Cutting Edge: Polymorphisms in the ICOS Promoter Region Are Associated with Allergic Sensitization and Th2 Cytokine Production

Rebecca A. Shilling, Jayant M. Pinto, Donna C. Decker, Daniel H. Schneider, Hozefa S. Bandukwala, Jeffrey R. Schneider, Blanca Camoretti-Mercado, Carole Ober and Anne I. Sperling

*J Immunol* 2005; 175:2061-2065; doi: 10.4049/jimmunol.175.4.2061

http://www.jimmunol.org/content/175/4/2061

References

This article cites 30 articles, 10 of which you can access for free at: http://www.jimmunol.org/content/175/4/2061.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The establishment of ICOS as an important regulator of Th2 development and effector function makes the ICOS locus an attractive candidate for Th2-mediated diseases, such as asthma and allergy. In evaluation of this candidate locus in humans, we identified 11 variants and determined that two in the putative promoter region are significantly associated with allergic sensitization and serum IgE levels. In addition, cultures of activated PBMCs from individuals homozygous for the associated polymorphisms produced increased levels of the Th2 cytokines, IL-4, IL-5, and IL-13, as well as TNF-α compared with controls. One of the polymorphisms, −1413G/A, demonstrated differential NF-κB binding in mobility shift analysis, suggesting that this polymorphism has functional consequences. Overall, these data demonstrate that ICOS is a susceptibility gene for allergic sensitization, perhaps through the promotion of Th2 differentiation. The Journal of Immunology, 2005, 175: 2061–2065.

The ICOS molecule, a member of the CD28 family, is an important regulator of T lymphocyte function and differentiation (1, 2). Although its ligand, B7RP-1, is expressed constitutively on B cells, dendritic cells, and macrophages, ICOS is T cell specific and induced after activation (2). We and others have found that in vivo ICOS blockade attenuates Th2-mediated pulmonary inflammation in murine models of allergic asthma and reduces Th2 effector cytokine production ex vivo (3–5). Furthermore, ICOS−/− mice have been shown to have markedly reduced levels of IgE, and ICOS−/− T cells produce lower levels of IL-4 compared with wild-type T cells (6–8). Although ICOS has also been shown to be important in Th1-mediated disease models, the literature establishes ICOS as an important regulator of Th2 immune responses (2).

Asthma and allergy are the most common chronic Th2-mediated diseases, affecting millions of people worldwide. ICOS and other members of the CD28 family are located on chromosome 2q33-4, a region that has been linked with asthma, allergy, and related phenotypes in several genome-wide scans (9). Other investigators have studied polymorphisms in the human ICOS gene, but none have examined associations with allergy or conducted functional studies of the variants (10–14). In our study, we surveyed the ICOS gene for variation and identified polymorphisms in the promoter region that are associated with increased Th2 cytokine production, allergic sensitization, and total serum IgE levels.

Materials and Methods
Identification of genomic structure and polymorphism detection
To detect polymorphisms in the ICOS gene, 30 DNA samples were selected without regard to disease status from 10 Hutterites (described below; Ref. 15), 10 unrelated European Americans, and 10 unrelated African Americans. ICOS PCR products were analyzed by denaturing HPLC (Varian Chromatography Systems), and variant denaturing HPLC patterns were sequenced on an ABI 3700 sequencer using the BigDye Terminator technology (Applied Biosystems). Approval from The University of Chicago Institutional Review Board was obtained for these studies.

Genotyping and association studies
To test for associations with asthma and atopy, we studied ~750 members of a 13-generation, 1623-person Hutterite pedigree who were previously evaluated for atopy by skin prick tests (SPTs)3 to 14 airborne allergens and measurements of total serum IgE, and for asthma and bronchial hyperresponsiveness to methacholine (15). The Hutterites are a founder population of European origin who now reside in North America; >35,000 Hutterites are descendants of 64 ancestors. Additional details on the Hutterite population are provided elsewhere (15). Selected single nucleotide polymorphisms (SNPs) in ICOS were genotyped in these individuals using standard methods (15, 16). To test for differences in genotype frequencies between Hutterite cases and controls, we used an association test that takes into account the relatedness between individuals (17); to test for associations with the quantitative trait, mean (ln) IgE, we used a general two-allele model test that also takes into account the relatedness between Hutterites (18).
EMSA

Nuclear extractions were prepared using a standard protocol (19). Double-stranded oligonucleotide probes were generated by annealing complementary synthetic oligonucleotides and end-labeling with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (Amersham Biosciences). The nuclear extract (5 μg) was incubated with 3000 cpm of labeled probe and 1 μg of poly(dl-dC). For the competition assays, 100- to 200-fold molar excess of un-labeled oligonucleotide was added. Abs used for supershifts included the following: GATA-3, NF-κB p50, NF-κB p52, c-rel (sc-268x, sc-7178x, sc-7386x, sc-6955x; Santa Cruz Biotechnology), and NF-κB p65 (gift from Dr. G. Fran- zoso, University of Chicago, Chicago, IL, and Roche Applied Science, Indianapolis, IN).

Cell preparation and analysis

PBMCs were obtained from individuals within the Chicago community who were homozygous for either the −1413A/−693A or the −1413G/−693G haplotype. Cells were analyzed by flow cytometry with anti-CD4, CD8, CD69, CD28 (BD Biosciences), and anti-ICOS (eBiosciences). PBMCs were stimulated on anti-CD3 (OKT3; 1 μg/ml)-coated plates, and after 48 h, the cell culture supernatants were tested for cytokine content by a cytometric bead array (BD Biosciences). IL-13 was measured by ELISA (R&D Systems). Significance was determined using the unpaired Student’s t test.

Results

SNPs in the 5’ region of ICOS are associated with allergic sensitization and serum IgE

We identified 11 variants that partially overlap with previously reported polymorphisms (10–13); three SNPs in the 5’ flanking sequence (−1413G/A, −868T/A, and −693G/A), one synonymous SNP in exon 2 (Q50Q; 18915A/G), one SNP in intron 3 (20873C/T), one SNP in intron 4 (22749T/G), four SNPs in the 3’ untranslated region (22790A/C, 23752T/C, 23925C/T, and 24195G/A), and a dinucleotide repeat polymorphism in intron 4 (22643GTA14).

Members of the Hutterite pedigree were genotyped for four SNPs (−1413, −693, 23752, and 24195). The −868 SNP was not present in the 10 Hutterites in the screening set and was therefore not typed in the larger sample. There was no association between any of the phenotypes and the two SNPs in the 3’ untranslated region (p > 0.10). However, the −1413A and −693A alleles were significantly associated with allergic sensitization to airborne allergens (more than one positive SPT), and the −693AA and −1413AA genotypes were associated with increased total serum IgE levels (adjusted for age and gender) (Table I). These two SNPs are in linkage disequilibrium (LD), so the results are nearly identical. In particular, allergic sensitization to pollens, house dust mite, and cockroach allergens was more common among Hutterites with the −1413A and −693A alleles (Table I), and the prevalence is markedly increased in individuals homozygous for these SNPs (Fig. 1). Moreover, the mean (ln) IgE values were 4.13 (SD, 1.51) among AA, 3.23 (SD, 1.67) among GA, and 2.94 (SD, 1.60) among GG individuals (p = 0.0014). Although we did not find an association with asthma or bronchial hyperresponsiveness in this population (p > 0.10), it should be noted that the Hutterites are farmers and generally have mild asthma that infrequently requires inhaled corticosteroids or hospitalizations. Because the prevalence of atopy (more than one positive SPT) among Hutterite asthmatics is only 50%, compared with outbred populations in which >70% of asthmatics are atopic (15), allergic sensitization may not be a risk factor for asthma in the Hutterites, as it is in other populations. Nonetheless, our data show that one or both promoter SNPs, or other polymorphisms that are in LD with the −1413 and −693 SNPs, are associated with an increased risk for atopic responses to common airborne allergens and IgE production in this population.

The −1413G allele represents an NF-κB p50 binding site

Using the TRANSFAC 4.0 database, we found that the −1413G allele contained a site that is 90% homologous to the consensus NF-κB p50 binding site, whereas the −1413A allele eliminated the homology. To test whether the −1413 SNP was within an NF-κB site, two 24-mer probes, identical except for the −1413 SNP, were used in EMSA analysis. The −1413G probe, but not the −1413A probe, bound nuclear proteins in a pattern identical to an NF-κB consensus probe using both CEM and HeLa nuclear lysates (Fig. 2, a and b). The unlabeled −1413A oligonucleotide failed to compete for protein binding to either the −1413G or consensus NF-κB probes, whereas the unlabeled −1413G and NF-κB oligonucleotides eliminated protein binding to the −1413G probe (Fig. 2a). Abs to both p50 and p65 subunits supershifted the −1413G bands in HeLa cells, suggesting that p50-p65 heterodimers were bound (Fig. 2b). In CEM cells, anti-p52, anti-c-rel, and the control Abs had no effect. EMSA analysis of the −693 SNP did not reveal a difference in nuclear

Table I. Association between the ICOS 5’ promoter region SNPs and allergic phenotypes in the Hutterites

<table>
<thead>
<tr>
<th>SNP</th>
<th>Asthma (n = 71)</th>
<th>BHR (n = 156)</th>
<th>IgE (n = 638)</th>
<th>Any (n = 311)</th>
<th>Cockroach (n = 148)</th>
<th>Mite (n = 158)</th>
<th>Mold (n = 75)</th>
<th>Pollens (n = 157)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1413G/A</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td>0.0014</td>
<td>0.0034 (34.0/24.6)a</td>
<td>0.00015 (39.0/24.8)</td>
<td>0.014 (35.1/26.0)</td>
<td>&gt;0.10 (28.7/28.0)</td>
<td>0.033 (36.0/26.0)</td>
</tr>
<tr>
<td>−693G/A</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td>0.0043</td>
<td>0.0042 (34.1/24.1)</td>
<td>0.0022 (39.1/25.5)</td>
<td>0.049 (33.8/26.8)</td>
<td>&gt;0.10 (30.3/28.2)</td>
<td>0.028 (36.2/26.5)</td>
</tr>
</tbody>
</table>

The p values for association with −1413A and −693A alleles are shown. For the quantitative trait, mean (ln) IgE, n = all individuals in the analysis. For the case-control analyses of the qualitative traits, n = the number of cases for each phenotype. BHR, bronchial hyperresponsiveness.

* The frequencies of the −1413A and −693A alleles in the Hutterites were 28.1% and 28.3%, respectively.

a The frequency (percentage) of the −1413A allele and −693A alleles in the cases/controls.
protein binding between the two alleles, and unlabeled oligonucleotides blocked binding to self-probe as well as the converse allele (data not shown). Thus, although the −693 SNP does not represent a site for variation in transcription factor binding, the −1413G allele represents a p50 NF-κB site that can bind p50 homodimers or p50-p65 heterodimers.

PBMCs homozygous for the −1413A allele have significantly higher Th2 cytokine production

The association of the promoter region SNPs with atopic responses, together with differences in transcription factor binding, led us to examine whether the −1413 alleles were associated with differences in ICOS expression on T cells and cytokine production. Individuals were recruited in the Chicago area, and five homozygous −1413A individuals were identified. PBMCs from these individuals, as well as eight homozygous for the −1413G allele, were stained for ICOS expression and other T cell markers before and after activation. A small but significant difference in ICOS expression was found on CD4 and CD8 T cells between the −1413AA and −1413GG genotypes before activation (Fig. 3a). Although there was a trend toward increased ICOS expression on the −1413AA C4+ T cells 2 and 7 days after stimulation, significance was not reached (Fig. 3b). Other markers, such as CD28 and CD69, did not show any significant differences between the genotypes before or after stimulation (data not shown). On day 2, supernatants from these cultures were analyzed for cytokines, and −1413AA cell cultures contained significantly higher levels of IL-4, IL-5, IL-13, and TNF-α compared with cultures from −1413GG cells (Fig. 3c). No differences in IL-10, IL-2, and IFN-γ levels were seen. To determine whether these findings were reproducible, we tested all of the −1413AA and six of the eight −1413GG individuals a second time and found similar results (data not shown). Although we have no clinical information with regard to Th2-skewed diseases or autoimmunity in these volunteers, we saw no differences in the ratio of CD4:CD8 T cells (2:1) to suggest gross differences in lymphocyte populations.

Discussion

To our knowledge, this is the first report of an association of ICOS variation with Th2-mediated phenotypes. Although we found no polymorphisms that affected the protein sequence of ICOS, our results suggest that subtle changes in ICOS expression may affect secondary Ig production by B cells leading to increased IgE levels and allergic sensitization. Complete ICOS deficiency has been studied in both mice and humans. In the mouse, ICOS deficiency leads to defects in CD40-mediated Ab class switching and particularly reduced levels of IgE production (6–8). In humans, individuals with a homozygous deletion of the ICOS gene have been identified who have common variable immunodeficiency (20). Their immunodeficiency is characterized by adult-onset panhypogammaglobulinemia with reduced numbers of mature B cells.

Given that the subjects used in the cytokine experiments were chosen without regard to allergic phenotypes, it is remarkable that significant differences in cytokine production were found based solely on genotype at a single locus. The finding of increased TNF-α is particularly interesting given its role as a proinflammatory cytokine that has been implicated in Th2 development in a murine system and in the pathogenesis of asthma in studies involving humans (21, 22). The mechanism by which the −1413 SNP affects ICOS expression and Th cytokine production may be explained by the potential differential transcription factor binding of NF-κB. p50 homodimers have been shown previously to be negative regulators of transcription by several groups, and mutations at these sites actually leads to increased gene expression (23– 26). However, we must stress that it is possible that the −1413/693 SNPs are in LD with other polymorphisms and that it is these unknown polymorphisms that are responsible for the associated phenotypes described. Although additional studies are necessary to prove that NF-κB plays a role in the transcriptional regulation of ICOS, our data support a model in which loss of NF-κB regulation with the −1413AA genotype may increase ICOS expression on the cell surface and lead to changes in T cell differentiation and function.
The increase in Th2 cytokines in our system is likely attributable to secretion by memory T cells, because several groups have shown that it is predominantly memory, not naive, human T cells that produce IL-4 mRNA and protein at early time points (27). Because we also found increased levels of ICOS expression on CD4+ and CD8+ T cells from −1413AA individuals before in vitro activation (A) but is not significantly higher on days 2 and 7 after activation with anti-CD3 (B). Cultures from PBMCs stimulated for 48 h with anti-CD3 were measured for cytokines by cytometric bead array or by ELISA (IL-13). Cultures of PBMCs from individuals homozygous for −1413A (filled bars) had increased levels of the Th2 cytokines, IL-13, IL-5, and IL-4, as well as TNF-α, compared with cultures from individuals homozygous for −1413G (open bars). No significant differences in the Th1 cytokines, IFN-γ, and IL-2 were found, nor was there a significant difference in the amount of IL-10. Student’s t tests are shown (**, p < 0.01; *, p < 0.05; ns, not significant).

The increase in Th2 cytokines in our system is likely attributable to secretion by memory T cells, because several groups have shown that it is predominantly memory, not naive, human T cells that produce IL-4 mRNA and protein at early time points (27). Because we also found increased levels of ICOS expression on −1413AA CD4+ and CD8+ T cells before activation, it is probable that increased ICOS expression is associated with an increased Th2 memory pool. Together, our data suggest that −1413AA individuals are more Th2 biased. Whether increased ICOS expression affects Th2 differentiation or is a marker of Th2 differentiation remains to be elucidated. Alternatively, increased ICOS expression may lead to decreased responses to Th1-type stimuli and tip the balance toward Th2 responses.

Whether the −1413/−693 variation per se alters the expression of ICOS and subsequently affects B cell Ig production remains to be directly proven. However, the type 1 diabetes susceptibility locus, Idd5.1 in NOD mice, contains ICOS (28, 29). Interestingly, ICOS expression is higher on activated NOD T cells than B6/B10 T cells, and this difference in expression was mapped to a 2.1-Mb region encompassing ICOS and CTLA4. This region also accounted for differences among strains in disease severity in murine experimental autoimmune encephalomyelitis, suggesting that the level of ICOS expression may directly influence T cell effector function. Additionally, variation in ICOS surface expression levels in a murine model has been shown to correlate with distinct populations of cytokine-producing cells (30). High ICOS-expressing T cells were IL-10 producing, whereas medium ICOS-expressing T cells were IL-4 and IL-13 producing. These data support the idea that the level of ICOS expression influences differentiation and cytokine production. Together, our study and these reports argue that genetic variation in the 5′ promoter region of ICOS may directly affect T cell effector function in humans and thereby bias immune responses.

Acknowledgments
We thank C. Bourgain for assistance with statistical analysis in the Hutterites, A. Welcher (Amgen Inc., Thousand Oaks, CA) for providing reagents for this study, G. Franzoso for anti-p65 Ab and technical advice, and A. M. Dumitrescu, S. Yoshinaga, A. Tesciuba, K. Blaine, J. Solway, and M. Alegre for helpful discussions and assistance during these studies.
Disclosures

The authors have no financial conflict of interest.

References

neous nucleic acid analysis. Genome Res. 9: 492–498.
18. Abney, M., C. Ober, and M. S. McPeek. 2002. Quantitative-trait homogeneity and association mapping and empirical genomewide significance in large, complex pedi-
smooth muscle-specific gene expression through regulated nuclear translocation of se-
ICOS is associated with adult-onset common variable immunodeficiency. Nat. Im-
munol. 4: 261–268.
22. Thomas, P. S. 2001. Tumour necrosis factor-α: the role of this multifunctional cyto-
transactivation of the VCAM-1 promoter through an NF-κB enhancer motif. J. Biol.
Chem. 270: 8976–8983.
NF-κB activity increases during the age-dependent desensitization of the lover. J. Biol.
Acad. Sci. USA 85: 9743–9747.
gene content, comparative sequencing, and expression analyses support Cdk4 and
Nrmpl as candidates for Idd4.1 and Idd4.2 in the nonobese diabetic mouse. J. Im-
munol. 177: 164–173.
regulates ICOS expression and modulates murine experimental autoimmune en-
effectors T cells with high inflammatory potential and a strong bias for secretion of