar biological effects on various cell types, including B cells, monocytes, and endothelial cells [1–3]. In human B cells, both cytokines upregulate the expression of surface IgM, MHC class II molecules, and CD23 and induce germline Cε transcription that precedes IgE isotype switching [1,2,4,5]. Although the biological functions of IL-4 also include the upregulation of CD40 expression, IL-13 fails to exhibit this effect [5]. Thus, IL-4 does not always share all functional characteristics with IL-13, despite many overlapping activities of these cytokines.

The functional receptors for IL-4 or IL-13 are a heterodimeric complex composed of the IL-4 receptor α chain (IL-4Rα, CD124)[1] and either the common γ chain (γc, CD132) or the IL-13 receptor α1 chain (IL-13Rα1, CD213a1) [6–8]. In addition, the IL-13 receptor α2 chain (IL-13Rα2, CD213a2), which binds IL-13 with much higher affinity than IL-13Rα1, has been shown to act as a decoy receptor because of its short cytoplasmic domain [9,10]. Among these four receptor chains, IL-13Rα2 is not expressed in primary human B cells, although the pattern of expression of each chain depends on the cell type. Human B cells, which express the three chains, IL-4Rα, γc, and IL-13Rα1, respond to both IL-4 and IL-13 with activation of several signaling pathways involving Janus

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[1] *Abbreviations used:* AID, activation-induced cytidine deaminase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; γc, common γ chain; IL-4Rα, IL-4 receptor α chain; IL-13Rα1, IL-13 receptor α1 chain; IL-13Rα2, IL-13 receptor α2 chain; JAK, Janus kinase; LPS, lipopolysaccharide; mAb, monoclonal antibody; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cells; RAG, recombination-activating gene; RT, reverse transcription; STAT, signal transducers and activators of transcription.

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kinases (JAK1, JAK3, and Tyk2), adaptor molecules (insulin receptor substrate-1/2 and Src homologous and collagen), and signal transducers and activators of transcription (STAT3 and STAT6) [11–15]. In particular, JAK-dependent activation of STAT6 induced in B cells by IL-4 and IL-13 is attributed to their shared activities, such as enhanced expression of CD23 and induction of germline Cε transcription. In this respect, the IL-4Rα that associates with STAT6 plays an important role in mediating the similar biological effects of IL-4 and IL-13.

Although IL-4 and IL-13 direct IgE isotype switching in mature human B cells by inducing germline Cε transcription, additional stimulation with CD154 or anti-CD40 monoclonal antibody (mAb) is required for induction of IgE switching that results in mature Cε transcription and IgE synthesis [2,5,16]. Ligation of CD40 in the presence of an appropriate cytokine has been shown to induce expression of activation-induced cytidine deaminase (AID), a B cell-specific RNA-editing enzyme, which is involved in the regulation of the DNA modification step of isotype switching [17–19]. Furthermore, several studies using mature mouse B cells have shown that IL-4 combined with anti-CD40 mAb or lipopolysaccharide (LPS) induces expression of recombination-activating gene (RAG)-1 and RAG-2 which permits secondary V(D)J rearrangement of Ig genes [20–23]. Although RAG-1 and RAG-2 are expressed in vivo at high levels during B cell development in the primary lymphoid organ, their expression is downregulated or absent in mature B cells [24]. Nevertheless, RAG-1 and RAG-2 reexpressed in mature mouse B cells activated in vitro are involved in secondary V(D)J recombination, termed receptor revision [25]. Reexpression of RAG-1 and RAG-2 can also be seen in germinal center B cells of secondary lymphoid organs in both mice and humans [20,21,26–28]. Although the nature of RAG-expressing B cell subsets in human tonsils is still controversial [29], no information on whether mature human blood B cells activated in vitro reexpress RAG-1 and RAG-2 has been provided to our knowledge.

In the present study, we examined both RAG and AID expression by mature human blood B cells stimulated in the presence of anti-CD40 mAb with IL-4 or IL-13. Our results show that RAG-1 and RAG-2 expression is induced in response to IL-4, but not to IL-13, whereas AID expression is expectedly induced in response to both IL-4 and IL-13, and provide evidence that IL-4-dependent RAG expression combined with AID and IgE expression is accompanied by receptor revision in the induced IgE^+ cells.

2. Materials and methods

2.1. B cell preparation and culture

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation from heparinized venous blood of healthy adult donors who had given consent to the blood donation, as described previously [30]. B cells were negatively isolated from PBMC by an indirect magnetic labeling system using mouse mAbs against CD2, CD14, CD16, CD36, and CD56 (Dynal, Oslo, Norway) according to the manufacture's instructions. This sorted population contained >99% $CD40^+$ cells, >98% IgD^+ cells, 60–70% κ^+ cells, 30–40% λ^+ cells, and <1% $CD3^+$ cells, as determined by flow cytometry. In some experiments, depletion of λ^+ cells was used to isolate κ^+ B cells (purity >98%). This isolation was done using biotinylated mouse anti-λ mAb (Becton–Dickinson, San Jose, CA) and streptavidin-coated magnetic beads (Dynal). Unfractionated or κ^+ B cells were suspended at a concentration of 1×10^6 cells/ml in Iscove's modified Dulbecco's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Aliquots of the cell suspension in a total volume of 0.2 ml were cultured in the presence of 1 μg/ml mouse anti-CD40 mAb (Ancell, Bayport, MI) in 96-well round-bottomed plates (Costar, Cambridge, MA) with appropriate concentrations of IL-4 or IL-13 (Genzyme, Minneapolis, MN) for the indicated time periods.

In separate experiments, tonsil tissue was obtained from one child undergoing tonsillectomy and finely minced. Suspensions of tonsil cells were prepared as described above. B cells were isolated from tonsil cells by negative selection using magnetic separation and simply used as a positive control for detection of AID and RAG mRNAs [18,28].

2.2. Flow cytometry

The following antibodies were used for flow cytometry: mouse anti-IL-4 Rα and anti-γc mAbs (Genzyme); mouse anti-CD40 mAb (BioSource, Camarillo, CA); mouse anti-CD3, anti-CD19, anti-κ, and anti-λ mAbs (Becton–Dickinson); mouse anti-IgM, anti-IgD, and anti-IgE mAbs (PharMingen, San Diego, CA); and goat anti-IL-13Rα1 and anti-IL-13Rα2 polyclonal antibodies (R&D Systems, Mineapolis, MN). Detection of IL-4 binding cells was performed at 4 °C using a combination of biotinylated IL-4 (15 ng/1 × 10^5 cells/35 μl) and fluorescein isothiocyanate (FITC)-labeled avidin (R&D Systems) according to the manufacture's instructions. Detection of IL-13 binding cells was performed at 4 °C using a combination of unlabeled IL-13 (50 ng/1 × 10^5 cells/35 μl), biotinylated mouse anti-IL-13 mAb, and phycoerythrin (PE)-labeled streptavidin. This procedure was established on the basis of several experiments using different concentrations of unlabeled IL-13. Biotinylated anti-IL-13 mAb with no interference with binding of IL-13 to IL-13Rα1 and PE-labeled streptavidin were obtained from PharMingen. Intact and IL-13Rα1-transfected

DND39 cells were used as positive control cells for IL-4 and IL-13 binding experiments [14,15]. The transfectant was provided by Dr. K. Izuhara (Saga Medical School, Saga, Japan). A combination of biotinylated anti-IgE mAb and PE-labeled streptavidin was used for detection of cell surface IgE, as described previously [31,32]. Additionally, expression of λ chains was analyzed by gating on IgE^+ population, which was also stained with FITC-labeled anti-λ mAb. In each assay, an isotype-matched control mAb, unimmunized goat IgG, or soybean trypsin inhibitor was used for negative staining. Samples were analyzed on a FACSCalibur using CellQuest software (Becton–Dickinson).

2.3. Reverse transcription-polymerase chain reaction

Extraction of total cellular RNA, cDNA synthesis by reverse transcription (RT), and polymerase chain reaction (PCR) were performed exactly as described previously [30]. The primer pairs used for the amplification of mRNA for germline Cε, mature Cε, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were the same as described previously [14,30]. The resultant cDNA was also amplified by PCR using following sense and antisense primers: IL-4Rα, 5′ GCTATGTCAGCATCACC AAGA 3′ and 5′ TTCTACTTCCTCCAGGTGTCT 3′; IL-13Rα1, 5′ GGAGCCAGCTCAATTTGTAG 3′ and 5′ CCACGGGAAGTTAAAGGCA 3′; IL-13Rα2, 5′ AT GGCTTTCGTTTGCTTGGCTA 3′ and 5′ TAGTCTG ATGCCTCCAAATAGG 3′; γc, 5′ TTTCTTCCTGAC C ACTATGCC 3′ and 5′ CAGTCCAGCTGTGGTCC CAG 3′; AID, 5′ GAGGCAAGAAGACACTCT GG 3′ and 5′ GTGACATTCCTGGAAGTTGC 3′; RAG-1, 5′ ATGGCAGCCTCTTTCCCACCC 3′ and 5′ ACATCT GCCTTCACATCGATC 3′; and RAG-2, 5′ CACTC TAGGGATTCAAAGATC 3′ and 5′ GATGTGTAGC TTTGGAAATCT 3′. The amplified products were verified to increase in proportion to the amount of template, electrophoresed on 2% agarose gel, and stained with ethidium bromide. The PCR using above primer pairs yields an 830-bp product (IL-4Rα), a 510-bp product (IL-13Rα1), a 620-bp product (IL-13Rα2), a 479-bp product (γc), a 647-bp product (AID), a 500-bp product (RAG-1), and a 500-bp product (RAG-2).

In some experiments, quantitative real-time PCR was performed using primer pairs optimized for the LightCycler (Roche Molecular Biochemicals, Heidelberg, Germany). The sequences of sense and antisense primers were as follows: AID, 5′ AGGCGTGACAGTGCTA CATC 3′ and 5′ CAGAAAGTCGGCCACATGTC 3′; RAG-1, 5′ GTGAGGGAAATGAGTCTGGT 3′ and 5′ CCTAATGGGTCCCCTAAGCT 3′; and RAG-2, 5′ GCCATGATCTACTGCTCTC 3′ and 5′ CTTTGGGG AGTGTGTAGAG 3′; and GAPDH, 5′ TGAACGG GAAGCTCACTGG 3′ and 5′ TCCACCACCCTGTTG CTGTA 3′. Thermal cycler conditions were set to 95 °C for 10 min, followed by 40 cycles (AID, RAG-1, and RAG-2) or 30 cycles (GAPDH) of 95 °C for 10 s, 62 °C (AID, RAG-1, and RAG-2) or 68 °C (GAPDH) for 10 s, and 72 °C for 7 s (AID), 8 s (RAG-1), 6 s (RAG-2), or 16 s (GAPDH). No amplification of unspecific products was observed when a melting curve analysis was carried out to control for specificity of the amplification products. The copy number of AID, RAG-1, and RAG-2 was normalized by GAPDH and expressed as the number of transcripts per 10^3 copies of GAPDH.

2.4. Confocal microscopy

Immunofluorescence confocal analysis of RAG-1 and RAG-2 protein expression was performed according to some modifications of the procedure described elsewhere [20]. Briefly, cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. To display RAG proteins, cells were incubated first with rabbit anti-RAG-1 or anti-RAG-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and then reacted with Alexa Fluor 568-labeled goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR). To visualize the nuclei, cells were stained with 4′,6-diamidine-2-phenylindole (DAPI; Kirkegaard & Perry Laboratories, Gaithersburg, MD), which is known to bind to double-stranded DNA [33]. Slides were mounted using FluoroGuard antifade reagent (Bio-Rad Laboratories, Hercules, CA). Samples were analyzed on a confocal laser scanning microscope (Bio-Rad Laboratories).

3. Results

3.1. Phenotypic characterization of blood B cells

We first compared the expression levels of IL-4 and IL-13 receptor chains in freshly isolated blood B cell population, which contained >98% IgD^+ cells and <1% $CD3^+$ cells (Fig. 1A), with those in intact and IL-13Rα1-transfected DND39 cells (Figs. 1B and C). RT-PCR analysis showed that mature blood B cells were positive for IL-4Rα, γc, and IL-13Rα1 mRNAs and not IL-13Rα2 mRNA. This pattern of receptor chain gene expression confirmed previous reports [7,8,10]. Furthermore, flow cytometric results revealed that IL-4Rα, γc, and IL-13Rα1 were expressed on 96.1 ± 1.9%, 95.5 ± 1.7%, and 32.4 ± 0.8% of the B cells from five different donors, respectively. Consistent with the frequency of IL-4Rα- or IL-13Rα1-bearing B cells, IL-4 or IL-13 binding experiments gave similar results. Our observation of low levels of IL-13Rα1 on blood B cells is not due to the poor sensitivity of the assay, because intact and IL-13Rα1-transfected DND39 cells which were examined for comparison showed the predicted pattern and level of IL-13Rα1 expression. Separate experiments

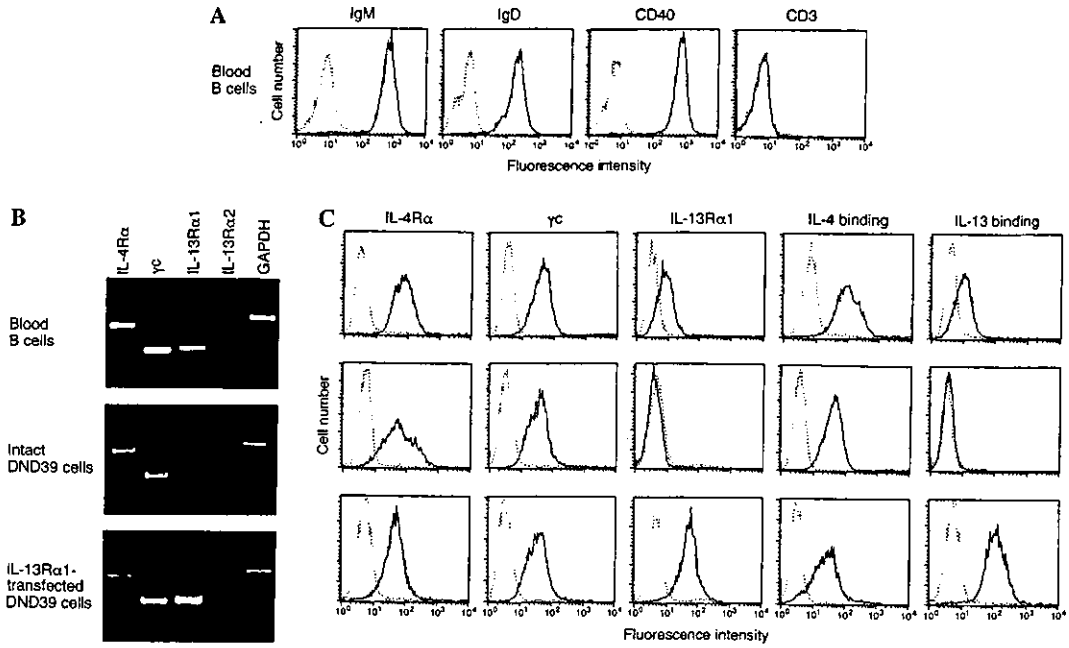

Fig. 1. Profile of IL-4 and IL-13 receptor chain expression in freshly isolated blood B cells, intact DND39 cells, and IL-13Rα1-transfected DND39 cells. Purity of the blood B cell preparation was assessed by flow cytometry (A). Cells were analyzed for the expression of IL-4Rα, γc, IL-13Rα1, and IL-13Rα2 mRNAs by RT-PCR (B). Cell surface expression of IL-4Rα, γc, and IL-13Rα1 and binding of IL-4 and IL-13 to cells were analyzed by flow cytometry (C). The dotted tracing in each flow cytometric assay indicates background staining. The results shown are representative of five different donors (blood B cells) and two independent experiments (intact and IL-13Rα1-transfected DND39 cells).

performed in unfractionated PBMC also showed that 30–40% of the $CD19^+$ cells were positive for IL-13Rα1 (data not shown).

We next examined RAG and AID mRNA expression in blood B cells by RT-PCR. Fig. 2A shows the results of nine different samples. Neither RAG-1 nor AID mRNA was found in all nine samples, whereas RAG-2 mRNA was detected in two samples. These three mRNAs, however, were detectable in tonsil B cells, which were used as a positive control (Fig. 2B). The results obtained in blood B cells accord with a previous report describing that about 20% of the cell preparations negative for RAG-1 mRNA were positive for RAG-2 mRNA [28].

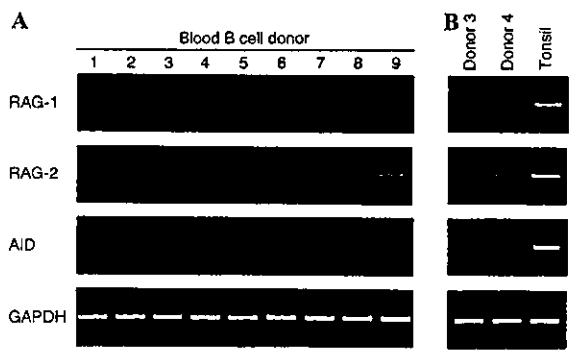

Fig. 2. RAG and AID mRNA expression in freshly isolated blood and tonsil B cells. Blood B cells from nine different donors were analyzed for the expression of RAG-1, RAG-2, and AID mRNAs by RT-PCR (A). To confirm the results obtained in two (donors 3 and 4) of the nine blood B cell preparations, tonsil B cells were used as a positive control and examined for comparison (B).

Thus, the RAG double-negative B cells were selected and used throughout the present experiment.

3.2. Kinetics of RAG, AID, and Cε transcription in stimulated B cells

Blood B cells were stimulated in the presence of anti-CD40 mAb with IL-4 (5 ng/ml) or IL-13 (50 ng/ml) for up to 72 h and analyzed for RAG, AID, and Cε transcription by RT-PCR. Results of one representative experiment among five identical experiments with different donors are shown in Fig. 3. IL-4 was effective in inducing RAG-1 transcription, which occurred by 6 h and persisted to at least 72 h. RAG-2 was also expressed in parallel with RAG-1 in response to IL-4. In contrast, IL-13 was ineffective for RAG-1 and RAG-2 transcription. Although germline Cε, AID, and mature Cε transcripts were expectedly expressed in response to both IL-4 and IL-13, IL-4-dependent RAG transcription was initiated before the induction of AID and mature Cε transcription.

3.3. Quantitative analysis of RAG and AID transcription in stimulated B cells

Quantitative real-time PCR was performed to determine the expression levels of RAG-1, RAG-2, and AID transcripts in blood B cells stimulated in the presence of anti-CD40 mAb with IL-4 (5 ng/ml) or IL-13 (5–100 ng/ml) for 3 days. Table 1 summarizes the results obtained

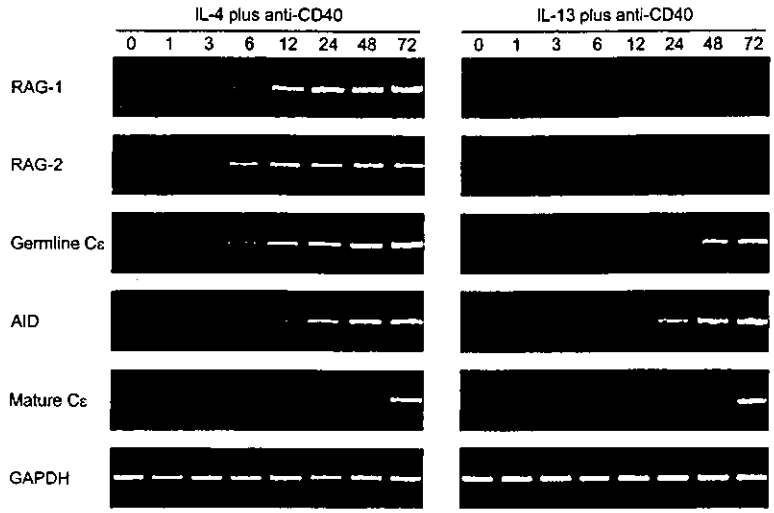

Fig. 3. Kinetics of RAG, AID, and Cε transcription in stimulated blood B cells. B cells were stimulated in the presence of anti-CD40 mAb with IL-4 (5 ng/ml) or IL-13 (50 ng/ml) for up to 72 h, followed by assessment of RAG-1, RAG-2, germline Cε, AID, and mature Cε transcription by RT-PCR. Results are representative of five experiments with different donors.

Table 1
Failure of IL-13 to induce RAG transcription in blood B cells activated via CD40

Stimulus	Relative expression (target/GAPDH)		
	RAG-1	RAG-2	AID
Vehicle	ND	ND	ND
IL-4			
5 ng/ml	6.8 ± 1.5	3.6 ± 0.9	201.5 ± 33.8
IL-13			
5 ng/ml	ND	ND	42.9 ± 10.1
50 ng/ml	ND	ND	78.2 ± 16.5
100 ng/ml	ND	ND	64.3 ± 12.9

B cells from five different donors were stimulated in the presence of anti-CD40 mAb with IL-4 (5 ng/ml) or IL-13 (5–100 ng/ml) for 3 days. Expression of RAG-1, RAG-2, and AID transcripts was determined by quantitative real-time PCR and evaluated as the number of transcripts per 10^3 copies of the housekeeping gene GAPDH. Results are expressed as mean values ± SEM. ND, not detected.

from five different donors. Moderate levels of RAG-1 and RAG-2 and marked levels of AID were expressed in response to IL-4. Furthermore, expression level of RAG-1 were about two times higher than that of RAG-2. Although IL-13, even when used at 100 ng/ml, failed to induce detectable RAG-1 and RAG-2 expression, considerable levels of AID were expressed in response to IL-13, regardless of its concentration. A dose–response study of IL-13 showed that maximal induction of AID expression was obtained at 50 ng/ml. Consistent with the RT-PCR analysis shown in Fig. 3, quantitative real-time PCR revealed that RAG transcription combined with AID transcription was induced in response to IL-4, but not to IL-13.

3.4. Immunofluorescence analysis of RAG protein expression in stimulated B cells

To examine RAG protein expression, confocal analysis was performed in blood B cells stimulated in the presence of anti-CD40 mAb with IL-4 (5 ng/ml) or IL-13 (50 ng/ml) for 3 days. As shown in Fig. 4, RAG-1 and RAG-2 proteins were expressed in response to IL-4, but not to IL-13. In addition, nuclear localization of RAG-1 and RAG-2 was found in the positive cells, as determined by a counterstaining with DAPI. Although RAG-1 plus RAG-2-positive cells were not examined, confocal analysis also confirmed that IL-4, but not IL-13, was effective in inducing RAG expression.

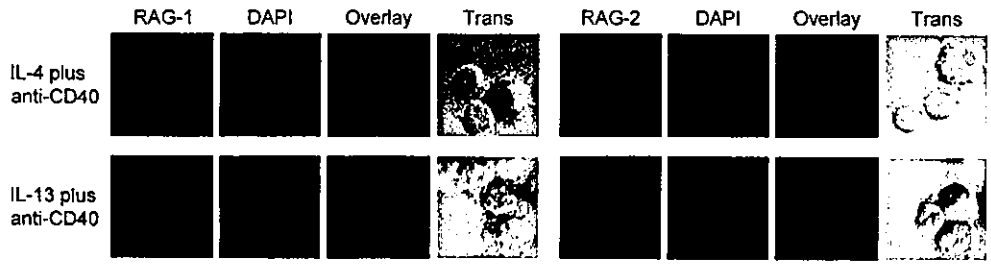

Fig. 4. Immunofluoresence analysis of RAG protein expression in stimulated blood B cells. Immunofluorescence confocal microscopy of RAG-1 and RAG-2 was performed in B cells stimulated in the presence of anti-CD40 mAb with IL-4 (5 ng/ml) or IL-13 (50 ng/ml) for 3 days. To determine the nuclear localization of the labelling with anti-RAG antibodies, DAPI was used as a counterstain for the nucleus. Stained cells were analyzed at ×600 magnification. Results are representative of three experiments with different donors.

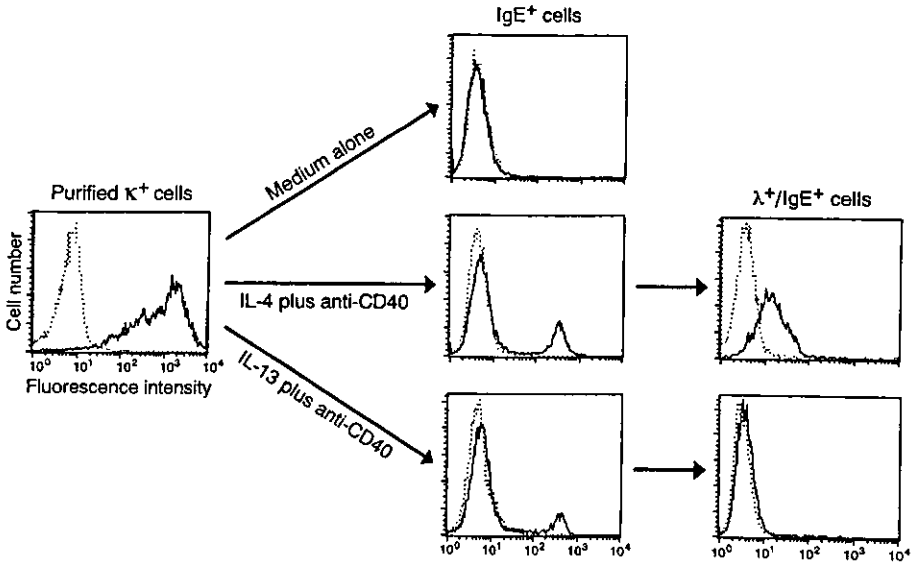

Fig. 5. Induction of λ chains in κ chain-bearing B cells. Purified κ^+ cells were prepared by an indirect magnetic labeling system using anti-λ mAb to deplete λ^+ cells from blood B cells. Purity of the κ^+ cell preparation was confirmed by flow cytometry (left panel). Purified κ^+ cells were cultured with medium alone, IL-4 (5 ng/ml) plus anti-CD40 mAb, or IL-13 (50 ng/ml) plus anti-CD40 mAb for 5 days, followed by assessment of the expression of surface IgE by flow cytometry (middle panels). Expression of λ chains was further analyzed by gating on the induced IgE^+ cells (right panels), as described in Materials and methods. The dotted tracing in each panel indicates background staining. Results are representative of five experiments with different donors.

3.5. Functional analysis of RAG gene products

To determine whether RAG-1 and RAG-2 proteins expressed in response to IL-4 were functional, we examined the induction of λ chains in κ chain-bearing cells, a phenomenon known as RAG-mediated light chain rearrangement [20,21,25]. For this purpose, B cells from five different donors were depleted of λ^+ cells by positive selection and the κ^+ cells obtained were then stimulated in the presence of anti-CD40 mAb with IL-4 (5 ng/ml) or IL-13 (50 ng/ml). After stimulation for 5 days, the cells were treated with acid buffer and analyzed for the surface expression of IgE by flow cytometry, as described previously [31,32]. IgE was expressed on $15.3 \pm 2.7\%$ and $9.3 \pm 1.5\%$ of the total cells in response to IL-4 and IL-13, respectively (Fig. 5). Since RAG expression was inducible in IL-4-responsive cells, but not in IL-13-responsive cells, the expression of λ chains was further analyzed by gating on the induced IgE^+ cells. As expected, λ chains were detected on $18.2 \pm 3.7\%$ of the IgE^+ cells induced by IL-4, but not on those induced by IL-13. These results showed that some κ^+ cells became positive for λ chains after stimulation with IL-4 plus anti-CD40 that led to RAG expression.

4. Discussion

The present study shows that IL-4 induces RAG-1 and RAG-2 expression in mature human blood B cells activated via CD40, whereas IL-13 does not. RAG-1 and RAG-2 expressed in response to IL-4 are functional because they were involved in secondary rearrangement of Ig genes leading to light chain replacement. AID was expectedly expressed in response to both IL-4 and IL-13 and the expression was followed by induction of mature Cε transcription, a phenomenon known to result from IgE isotype switching [16]. In addition to these functional features of mature blood B cells, there is a quantitative difference between IL-4Rα- and IL-13 Rα1-bearing cells. Indeed, expression level of IL-4Rα was high compared with that of IL-13 Rα1. This difference might also reflect the magnitude of IL-4- and IL-13-dependent AID and IgE expression. Furthermore, predominant expression of newly rearranged λ chains was detectable on IgE^+ cells induced by IL-4 plus anti-CD40, but not on those induced by IL-13 plus anti-CD40. The results obtained indicate that RAG expression combined with AID and IgE expression is inducible in IL-4-responsive cells, but not in IL-13-responsive cells.

In mature blood B cells that were stimulated with IL-4 plus anti-CD40, RAG transcription was followed by AID and mature Cε transcription. Thus, these three events require a similar B cell activation status, in which revision of antigen receptors may precede mature Cε transcription. The induction of λ chains in κ^+ cells has been shown to result from new rearrangement of λ chain genes which is mediated by the products of RAG-1 and RAG-2 reexpressed in mature B cells [21,22,27,34]. When κ^+ cells purified from mature blood B cells were stimulated in the presence of anti-CD40 with IL-4, about 20% of the induced IgE^+ cells became positive for λ chains possibly due to RAG-dependent λ chain gene rearrangement. In contrast, IgE^+ cells induced in response to IL-13