Assigning the phenotype of a natural regulatory T-cell to the human T-cell line, KARPAS-299

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Abstract. The human CD30-positive anaplastic large (T-) cell lymphoma cell line, KARPAS-299 (DSM ACC31), was established from blast cells in the peripheral blood from a case of non-Hodgkin lymphoma in 1988. We describe the mRNA and surface expression in KARPAS-299 cells of a panel of markers highly restricted to human natural regulatory T-cells and associated with their suppressive activity, including FOXP3, CD25, IL-10, TGF-ß1, CD62L, and LAG-3. Results obtained from co-culturing human peripheral blood leukocytes with KARPAS-299 cells assigned a suppressive phenotype to the latter ones. In conclusion, KARPAS-299 cells show characteristics typical of natural regulatory T-cells and, thus, represent a valuable model for studying regulatory T-cell function, which may also facilitate drug development aimed at the modulation of regulatory T-cell activity for the pharmacological therapy of, for example, autoimmune diseases.

Introduction

In the ten years since $CD4^+CD25^+$ regulatory T-cells (Treg) and their pivotal role in regulating immune tolerance were first described (1), the underlying mechanisms of suppression and possible applications to control pathogenic T-cell responses in autoimmune, graft versus host, and inflammatory diseases has been the focus of enormous research efforts. As a consequence of this research, it is now clear that Treg represent a unique population of thymus-derived $CD4^+$ T-lymphocytes that constitutively expresses the α-chain of the IL-2 receptor (CD25), CTL-associated antigen-4 (CTLA-4), and L-selectin (CD62L). The Treg population comprises 5-10% of circulatory $CD4^+$ T-cells and acts to powerfully suppress responder T-cells *in vitro* and *in vivo* (2-4).

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The mechanisms responsible for Treg-mediated suppression of the immune response remain to be elucidated fully. In particular, little is known about Treg-specific surface markers and related co-stimulatory pathways required for the acquaintance of full suppressive activity. CD62L, neuropilin-1, lymphocyte-activating gene-3 (LAG-3), and the Ki1-antigen (CD30) are among the surface molecules that have been assigned to the Treg phenotype (5-7). Treg are also unique in the expression at their cell surface of both latent and active transforming growth factor (TGF)-ß1 (8), a feature that is believed to facilitate contact-dependent inhibition of responder T-cells. However, the role of TGF-ß1 in the suppressive activity remains controversial, as is the role of CTLA-4 signalling (9). *In vitro* generation of Treg is achieved by activation in the presence of the immunosuppressive cytokines, TGF-ß1 and interleukin (IL)-10 (10). Previous studies imply that TGF-ß1-induced gene expression of FOXP3, a transcription factor that is believed to be exclusively expressed in $CD4^+CD25^+$ and $CD8^+CD25^+$ T-cells, which correlates with their suppressive phenotype in humans and mice (11,12), converted naive $CD4^+CD25^-$ T-cells into anergic/suppressor cells upon T-cell receptor (TCR) activation (13). In both mice and humans, $CD4^+CD25^+$ cells have been shown to constitutively express high levels of TGF-ß1, and application of anti-TGF-ß1 antibodies leads to a dose-dependent decrease in suppressive activity in humans and mice (8). However, tools to support the Treg function *in vivo* and *in vitro* remained elusive.

Results and Discussion

We show that the human T-cell lymphoma cell line, KARPAS-299, exhibits a 'Treg-specific' mRNA and surface expression pattern. Furthermore, KARPAS-299 cells are capable of inhibiting the growth of co-cultured PHA/PMA-stimulated MNC.

The human cell line, KARPAS-299, was established from the peripheral blood of a 25-year-old man with T-cell non-Hodgkin lymphoma by Fischer *et al* (14) in 1988 and is now classified as '$CD30^+$ anaplastic large cell lymphoma' (ALCL). It is known as CD25-positive. As the CD25 cell surface protein is also constitutively expressed on natural regulatory T-cells and is widely used to identify and enrich Treg, we investigated by RT-PCR the presence of this and other 'Treg-specific'

Table I. Primers used for RT-PCR analyses.

Specific mRNA	Upstream primer 5'→3'	Downstream primer 5'→3'
TGF-ß1	CAGAAATACAGCAACAATTCCTGG	TTGCAGTGTGTTATCCGTGCTGTC
IL-10	GCCTAACATGCTTCGAGATC	TGATGTCTGGGTCTTGGTTC
CD25	GACGATGACCCGCCACAGATCCCA	CCTGGACGCACTGATAATAAACCA
Neuropilin-1	CTTACATCTCCTGGTTATCC	TTCTGGGAACATTCAGGACC
CD62L	CACCTGCAACTGTGATGTGG	GAGCAGATGAAGGTACATGC
LAG-3	CTCAGTTCCTGGGCTTGCTG	CTGGCTGATGCTGCCAAGTG
FOXP3	TGTCAGTCAACTTCACCAAG	AGCTGGTGCATGAAATGTGG
mAAP/CD13	GGTGGTGCACCTCAAGGG	GGAAGCATGTTGGACAGGG
DPIV/CD26	GATGCTACAGCTGACAGTCGC	TGGTGACCATGTGACCCACTG

Table II. mRNA and surface expression of various human T-cell lines.

	KARPAS-299		P12/Ichikawa	Jurkat E6.1	H9	Molt-4	Treg
	mRNA	FACS	mRNA	mRNA	mRNA	mRNA	mRNA
IL-2R/CD25	+++	100%	-	-	(+)	-	+++
FOXP3	++	n.d.	-	-	-	-	+++
TGF-ß1	+++	n.d.	+++	++	+++	+	+++
IL-10	++	n.d.	+	(+)	(+)	-	++
CD62L	++	11-36%	+++	++	+++	-	+++
LAG-3/CD223	++	n.d.	+	++	++	+++	+++
Neuropilin-1	++	n.d.	+	++	+	-	++
mAAP/CD13	++	9-51%	+	+	+	+	+++
DPIV/CD26	+++	100%	(+)	-	(+)	-	++

+++, strong expression; ++, moderate expression; +, low expression; (+), marginal expression; -, no expression; n.d., not determined. KARPAS-299 (DSM ACC31) and P12/Ichikawa (DSM ACC34) cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). H9 (ECACC No. 85050301), Jurkat E6.1 (88042803), and Molt-4 (85011413) were obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in RPMI/10% FCS at a maximum density of $0.5x10^6$ cells/ml. Cytofluorimetric analyses were performed with a FACS Calibur (Becton-Dickinson, San Jose, CA) using polyclonal chicken anti-TGF-ß1 (R&D Systems, Minneapolis, MN), anti-CD13, anti-CD25 (clone 123-13, Becton-Dickinson), anti-CD26 (Becton-Dickinson), and anti-CD62L (Coulter/Immunotech, Miami, FL). TRITC-rabbit anti-chicken IgG (Sigma, Deisenhofen, Germany) was used for TGF-ß1-staining and donkey-anti-rabbit PE-conjugate (Dianova, Hamburg, Germany) was used for all other unlabelled antibodies.

markers in KARPAS-299 cells in comparison to four other T-cell lines (Tables I and II).

CD25 appeared to be weakly expressed in H9-cells, but was completely absent in the other T-cell lines. TGF-ß1 and IL-10, both being immunosuppressive cytokines typically produced by Treg and functionally linked with its suppressive activity, showed highest expression levels in KARPAS-299 cells. Molt-4 cells completely lacked TGF-ß1 expression and only marginally expressed IL-10, whereas IL-10 was practically absent also from H9 or Jurkat E6.1 cells (Table II).

A 100% 'Treg-specific' marker has not yet been identified. The transcription factor, FOXP3, however, is regarded as rather selectively expressed by Treg (15). We show here that, among the various T-cell lines analysed, KARPAS-299 cells are unique in their expression of FOXP3. To further support this finding, which is suggestive of KARPAS-299 cells exhibiting a Treg phenotype, the expression of other recently proposed Treg-markers was studied. Huang *et al* (7) reported that surface-bound LAG-3 is required for proper Treg function, whereas another study (5) proposed neuropilin-1 as Treg surface marker. Fu *et al* (6) identified CD62L-positive Treg as more potent suppressors than the CD62L-negative fraction. Our results show that all of these three surface molecules are widely expressed among different T-cell lines. Thus, despite their clear association with the Treg phenotype, these markers on their own are not sufficient to define Treg.

Recently, we demonstrated the expression of membrane alanyl-aminopeptidase (mAAP) mRNA in human Treg of healthy volunteers. Among the various leukocyte and T-cell subsets analysed, Treg showed by far the highest mAAP mRNA levels. In accordance with this observation, KARPAS-299 cells exhibit strong mAAP mRNA and surface (CD13) expression. The surface expression of CD13 appeared variable, depending on the proliferation state. Inhibitors of the enzymatic activities of both mAAP/CD13 and dipeptidyl peptidase IV (CD26) exhibit strong immunosuppressive activity which, at

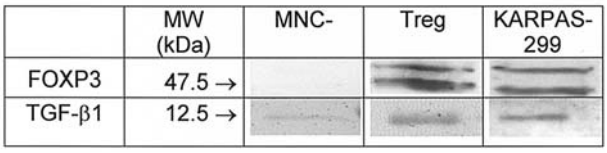

Figure 1. Detection of FOXP3 and TGF-ß1 in 2x10^6 KARPAS-299 cells by immunoblot analysis. Expression in KARPAS-299 cells was comparable to that of human Treg, whereas equal numbers of MNC depleted of Treg (MNC-) practically lacked FOXP3 and showed only weak TGF-ß1 expression. Immunodetection of FOXP3 and TGF-ß1 was performed as described (20) using the following antibodies: polyclonal rabbit anti-FOXP3 (ab10563, 1:500, abcam, Cambridge, UK), anti-rabbit-IgG-horseradish peroxidase (1:5000, Cell Signaling, Beverly, USA), polyclonal chicken anti-TGF-ß1 (1:500, R&D Systems, Minneapolis, MN), rabbit anti-chicken-alkaline phosphatase (1:2000, R&D).

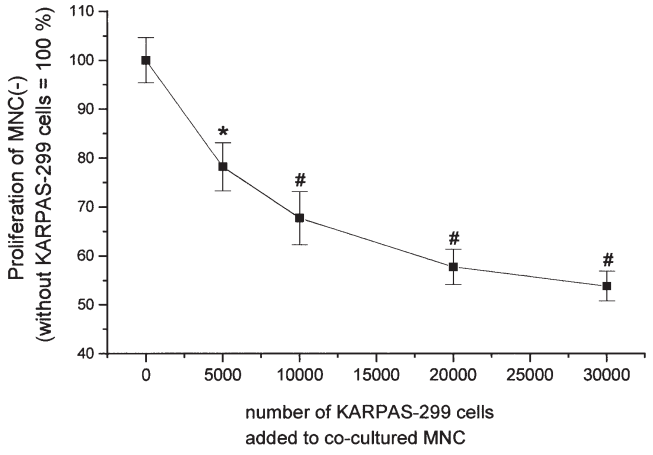

Figure 2. KARPAS-299 cells suppress the growth of co-cultured MNC depleted of Treg (MNC-). Peripheral blood mononuclear cells (MNC) were isolated from venous blood of healthy donors as described (21). Treg were positively selected from human MNC using CD25-Microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). The MNC depleted of Treg were seeded in 96-well plates at 30000 cells/well/150 μl and co-cultured with different numbers of KARPAS-299 cells placed in 0.2 μm Nunc tissue culture inserts. After 96 h, the inserts were removed and proliferation of the MNC was measured by using the Cell Proliferation kit (Promega, Mannheim, Germany). Mean of 3 experiments ± SD, $^{\#}$P<0.021; *P<0.035.

least partly, is due to an induction of TGF-ß1 production and release (16-19). As both ectopeptidases appear to be strongly expressed in KARPAS-299 cells and human Treg, we conclude that mAAP and DPIV expression is another, although not exclusive, feature of human natural Treg.

Collectively, the available data imply that the Treg phenotype is established by the simultaneous expression of a panel of surface markers, which alone are not exclusive for these cells. Essential prerequisites for a functionally active Treg are the presence of FOXP3, TGF-ß1, IL-10, LAG-3, CD25, CD62L, and, probably, CD13. All of these markers appear highly expressed in KARPAS-299 cells, which are thereby clearly distinguished from other T-cell lines. Taking into account the special role of FOXP3 and TGF-ß1 for the suppressive activity of Treg, the expression of both Treg markers was also assessed at the protein level by immunoblot analysis. As shown in Fig. 1, TGF-ß1 and FOXP3 protein expression in KARPAS-299 cells was as high as in purified human Treg, whereas equal numbers of MNC that had been depleted of Treg contained no FOXP3 and traces of TGF-ß1 only. To verify that also the immunosuppressive cytokine, IL-10, is expressed and secreted as a mature protein, IL-10 contents in the supernatant of KARPAS-299 cells were determined 24 and 48 h after seeding by a commercially available ELISA (R&D Systems). Thereby, we detected significant amounts (24 h, 49.8 pg/ml; 48 h, 136 pg/ml) of IL-10 in the supernatant of KARPAS-299 cells.

Both the mRNA and protein expression pattern of KARPAS-299 cells fully concur with a regulatory T-cell phenotype. In contrast, all other investigated T-cell lines do not show the complete characteristics of Treg.

We then analysed whether KARPAS-299 cells also exhibit the functional, suppressive properties of Treg. In co-culture experiments, KARPAS-299 cells were proven capable of suppressing the proliferation of MNC, previously depleted from CD4$^+$CD25$^+$ cells, in a dose-dependent manner (Fig. 2). These results are in full accordance with data obtained by using human Treg in analogous experiments (data not shown).

In summary, the data presented here imply that the human T-cell lymphoma cell line, KARPAS-299, represents a functionally active regulatory T cell. Considering the important role that CD4$^+$CD25$^+$ T-cells play in maintaining immune-tolerance, there is a great demand for studies on the identification of regulatory mechanisms and possible ways to attenuate Treg function. Using KARPAS-299 cells as a model of Treg function might greatly facilitate such studies.

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