Background: Cytotoxic T lymphocyte antigen-4 (CTLA-4) is an important downregulatory molecule expressed on both T and B lymphocytes. Numerous population genetics studies have documented significant associations between autoimmune diseases and single nucleotide polymorphisms (SNPs) within and around the CTLA-4 region of chromosome 2 in man. Furthermore, circulating levels of a soluble form of CTLA-4 (sCTLA-4) have been reported in a variety of autoimmune mediated diseases. Despite these findings, the relationship between levels of sCTLA-4 protein, mRNA transcript levels, and SNPs within the CTLA-4 region have not been clearly defined. In order to further clarify this relationship, we have tested four different SNPs within the CTLA-4 region among subjects whom are negative (n = 53) versus positive (n = 28) for sCTLA-4.

Results: Our data do not support a clear association between sCTLA-4 levels and any of the four SNPs tested.

Conclusion: The variation in the SNPs tested does not appear to effect sCTLA-4 protein levels, despite reports that they affect sCTLA-4 mRNA.
cations between SNPs within and around the CLTA-4 region and rheumatoid arthritis [10,11], celiac disease [12-14], type I diabetes, [15], myasthenia gravis [16,17] and autoimmune pancreatitis [18]. At the protein level, a variety of studies have implicated elevated levels of the sCTLA-4 protein in the plasma of patients with a variety of immunologically mediated diseases including autoimmune thyroid disease [6,19], systemic lupus erythematosus [20] cutaneous systemic sclerosis [21], allergic asthma [22,23], psoriasis vulgaris [24], and autoimmune pancreatitis [25].

In a landmark study of SNP analysis within a 330 kb region of chromosome 2q33 containing CD28, CLTA-4 and the ICOS gene regions in type I diabetics, Ueda et al [15] implicated the CT60 SNP (rs3087243) as playing an important role in the risk of development of diabetes. Interestingly, the “G” susceptibility allele appeared to be related to decreased levels of the sCTLA-4 mRNA relative to those of the full-length (transmembrane encoding) transcript. Subsequent to this report, a SNP within the ICOS gene region (IVS+173, also on chromosome 2q33), was reported to influence alternate splicing of CLTA-4 isoforms [26].

Despite the interesting associations between genetic variation near these immunoregulatory gene regions, mRNA transcript levels, and blood levels of sCTLA-4, a clear functional relationship between them and the pathogenesis of autoimmune disease have not been elucidated. We speculated that if the CT60 SNP or other SNPs within and in proximity of the CLTA-4 gene region were associated with changes in sCTLA-4 mRNA levels, the same SNPs might also be associated with changes in the amount of sCTLA-4 protein in blood plasma. To this end, we selected both positive and negative (undetectable) plasma samples for sCTLA-4 and performed SNP analysis for four commonly tested SNPs within and around the CLTA-4 region. We found no statistically significant differences in observed vs. expected genotypic frequencies for these SNPs when comparing positive vs. negative blood levels of sCTLA-4. Thus, our data do not support a relationship between these commonly tested SNPs and circulating levels of sCTLA-4 in the presence or absence of autoimmune disease.

**Methods**

**Study Population**

The sample set consisted of 81 serum samples from patients with a variety of autoimmune disease (n = 54) or normal adult volunteers without a history of autoimmune disease (n = 27). They were segregated without reference to disease status on the basis of the presence or absence of elevated levels of sCTLA-4 as described below. Blood samples were obtained following informed consent, and the study was done under the oversight of our local Institutional Review Board.

**Laboratory Analysis**

Sera from human subjects were tested in a sandwich ELISA for sCTLA-4 as previously described [6]. Samples were categorized as positive or negative for sCTLA-4 based upon a cutoff optical density of 2.5 fold increase over the OD450 nm observed when tested against an irrelevant capture antibody. In general, this corresponded to sCTLA-4 levels on the order of > 10 ng/ml as defined by commercially available test kits. Triplicate determinations were made with both anti-CTLA4 and irrelevant capture antibodies.

SNP genotyping was performed on DNA samples obtained from white blood cell pellets using the Qiagen mini kit (Chatsworth, CA) as described in the manufacturer's instructions. Polymerase chain reaction was used to amplify DNA fragments including SNPs. PCR products were digested with appropriate restriction enzymes and subjected to standard agarose gel electrophoresis for analysis.

CT60 (rs3087243) genotyping was performed as described in Vigano et al. [27]. The + 49 A/G (rs231775) and -318 (rs5742909) SNPs were determined as described by Harbo et al. [28]. IVS+173 (rs10932029) T/C genotyping was performed as described by Hunt et al. [14].

**Statistical Analysis**

The Freeman-Halton Extension of the Fisher Exact Test (two tailed) was used for comparison of the distribution of observed genotypes for each polymorphism when compared to expected genotypes based upon previously published allele frequencies. The following allele frequencies were used to calculate expected genotypic frequencies: CT60 A = 0.477, G = 0.523; +49A/G A = 0.642, G = 0.358; -318 C = 0.91, T = 0.09; IVS+173 T = 0.86, C = 0.14. Allele frequencies are from Ueda et al. [15], with the exception of IVS+173, which is from Haimila et al [29]. Expected frequencies were calculated based on the Hardy-Weinberg formula.

**Results and Discussion**

We tested 28 individuals who were positive and 53 who were negative for sCTLA-4 in blood plasma for the purpose of determining whether there was an association with common SNPs within the CLTA-4 and ICOS regions of human chromosome 2q33. No evidence of an association between levels of sCTLA-4 and SNP genotypes were found (Table 1.). Furthermore, there were no statistically significant differences in absolute allele counts between positive and negative sera (data not shown). Although the number of samples is rather small, there were no clear cor-
relations between absolute levels of sCTLA-4 protein and SNP genotypes.

Our data confirm and extend the findings of Purohit and co-workers [30], who reported a lack of association between CT60 genotype and sCTLA-4 levels. On the other hand, our findings appear to be at odds with the speculation that the CTLA-4 CT60-A/G SNP may determine the alternate splicing and production of the sCTLA-4 mRNA [15]. In the Ueda model, the CT60-G susceptibility allele appears to produce lower relative amounts of the sCTLA-4 mRNA; thus, one would expect that subjects at risk for autoimmune disease to have reduced levels of sCTLA-4 protein. It seems paradoxical given that lower levels of CTLA-4 message are present in susceptible individuals whereas higher levels of sCTLA-4 protein are observed in plasma of individuals with autoimmune disease. Possible explanations for the appearance of this discrepancy may include the possibility that there is no direct relationship between message levels at the cellular level and circulating protein in plasma. For example, elevated circulating sCTLA-4 levels may simply be due to increased half-life and/or decreased turnover of protein despite increased levels of synthesis. Also, it is possible that lower levels of sCTLA-4 message reflect a feedback regulatory loop in which mRNA levels are reduced in the face of higher levels of sCTLA-4 protein. Finally, it is possible that immunoreactive CTLA-4 material detected in human serum is not the direct gene product of the sCTLA-4 mRNA transcript. While our lab [5,6] has previously reported the presence of a novel epitope (which is predicted to arise from a frameshift due to alternate splicing) in immunoprecipitates from CTLA-4 monoclonal antibodies, only a minority of the material from these immunoprecipitation experiments is of the predicted molecular mass of the sCTLA-4 monomer (23 kDa). Thus, it is possible that ELISA based assays for circulating CTLA-4 levels cannot distinguish sCTLA-4 monomer produced directly by the sCTLA-4 transcript within a heterogeneous population of CTLA-4 immunoreactive material derived from other sources, such as that derived from proteolytic cleavage from cells that express the transmembrane protein. There are numerous examples of soluble receptors that are derived from such a mechanism including many of the members of the tumor necrosis factor receptor family as well as other cytokine receptors and adhesion molecules [reviewed in [31]]. Despite the finding that the IVS+173 SNP appears to affect the relative level of sCTLA-4 mRNA [26], our data suggest that the same SNP does not directly control circulating levels of sCTLA-4 protein. In any case, the precise mechanism that controls levels of the sCTLA-4 transcript and sCTLA-4 immunoreactive material needs to be further investigated, but there does not appear to be a simple relationship between the SNPs that are the object of study in this report and the sCTLA-4 protein.

Abbreviations
CTLA-4: Cytotoxic T-lymphocyte antigen-4; sCTLA-4: soluble CTLA-4; SNP: single nucleotide polymorphism; rs: reference SNP (from NCBI dbSNP database: http://www.ncbi.nlm.nih.gov/projects/SNP).

Competing interests
The authors declare that they have no competing interests.
Authors' contributions
MKO wrote the manuscript, participated in designing the study, and performed statistical analysis. AB performed SNP testing, data organization, and analysis. MT participated in designing the study and drafting of the manuscript.

Acknowledgements
We thank Aurora St. Luke's Medical Center Medical Staff Internship Program for support of Andrew Berry's internship. We also thank the research subjects who provided samples for these studies. The authors acknowledge the technical assistance of Karen Kozinski and Kate Dennert in performing ELISA and providing technical oversight of the study.

References
1. Coyle AJ, Lehar S, Lloyd C, Tian J, Delaney T, Manning S, Nguyen T, Burdwell T, Schneider H, Gonzalez JA, Gosselin M, Owen LB, Rudd CE, Gutierrez-Ramos J-C: The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity* 2000, 13:95-105.

2. Waterhouse P, Pennington JM, Timms E, Wakeham A, Shahinian A, Lee KP, Thompson CB, Greissier H, Mak TW: Lymphoproliferative disorders with early lethality in mice deficient in Cta-4. *Science* 1995, 270:985-988.

3. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH: Loss of CTA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTA-4. *Immunity* 1995, 3:51-54.

4. Teft WA, Kirchhof MG, Madrenas J: A molecular perspective of CTA-4 function. *Annu Rev Immunol* 2006, 24:65-97.

5. Oaks MK, Hallett KM, Penwell RT, Stauber EC, Warren SJ, Tector AJ: A native soluble form of CTA-4. *Cell Immunol* 2000, 201:144-153.

6. Oaks MK, Hallett KM: Cutting edge: a soluble form of CTA-4 in patients with autoimmune thyroid disease. *J Immunol* 2000, 164:5015-5018.

7. Magistrelli G, Jeannin P, Herbault N, Benoist De Coignac A, Gauchat JF, Bonnefoi JY, Kendall-Taylor P, Cawston TE, Young-Min S: Association of a T-cell regulatory gene CTA4 with susceptibility to autoimmune disease. *Nature* 2003, 423:506-511.

8. Huang WY, Strobel P, Gold R, Nix W, Schalke B, Kiefer R, Ospitz A, Klinker E, Muller-Hermelink HK, Marx A: A CTA4 high-type Is Associated with Myasthenia Gravis in Thymoma Patients. *Ann Neurol* 2005, 58:644-648.

9. Wang XB, Pirskanen R, Giscombe R, Leffert AK: Two SNPs in the promoter region of the CTA4 gene affect binding of transcription factors and are associated with human myasthenia gravis. *J Intern Med* 2008, 263:61-69.

10. Chang M-C, Chang Y-T, Tien Y-W, Liang P-C, Jan I-S, Wei S-C, Wong J-H: T-Cell Regulatory Gene CTA4 Polymorphism/Haplotype Association with Autoimmune Pancreatitis. *Clin Chem* 2007, 53:1700-1705.

11. Savenerino D, Brizzolara R, Simone R, Chiaiporti A, Milintenda-Floriani F, Pesce G, Bagnasco M: Soluble CTA4 in autoimmune thyroid diseases: Relationship with clinical status and possible role in the immune response regulation. *Clin Immunol* 2007, 123:190-198.

12. Wang CK, Lit LC, Tam LS, Li EK, Lam CW: Absent production of soluble costimulatory molecules CTA4, CD28, and CD86 in patients with systemic lupus erythematosus. *Rheumatology* 2005, 44:989-994.

13. Sato S, Fujimoto M, Hasegawa M, Komura K, Yanaba K, Hayakawa I, Matsushita T, Takehara K: Serum soluble CTA4 levels are increased in diffuse cutaneous systemic sclerosis. *Rheumatology* 2004, 43:1261-1266.

14. Ip WK, Wong CK, Leung TF, Lam CW: Elevation of plasma soluble T cell costimulatory molecules CTA4, CD28 and CD80 in children with allergic asthma. *Int Arch Allergy Immunol* 2005, 137:45-52.

15. Wong CK, Lun SW, Ko FW, Ip WK, Hui DS, Lam CW: Increased expression of plasma and cell surface co-stimulatory molecules CTA4, CD28 and CD86 in adult patients with allergic asthma. *Clin Exp Immunol* 2005, 141:122-129.

16. Luszczek W, Kubicka W, Jasek M, Baran E, Cislo M, Nockowski P, Luczyno-Rudy M, Witkiewicz A, Nowak I, Kubiarczyk P: CTA4 gene polymorphisms and natural soluble CTA4 protein in psoriasis vulgaris. *Int J Immunogenet* 2006, 33:217-224.

17. Umemura T, Ota M, Hamano H, Katsuyama Y, Muraki T, Arakura N, Kawa S, Kiyosawa K: Association of Autoimmune Pancreatitis With Cytotoxic T-lymphotye Antigen-4 Gene Polymorphisms in Japanese Patients. *Am J Gastroenterology* 2008, 103:588-594.

18. Kaartinen T, Lappalainen J, Haimila K, Autero M, Parkkonen K: Genetic variation in ICOS regulates mRNA levels of ICOS and splicing isoforms of CTA4. *Mol Immunol* 2007, 44:1644-1651.

19. Viganò P, Battadà D, Somigliana E, Abbati A, Candiani M, Di Blasio AM: Variants of the CTA4 gene that segregate with autoimmune diseases are not associated with endometriosis. *Molecular Human Reproduction* 2005, 11(10):745-749.

20. Harbo HF, Celus EG, Vandall F, Sparkland A: CTA4 promoter and exon 1 polymorphisms in multiple sclerosis. *Tissue Antigens* 1999, 53:106-110.

21. Haimila KE, Parkkonen JA, Holopainen PM: Genetic polymorphism of the human ICOS gene. *Immunogenetics* 2002, 53:1082-1032.

22. Purohit S, Podolsky R, Collins C, Zheng W, Schatz D, Muir A, Hopkins D, Huang YH, She JX: Lack of correlation between the levels of soluble cytotoxic T-lymphotye associated antigen-4 (CTA4) and the CT-60 genotypes. *J Autoimmune Diseases* 2005, 2:8.

23. Levine SJ: Mechanism of soluble cytokine receptor generation. *J Immunol* 2004, 173:5343-5348.