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A polymorphism in the human cytotoxic T-lymphocyte antigen 4 (*CTLA4*) gene (exon 1 +49) alters T-cell activation

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Abstract The cytotoxic T-lymphocyte antigen 4 (CTLA4) is an important modifier of T-cell activation with down-regulatory properties upon B7 engagement. An allelic polymorphism in exon 1 of the *CTLA4* gene coding for the peptide leader sequence of CTLA4 was recently described. This polymorphism was detected in association with several autoimmune diseases. In this study, we investigated the functional impact of the *CTLA4* exon 1 +49 A/G dimorphism on T-cell activation and cellular localization. We examined the T-cell response from healthy donors either homozygous for A or G at position +49 of the exon 1. Under suboptimal stimulation conditions we found a greater proliferative response of cells from donors homozygous for G at position +49. FACS analysis of CTLA4 expression revealed a reduced up-regulation of CTLA4 from G/G donors upon T-cell activation, if compared with wild-type cells. Intracellular CTLA4 distribution demonstrated qualitatively different staining patterns between the two genotypes as determined using confocal fluorescence microscopy. Our results suggest that the *G* allele at position +49 of exon 1 affects the CTLA4-driven down-regulation of T-cell activation and may be an important factor in the pathogenesis of autoimmune diseases.

Keywords CTLA4 · Genetic polymorphism · T cells

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Introduction

Activation of naive T cells is critically dependent on two signals: (1) antigen-recognition via the peptide-MHC complex and (2) an antigen-independent signal provided by interaction between CD28 on the T-cell surface and its ligands B7–1 (CD80) and B7–2 (CD86) on the antigen-presenting cell (APC). These co-stimulatory signals enhance production of several cytokines, in particular IL2, and increase proliferation (Anderson et al. 1999). Recent findings suggest that the CD28 homologue CTLA4 which, like CD28, binds members of the B7 family, plays an essential role in the down-regulation of T-cell responses (Krummel and Allison 1995;Walunas et al. 1994). Indeed, soluble anti-CTLA4 monoclonal antibodies (mAbs) that block CTLA4/B7 interaction augmented the proliferation of T cells stimulated with anti-CD3 and anti-CD28 antibodies in vitro. In contrast, under conditions of FcR cross-linking, anti-CTLA4 antibodies inhibited T-cell proliferation by inducing CTLA4 signaling (Thompson and Allison 1997). These data were further supported by studies with CTLA4-deficient mice, which rapidly develop lymphoproliferative disease with multiorgan lymphocytic infiltration and tissue destruction (Tivol et al. 1995). Concerning the mechanism by which CTLA4 ligation has a down-regulatory effect, it has been demonstrated that CTLA4 binding to B7 inhibits proliferation of T cells and accumulation of IL2, with subsequent arrest in cell-cycle progression (Krummel and Allison 1996; Walunas et al. 1996)

The human *CTLA4* gene (Chr 2q33) contains an A/G dimorphism at position +49 in exon 1 which causes an amino acid exchange (threonine to alanine) in the peptide leader sequence of the CTLA4 protein (Harper et al. 1991). Several autoimmune diseases including insulin-dependent diabetes mellitus, Graves disease, Hashimoto thyroiditis, rheumatoid arthritis, and multiple sclerosis have been shown to be associated with the *CTLA4* G^{49} allele (Braun et al. 1998; Donner et al. 1997; Harbo et al. 1999; Ligers et al. 1999; Seidl et al. 1998; Vaidya et al. 1999; Yanagawa et al. 1995). However, little information

is available about the functional relevance of this polymorphism.

In this study, we investigated the expression and function of CTLA4 in groups of individuals either homozygous for A or G at position +49. We demonstrated that the position 49 A/G dimorphism affects T-cell response, IL2 regulation, and cellular distribution of CTLA4, which may contribute to the pathogenesis of autoimmune diseases.

Materials and methods

Allelic-discrimination polymerase chain reaction

After written informed consent was obtained, we screened DNA samples from 64 healthy individuals (mean age 32±5 years, range 23–48 years) for different *CTLA4* exon 1 +49 genotypes. Chromosomal DNA was isolated from 1 ml EDTA-treated blood using the QIAamp Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The exon 1 dimorphism at position +49 of the *CTLA4* gene was analyzed by a nick translation-based PCR system, capable of differentiating single nucleotide exchanges (Mäurer et al. 2000). PCR reactions were performed on an ABI PRISM 7700 Sequence Detection System (Perkin Elmer, Foster City, USA). Genomic DNA derived from whole blood samples was used as template. Standard plasmids containing either *CTLA4* +49 A/A or *CTLA4* +49 G/G were included. Each PCR reaction contained 30 ng of chromosomal DNA or 10^4 plasmid copies.

A fragment of 89 bp (+17 to +105) of exon 1 of the *CTLA4* gene was amplified using the primers 5′-TTCAGCGGCACAAGGCTC-3′ and 5′-GCAGAAGACAGGGATGAAGAGAAG-3′ (PE Applied Biosystems, Weiterstadt, Germany).

Additionally, the PCR reaction contained two oligonucleotide probes (CTLA-4AlleleA: 5′-FAM-CTGAACCTGGCT**A**CCAGGACCTGG-TAMRA-3′; CTLA-4AlleleG: 5′-VIC-CTGAACCTGGCT**G**CCAGGACCT-TAMRA-3′; PE Applied Biosystems, Weiterstadt, Germany) labeled with fluorescent dyes (FAM: 6-carboxy-flourescein, TAMRA: 6-carboxy-tetramethyl-rhodamine) to spectrally distinguish the genotypes by different emission wavelength maximums.

Isolation of human dendritic cells

Mature and immature human dendritic cells (DC) were isolated from heparinized leukocyte-enriched buffy coats of healthy donors as described previously (Kolb-Mäurer et al. 2000, 2001).

Purification of T lymphocytes and mixed lymphocyte reaction

Peripheral mononuclear cells (PBMC) were purified by Lymphoprep gradient centrifugation (Nycomed, Oslo, Norway) according to the manufacturer's instructions. T lymphocytes were isolated by rosetting with sheep erythrocytes. After erythrocyte lysis, T cells were washed twice in ice-cold PBS and resuspended at a concentration of 1×10^6 viable cells/ml in RPMI 1640 supplemented 2 mM glutamine (Life Technologies, Karlsruhe, Germany) with 10% FCS (PAA Laboratories, Cölbe, Germany), 100 µg streptomycin/ml, and 100 units penicillin/ml (Biochrome, Berlin, Germany). Primary mixed lymphocyte reaction (MLR) was performed in triplicate by incubating 10^5 T lymphocytes with 5×10^4 mature/immature DCs or with irradiated PBMC in a 1:1 stimulator-responder ratio in 96-well round-bottom microtiter plates. Control experiments were performed in the presence of mAb against human CTLA4 (2 µg/ml, Clone BNI3, Pharmigen, San Diego, USA). Cellular proliferation was determined at 48 h, 72 h and 96 h after stimulation by [^{3}H] thymidine incorporation. Cells were collected on glassfiber filters by using a Betaplate 96-well harvester (Pharmacia-LKB, Uppsala, Sweden) and radioactivity was quantified with a 96-well Betaplate liquid scintillation counter (Pharmacia-LKB). Results are expressed as mean counts per minute.

Purification of total RNA and reverse transcription

Cells were harvested for IL-2 mRNA analysis at 24, 48 and 96 h after stimulation. Total RNA was prepared using the Qiagen RNeasy kit (Qiagen GmbH, Hilden, Germany). Purified RNA was eluted with 33 µl DEPC-treated water. For reverse transcription, 3 µl oligo(dT) (Pharmacia Biotech, Freiburg, Germany) were added, incubated for 10 min at 70°C and chilled on ice. After mixing with 12 µl first strand buffer, 6 µl 0.1 M DTT (Life Technologies), 10 mM dNTPs (Pharmacia Biotech) and 3 µl Superscript II reverse transcriptase (200 units per microliter, Life Technologies) the specimens were incubated for 50 min at 42°C. Reverse transcriptase was denaturated by incubation for 10 min at 95°C.

Quantification of beta-actin and IL-2 mRNA expression

PCR reactions for beta-actin and IL-2 mRNA were performed as previously described (Kruse et al. 1997). All PCR reactions were performed on an ABI Prism Sequence Detection System (Perkin Elmer). Composition of the PCR assay and oligonucleotide sequences have been published previously (Kruse et al. 1997, 2000).

Quantification of IL-2 protein expression

For measurement of protein concentration in culture supernatants we used the Quantikine HS Human IL-2 immunoassay (R&D Systems, Wiesbaden, Germany). At the indicated time points, 100 µl of culture supernatant were taken from each well. Samples from parallel stimulations were pooled. The supernatants were added to pre-coated 96-well plates at the recommended dilutions. The ELISA assay was performed according to the manufacturer's instructions.

Flow cytometry

Flow cytometry was used to monitor the expression of CTLA4 on T lymphocytes after stimulation. Immunofluorescence was performed according to standard techniques using phycoerythrin (PE)-conjugated mAb against CTLA4 (Clone BNI3, No. 34585, Pharmigen, San Diego, USA). Appropriate isotype-matched immunoglobulin G was used as control. Stained cells were analyzed on an EPICS XL-MCL (Coulter Immunotech Diagnostics, Krefeld, Germany).

Immunostaining

T cells were fixed for 10 min with PBS containing 4% paraformaldehyde. Cells were then washed with PBS, blocked with PBS containing 1% BSA and 0.1% saponin, and stained within the same medium. Staining with mAb against CTLA4 (Pharmigen, San Diego, USA, 1:100) and calreticulin (R&D Systems, 1:1000) was followed by addition of anti-mouse Cy2 (1:200) and anti-rabbit Cy3-conjugated (1:100) secondary antibodies (Sigma, St. Louis, USA). Cell nuclei were counterstained with DAPI (Sigma). After staining, cells were washed in PBS, centrifuged on coverslips and examined with a Zeiss Axiophot fluorescence microscope (100× oil-immersion objective). In addition, digital micrographs were taken using a Leica TCS 4D confocal laser microscope with a 50× objective in a 512×512 pixel format as described in previous studies (Wiese et al. 1999).

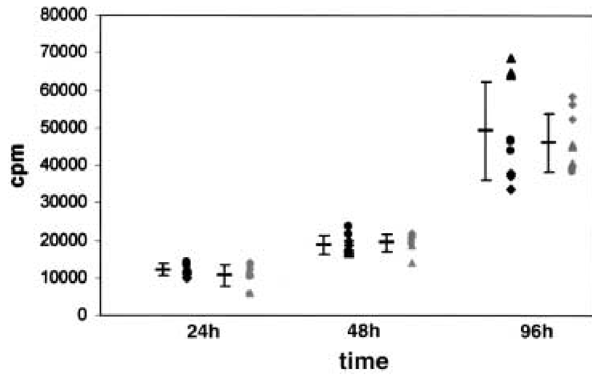

Fig. 1 Stimulation of T cells from healthy donors either homozygous for A or G at position + 49 of the *CTLA4* gene with 5×10^4 mature CD83 positive dendritic cells (DCs). Three individual T-cell cultures from different donors (n=3, *rhombus* donor 1, *triangle* donor 2, *circle* donor 3, A/A black, G/G gray) are shown. *Bars* represent mean value with standard deviation. Proliferation was assessed by [^{3}H] thymidine incorporation for 16 h and reported as cpm

Statistical methods

The results of the experiments are expressed as means ± standard deviation. Significant differences between experimental groups were analyzed by the Students *t*-test or Mann-Whitney *U*-test when appropriate. A *P* value of <0.05 was considered significant.

Results

CTLA4 exon 1 polymorphism in healthy individuals

We examined the genomic DNA from 64 healthy individuals with allelic-discrimination PCR (AD-PCR). At position +49 of exon 1 of the *CTLA4* gene, the genotype A/A was found in 29 individuals (45%), the genotype G/G in 7 individuals (11%), and 28 individuals (44%) were heterozygous (A/G). This distribution of genotypes corresponds to that described in the literature (Ligers et al. 1999). For analysis of differences in T-cell activation, we compared individuals homozygous for G with matched individuals homozygous for A at position +49 exon 1 of the *CTLA4* gene.

CTLA4 exon 1 polymorphism and T-cell proliferation

To test the T-cell response in the different groups, we used a primary MLR assay with allogenic DCs. The functional properties of DCs are strictly dependent on their maturational state: (1) immature DCs are characterized by high phagocytotic capacity, but a low expression of co-stimulatory molecules and a low avidity to T cells; in contrast (2) mature CD83$^+$ DCs show a high expression of MHC class II and co-stimulatory molecules and play the most critical role in activation of naive CD4 T lymphocytes (Banchereau and Steinman 1998). Only a few mature DCs are needed to provoke a potent T-cell immune response with a strong expansion of allo-

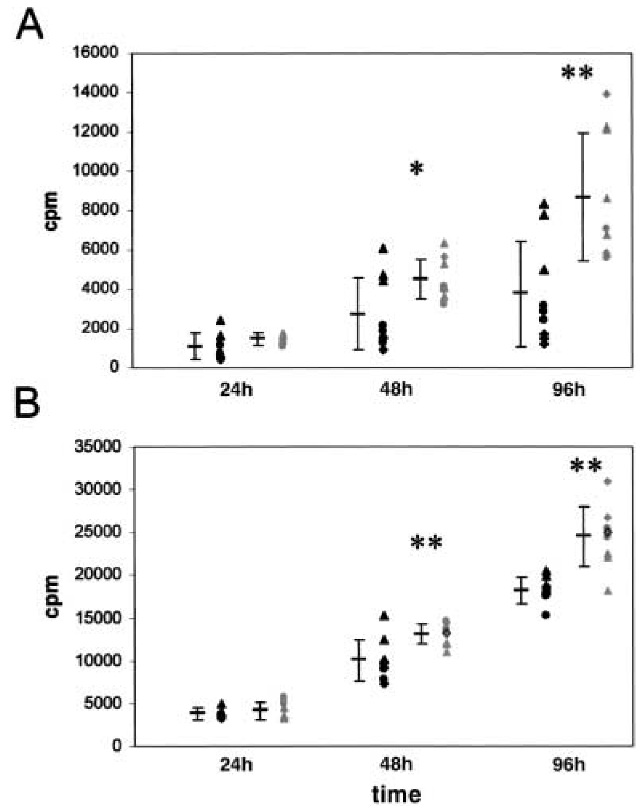

Fig. 2 Stimulation of T cells from healthy donors either homozygous for A or G at position + 49 of the *CTLA-4* gene with **A** 5×10^4 immature DCs and **B** 1×10^5 allogenic irradiated PBMCs. Three individual T-cell cultures from different donors (n=3, *rhombus* donor 1, *triangle* donor 2, *circle* donor 3, A/A black, G/G gray) are shown. *Bars* represent mean value with standard deviation. Proliferation was assessed by [^{3}H] thymidine incorporation for 16 h and reported as cpm. * P<0.05, ** P<0.01

reactive T cells and the exclusive development of Th1 cells.

Stimulation of purified T lymphocytes with mature CD83$^+$ allogenic DCs resulted in a strong T-cell proliferation. However, we could not detect any differences at different time points between the cells from donors homozygous for A or G at position +49 of the *CTLA4* gene under this stimulation condition (Fig. 1).

As expected, stimulation with immature dendritic cells elicited only a weak T-cell proliferation. However, under these conditions cells from individuals with the G/G genotype showed a higher proliferation than cells from donors with the A/A genotype. The differences were statistically significant after 48 h (Fig. 2A).

In addition, we performed a primary MLR with allogenic peripheral blood mononuclear cells (PBMCs). T-cell proliferation was clearly higher than after stimulation with immature DCs, but in comparison to stimulation with mature DCs the conditions still can be regarded as suboptimal. Again cells from subjects with the G/G genotype showed a significant higher proliferation 48 h and 96 h after stimulation (Fig. 2B).

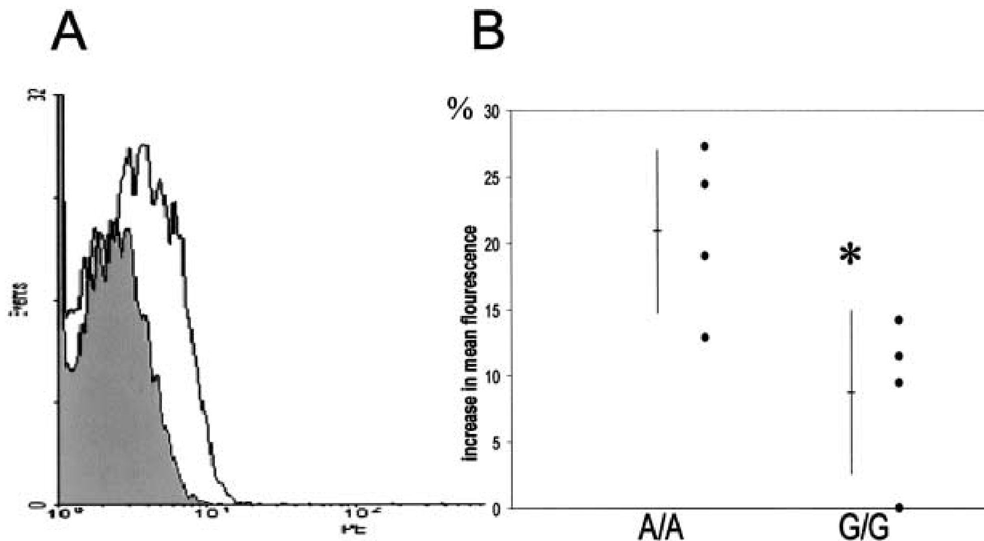

Fig. 3 **A** CTLA4 surface expression 48 h after primary mixed lymphocyte reaction (MLR) with mature DCs. T cells were gated and analyzed on a histogram. The *x axis* represents the logarithm of PE-fluorescence, the *y axis* represents cell number. The *shaded area* shows the result of unstimulated cells stained with anti-CTLA4 mAb, the *white area* shows results of stimulated cells stained with anti-CTLA4 mAb. **B** Comparison of increase in CTLA4 surface expression between cells from different donors with genotype A/A (n=4) or G/G (n=4) at postion +49 of the *CTLA4* gene after primary MLR against allogenic blood-derived dendritic cells. Cells from three individual T-cell cultures/donor were pooled. Experiments were run in duplicate. *Bars* represent mean value with standard deviation, *black dots* represent values of different donors. * P<0.05

Surface and intracellular expression of CTLA4

To analyze cellular localization of CTLA4 in the two genetically distinct groups, we investigated CTLA4 surface expression as early as 48 h after stimulation of purified T cells with mature allogenic human DCs. Low levels of surface CTLA4 expression were induced on T cells in our MLR system (Fig. 3A, B). However, the increase in mean fluorescence was less pronounced in cells from donors homozygous for G at position +49 exon 1 of the *CTLA4* gene, indicating that the G^{49} allele resulted in a reduced up-regulation of CTLA4 surface expression (Fig. 3B).

These quantitative differences in surface expression were not detected if permeabilized cells were analyzed (determination of total – surface and intracellular – level of CTLA4) (data not shown). Therefore, we looked for qualitative differences in the intracellular CTLA4 staining pattern between cells from subjects with different genotypes at position +49. As shown in Fig. 4A, B, fluorescent staining of lymphocytes from subjects homozygous for A revealed a more circular and homogeneous staining pattern. These results were further confirmed by confocal microscopy (Fig. 4C, D). Thus, it appears that the gene polymorphism at position +49 affects subcellular distribution and localization of CTLA4. Performing a double staining of CTLA4 and the endoplasmic reticulum with an antibody against calreticulin, we found no co-localization of CTLA with the endoplasmic reticulum (Fig. 4E, F).

CTLA4 exon 1 polymorphism and IL-2 regulation

Primary MLRs against irradiated allogenic PBMCs were performed with T cells from healthy donors either homozygous for A or G at position +49 of the *CTLA4* gene. We determined IL-2 mRNA expression and IL-2 cytokine secretion at 24, 48 and 96 h after stimulation. Quantitative PCR revealed a comparable IL-2 mRNA expression in both groups 24 h after stimulation; 48 h after primary MLR a higher IL-2 mRNA expression could be observed in cells with the +49 G/G genotype; 96 h after primary MLR, both groups showed a comparable decrease of IL-2 mRNA levels (Fig. 5A). The kinetics of IL-2 cytokine secretion revealed low IL-2 levels in both groups 24 h after primary MLR. 48 h after primary MLR, an elevated IL-2 secretion could be observed, which was more pronounced in cells from donors homozygous for G at position +49. 96 h after primary MLR, cells homozygous for G at position +49 still showed an increased IL-2 secretion, while the IL-2 levels in the A/A group remained at a similar level. Statistical analysis revealed a trend towards a higher IL-2 secretion after 96 h in cells with the +49 G/G genotype (Fig. 6A, P=0.08, t-test).

As expected, blocking anti-CTLA4 antibodies resulted in an IL-2 mRNA up-regulation and an increased IL-2 secretion. The up-regulation of IL-2 mRNA in cells with the exon 1 +49 G/G genotype was stronger than cells with the +49 A/A genotype. The difference was significant 48 h after primary MLR (Fig. 5B). In addition, the increase in IL-2 protein secretion was more pronounced in cells from individuals with the G/G genotype than in individuals with the A/A genotype, although the differences did not reach statistical significance (Fig. 6B).

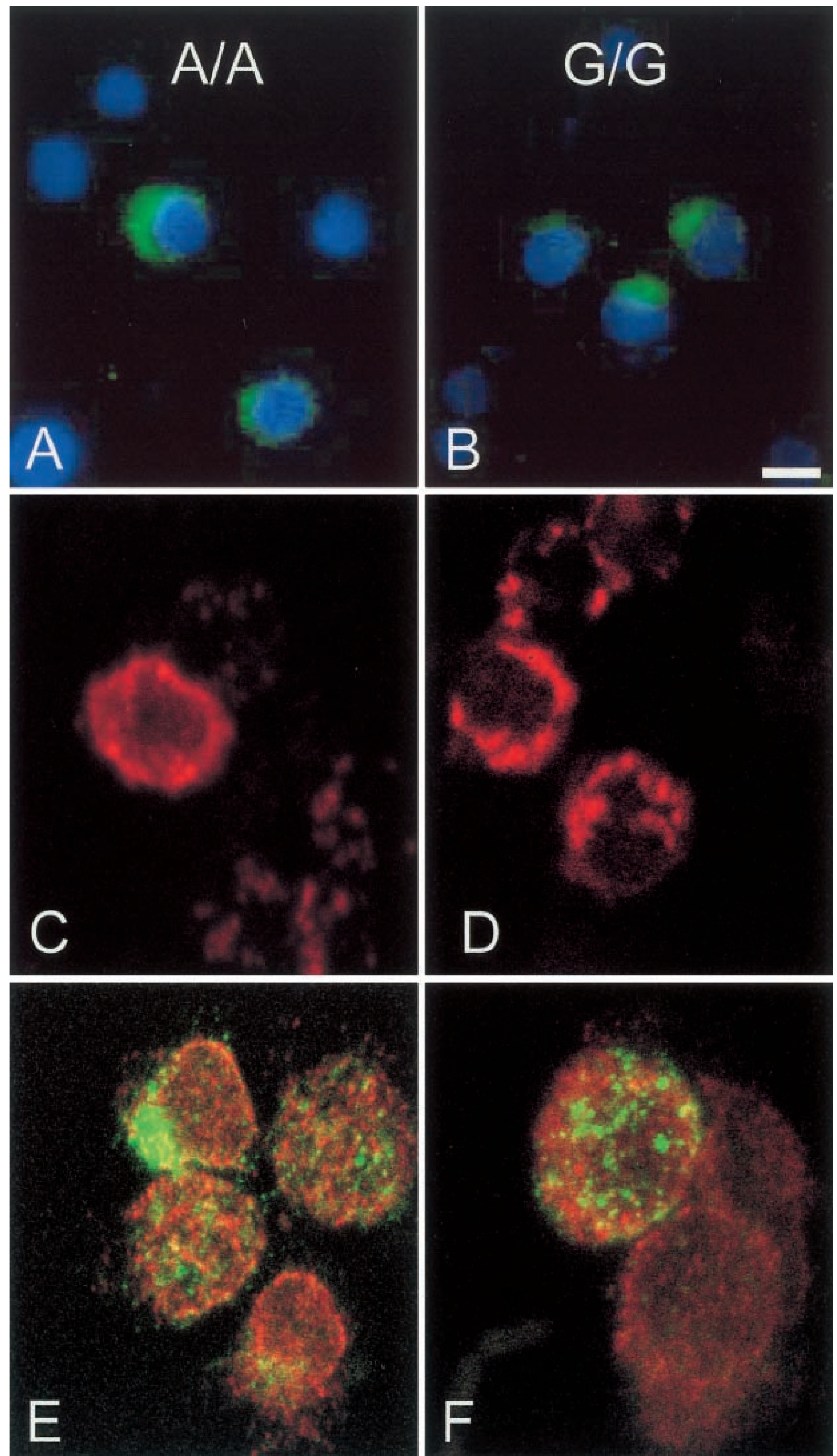

Fig. 4 A, B Cellular localization of CTLA4 immunoreactivity (*green*) of PHA (5 µg/ml) activated T cells 48 h after stimulation. Comparison of cells with genotype A/A or G/G at postion +49 of the *CTLA4* gene. Cell nuclei were counterstained with DAPI. Bar 10 µm. **C, D** Cellular localization of CTLA4 immunoreactivity (*red*) depicted with confocal microscopy. It appears that T cells from subjects homozygous for A at position +49 demonstrate a more homogenous, circular labelling. **E,F** Confocal images of T cells from subjects homozygous for A/A or G/G at postion +49 of the *CTLA4* gene after double-labelling of calreticulin (*red*) and CTLA4 (*green*). The images demonstrate that CTLA4 is not localized within the endoplasmic reticulum

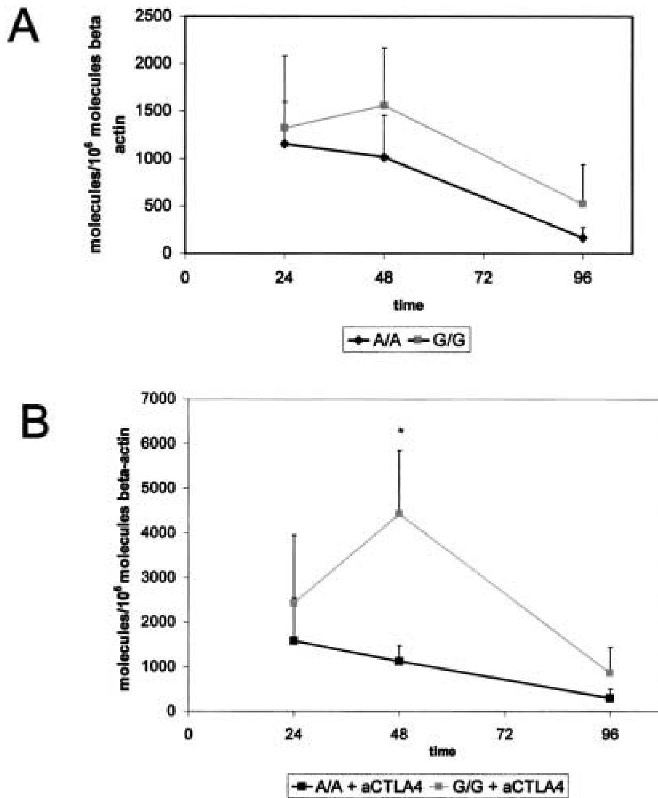

Fig. 5 Kinetics of IL-2 mRNA expression after primary MLR against allogenic irradiated PBMC in the absence (**A**) or presence (**B**) of 2 μg/ml of mAb against CTLA4. Comparison between cells from healthy donors either homozygous for A (n=5) or G (n=4) at position +49 of the *CTLA4* gene. Experiments were run in triplicate; values are given as mean with standard deviation. * P<0.05

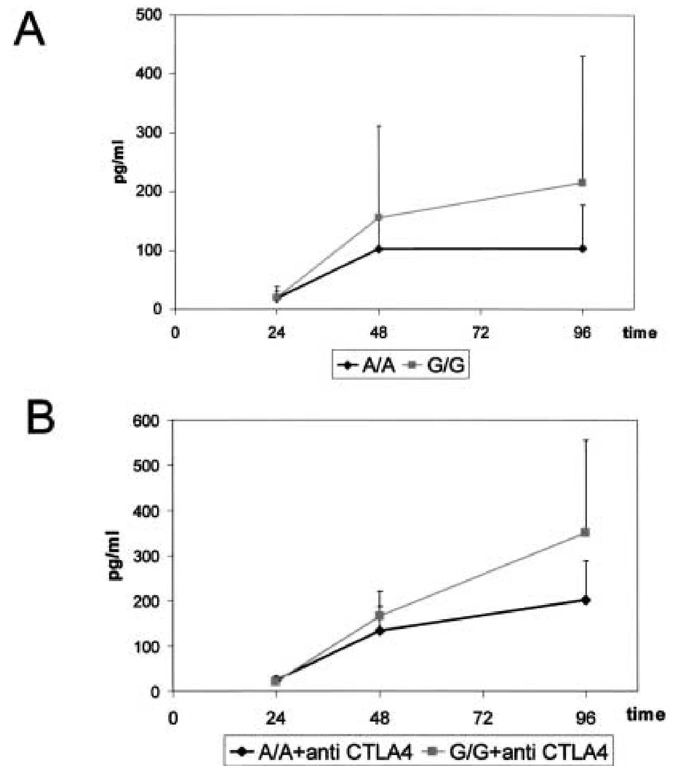

Fig. 6 Kinetics of IL-2 protein secretion after primary MLR against allogenic irradiated PBMC in the absence (**A**) or presence (**B**) of 2 μg/ml of mAb against CTLA4. Comparison between cells from healthy donors either homozygous for A (n=5) or G (n=4) at position +49 of the *CTLA4* gene. Experiments were run in triplicate; values are given as mean with standard deviation

Discussion

In the present study, we demonstrated an impact of the *CTLA4* exon 1 +49 A/G dimorphism on immune regulation after T-cell stimulation. CTLA4 plays a critical role in the regulation of the immune response and delivers inhibitory signals when interacting with co-stimulatory B7 molecules on antigen presenting cells (APCs).

The current hypothesis suggests that CTLA4 functions late in the immune response. CTLA4 is not detectable on the surface of resting T cells. Instead, CTLA4 expression was observed 24–48 h after activation, while CD28 is already expressed on resting T cells. The initial role for the co-stimulatory molecules B7–1 and B7–2 is to provide CD28 co-stimulation to T cells activated by engagement of the antigen receptor. Full activation would result in maximal expression of CTLA4 and, in subsequent encounters, CTLA4/B7 interactions would dominate. However, with activated human T cells, maximal CTLA4 expression was 2–3% of CD28. CTLA4 is therefore a high avidity, low abundance receptor for B7 molecules (Linsley et al. 1992).

It was demonstrated that CD28 and CTLA4 were co-expressed at the mRNA level as early as 1 h after T-cell receptor activation (Lindsten et al. 1993), suggesting a role of CTLA4 also in early stages of the immune response. In addition, it has been shown that CTLA4 is already located intracellularly. Redistribution of this pool to the cell membrane would limit ligand-mediated CD28 activation. Because of the high avidity of CTLA4, small numbers of these intracellular CTLA4 molecules, if mobilized to the surface of the cell, might have profound effects. Under conditions of limited B7 availability (e.g., after stimulation with immature DCs or allogenic PBMCs), CTLA4 competes with CD28 early in the immune response and delivers inhibitory signals. Therefore, under suboptimal activation conditions, CTLA4 might serve to attenuate weak signals mediated by the antigen receptor and CD28, and thereby contribute to peripheral tolerance (Chambers et al. 1996). In contrast, under optimal conditions with APC expressing high levels of B7, like mature DCs, CTLA4 would be limiting, and the co-stimulatory signals generated by CD28 would dominate, resulting in full T-cell activation.

Disturbance of peripheral tolerance might be an important factor in many autoimmune disorders. Interestingly, many studies have described an association of the CTLA4 +49 dimorphism with autoimmune diseases (Braun et al. 1998; Donner et al. 1997; Harbo et al. 1999; Ligers et al. 1999; Seidl et al. 1998; Vaidya et al.

1999; Yanagawa et al. 1995), but little is known about the functional relevance of this dimorphism. In our study, we found a different CTLA4 surface expression and intracellular distribution of CTLA4 in the two *CTLA4* +49 genotypes. This might cause dysregulation of the CTLA4-driven down-regulation of T-cell activation especially under suboptimal stimulation conditions and might therefore contribute to a reduced immune tolerance and development of autoimmune disease. Our findings are in line with observations from other studies showing that the expression of the G/G allele correlates with increased T-cell proliferation after stimulation with an allogenic cell line and demonstrating a significantly increased CTLA4 surface expression in cells from donors homozygous for adenine at position +49 (Kouki et al. 2000; Ligers et al. 2001).

According to recent studies, the majority of CTLA4 is found intracellularly and can be rapidly mobilized from these intracellular stores to the site of T-cell receptor engagement on the cell surface (Linsley et al. 1996). These data suggest that CTLA4 cell surface expression is dynamically regulated with transition of the molecules between intracellular stores and the cell surface in response to environmental stimuli. Therefore, differences of intracellular CTLA4 might be the reason for a different surface CTLA4 expression. Although, we found differences in the subcellular distribution pattern of CTLA4 between cells from subjects with different genotypes at position +49, the question is still open of how a dimorphism in exon 1 of the *CTLA4* gene can cause this. Exon 1 of the *CTLA4* gene encodes for the hydrophobic 37 amino acid leader sequence of the protein (Harper et al. 1991; Khatlani et al. 2000). The *CTLA4* exon 1 +49 A/G dimorphism causes an amino acid exchange from threonine to alanine at position 17 in the leader sequence. The leader peptide serves as a signal peptide that directs the secreted protein to the endoplasmic reticulum. We hypothesized that the amino acid exchange at position 17 results in an “altered address” for the CTLA4 protein, and might interfere with the intracellular storage pool (Nicchitta and Blobel 1993; Simon and Blobel 1991). This could result in an altered transition between intracellular stores and the cell surface Therefore, we performed double staining of CTLA4 and the endoplasmic reticulum with an antibody against calreticulin. However, CTLA4 does not co-localize with the endoplasmic reticulum and further investigations are needed to examine the exact intracellular CTLA4 localization.

In order to substantiate the functional relevance of the *CTLA4* +49 polymorphism, not only in a qualitative but also in a quantitative way, we examined IL-2 regulation. CD28/B7 interaction resulted in increased IL-2 production and subsequent T-cell proliferation. Recent studies have demonstrated that CTLA4 ligation inhibits IL-2 production and results in subsequent arrest of cell cycle progression, rather than induction of apoptotic cell death (Krummel and Allison 1996; Walunas et al. 1996). This reversal of CD28-dependent T-cell activation suggests that CTLA4 acts as a competitive antagonist of CD28. Moreover, blocking anti-CTLA4 antibodies result in an increased T-cell proliferation which correlates with an increased IL-2 secretion beginning 24 h after stimulation and reaching a maximum 72 h after stimulation (Krummel and Allison 1996; Walunas et al. 1996). Following MLR, cells with the G/G genotype showed an increased mRNA and protein expression of the primary T-cell growth factor IL-2 in comparison to cells with the A/A genotype, however the differences fail to reach statistical significance. Nevertheless, our results with CTLA4 blocking antibodies suggest that the differences in IL-2 secretion are truly related to differences in CTLA4 function.

In conclusion,we suggest that the G^{49} allele of the *CTLA4* gene should be considered as a disease modifying factor in autoimmune diseases. Our results support the hypothesis that the G^{49} allele causes an imbalance in the competition between CTLA4 and CD28 on the cell surface. The observed differences in intracellular CTLA4 staining might point to an alteration of intracellular storage pools in individuals carrying the G^{49} allele, with consequent problems in the redistribution of CTLA4 to the cell surface upon T-cell activation. This antagonism is of particular importance early in the immune response under suboptimal stimulation conditions (often present in autoimmune reactions), because CTLA4 can attenuate weak signals delivered by the T-cell receptor and CD28, and might in that way contribute to peripheral tolerance.

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