CHAPTER 1 GENERAL INTRODUCTION

1.1 T cell immune responses

T cells play a central role in adaptive immune responses. Upon activation, antigen-specific T cells undergo clonal expansion (1), resulting in an increase of cells recognising the same peptide antigens presented by major histocompatibility complex (MHC) encoded cell surface proteins (2), and contributing to the elimination of dangerous invaders. Furthermore, memory T cells, which arise from activated T cells, respond faster than naïve T cells to a second encounter of foreign antigens (3, 4). In addition, unlike innate immune cells, which recognise evolutionarily conserved antigen motifs such as proteoglycan from gram positive bacteria (5), T cells recognise a virtually unlimited number of diverse antigens with their T cell receptors (TCR). This is due to the generation of an equally huge number of different T cell receptors by random recombination of gene fragments during their development in the thymus (4, 6), while antigen-recognising receptors such as Toll like receptors (TLRs, (7, 8)) on innate immune cells are germ-line encoded (9). Consequently, autoreactive T cells recognising self-antigens with high affinity are also inevitably generated.

To avoid the potential danger caused by autoaggressive T cells, complex regulatory mechanisms to control T cell responses have evolved. During T cell development in the thymus, most T cells with high affinity to self-antigens are eliminated by “negative selection” (central tolerance, (10-13)). However, not all self antigens are present in the thymus (14). Consequently, autoreactive T cells can still escape negative selection and enter into the periphery. Thus, in addition to central

tolerance, T cells in the periphery are subject to regulatory mechanisms to maintain self-tolerance.

Firstly, signalling transduced by the TCR through interaction with antigen/MHC molecules is not sufficient to activate naïve T cells. A second signal, coming from ligation of costimulatory molecules with their corresponding ligands expressed on antigen presenting cells (APC) from the innate immune system, is necessary (15, 16). Thus, T cell activation is under control by innate immune cells, which are evolutionarily dedicated to recognise dangerous invaders. T cells receiving only antigen-specific signals through TCRs become hyporesponsive or anergic (15), or are depleted from the periphery through apoptosis (17). Furthermore, differentiation of naïve T cells into effector or memory T cells is controlled by innate immune cells directly through interaction between surface molecules or indirectly through cytokines secreted in the microenvironment (18, 19).

Once T cells become activated, several negative feedback mechanisms arise to restrict the extent of T cell responses. In this context, recent evidences suggest that cytotoxic T cell antigen-4 (CTLA-4) molecules in T cells and $CD25^{+}CD4^{+}$ regulatory T cells play an important role in the downregulation of T cell responses.

1.2 CTLA-4: a negative regulator for T cell responses

CTLA-4 and its homologous molecule CD28 belong to the immunoglobulin superfamily. Both CD28 and CTLA-4 are encoded on the same chromosome in humans and mice (20, 21), share the same ligands B7-1 (CD80) and B7-2 (CD86) (22, 23), and have presumably similar protein structures (24). This strongly suggests

that CD28 and CTLA-4 arose as a result of gene duplication. However, CD28 and CTLA-4 differ in their expression pattern and functions (25, 26). Unlike CD28, which is constitutively expressed by most T cells (27) and is the most important costimulatory molecule for the activation of naïve T cells, CTLA-4 is expressed only by $CD25^+CD4^+$ regulatory T cells in unprimed mice (28). The majority of resting $CD4^+$ T cells has no serologically detectable CTLA-4 expression (29). Furthermore, the intracellular domain of CTLA-4 is coupled to the endocytosis machinery by interaction with the clathrin-associated adaptor complex AP-2 (30). Thus, in contrast to CD28, CTLA-4 is predominately located in intracellular compartments. Upon activation, surface CTLA-4 expression of T cells increases and CTLA-4 is transported to the site where the interaction of surface molecules such as the TCR on T cells with those on APCs occurs (31, 32). Consequently, this enables CTLA-4 to access and modulate TCR and /or CD28 signalling.

In mice and humans, CTLA-4 was shown to function as a negative regulator of T cell responses (25, 33, 34). CTLA-4 knockout mice have massive lymphoproliferation and die of multi-organ autoimmune diseases at 3-4 weeks after birth (35). In mice (36) and humans (37), blockade of CTLA-4 with monoclonal antibodies enhances anti-tumor immunity. Furthermore, polyclonal T cell activation in vitro is inhibited by intensive crosslinking of CTLA-4 with monoclonal antibodies. The mechanisms by which CTLA-4 mediates deactivation of T cell responses remain controversial. According to the current literature, several mechanisms may indeed be involved (38). Under conditions of limited ligand expression, CTLA-4 can enhance the activation threshold for T cells by effectively competing for ligands with CD28, since CTLA-4 has higher affinity to CD80 and CD86 (39). In addition, CTLA-4 can also deactivate T cell responses by transducing negative signals which may involve recruitment of

negative regulators, such as the phosphatase SHP-1, to the site of the TCR/CD28 signalling complex (40). Recently, CTLA-4 was shown to influence TCR/CD28 signalling by affecting formation of lipid “rafts”, which are important for T cell activation (41).

Comparison of responses of CTLA-4 deficient- with CTLA-4 expressing T cells reveals that T cell size and expansion upon stimulation increase in the absence of CTLA-4 (42). These findings indicate that CTLA-4 mediated deactivation is T cell autonomous, and restrict the extent of clonal T cell responses (43). However, *in vivo* mixed bone marrow chimera experiments also show that responses of CTLA-4 deficient T cells can be under control by other CTLA-4 expressing T cells (44). This suggests that CTLA-4 mediated regulation can also function in trans, probably through $CD25^+CD4^+$ regulatory T cells.

1.3 $CD25^+$ $CD4^+$ regulatory T cells

$CD25^+CD4^+$ regulatory T cells (Treg cells), constituting about ten percent of mouse and human $CD4^+$ T cells (45, 46), play a crucial role in maintaining peripheral tolerance. Depletion of $CD25^+CD4^+$ regulatory T cells, either by thymectomy three days after birth or by injection of CD25 mAb, causes autoimmune diseases, such as inflammatory bowel disease, diabetes and thyroiditis (47-49). Also CD25 or IL-2-deficient mice, which are unable to generate and/or maintain $CD25^+CD4^+$ regulatory T cells, develop multi-organ specific autoimmune diseases (50). In addition, introduction of $CD25^+CD4^+$ regulatory T cells into susceptible mice inhibits autoimmune diseases such as colitis caused by transferring $CD25^-CD4^+$ T cells alone (51). Thus, $CD25^+CD4^+$ regulatory T cells are involved in controlling autoreactive

responses, and the balance between Treg cells and activated T cells determines the outcome of immune responses.

Although the mechanisms by which $CD25^+CD4^+$ regulatory T cells act to control autoimmune responses remain unclear, several studies from mouse autoimmune disease models suggest that IL-10 and TGF-ß are important for the regulatory functions mediated by $CD25^+CD4^+$ T cells *in vivo* (52, 53). Unexpectedly, *in vitro* experiments show that control of the activation and proliferation of other T cells ($CD25^-CD4^+$ and $CD8^+$ T cells) by Treg cells may be cytokine-independent in cell culture (45, 54). Rather, membrane contact between $CD25^+CD4^+$ regulatory T cells and indicator cells is essential for the Treg suppressive function (45, 55). However, the relevance of *in vitro* suppressor activities for the *in vivo* regulatory functions of Treg needs to be established.

In vitro, mouse and human $CD25^+CD4^+$ regulatory T cells are hyporesponsive to anti-TCR stimulation and sensitive to cell death (56). Since naturally occurring $CD25^+CD4^+$ regulatory T cells are rare, and conditions for in vitro expansion of $CD25^+CD4^+$ regulatory T cells remain largely unknown, strategies for the expansion of Treg cells *in vitro* are currently the subject of intensive investigation.

Although the mechanisms and functions of the mouse and human negative regulator CTLA-4, and of $CD25^+CD4^+$ regulatory T cells are under intensive investigation, little is known about rat CTLA-4 and $CD25^+CD4^+$ T cells. Knowledge about the mechanisms controlling autoimmunity in this species is of particular importance because the rat provides several very robust models for human autoimmune diseases. To characterise CTLA-4, monoclonal antibodies specific for

rat CTLA-4 are a necessary tool, as shown by anti-mouse CTLA-4 mAbs. Furthermore, since one characteristic of mouse and human $CD25^+CD4^+$ regulatory T cells is the constitutive expression of CTLA-4, once generated, rat anti-CTLA-4 monoclonal antibodies would allow the examination of rat $CD25^+CD4^+$ T cells in detail.

1.4 CD28 superagonists

As mentioned above, two signals are required to activate naïve T cells: one antigen-specific signal induced by ligation of TCR, and a second signal, from the engagement of costimulatory molecules. CD28 is the most important costimulatory molecule for naïve T cells. Engagement of CD28 with ligands or with monoclonal antibodies, together with TCR ligation, activates naïve T cells and prevents T cell anergy and apoptosis. Although no activation and proliferation are induced by ligation of CD28 alone with its natural ligands B7 or conventional anti-CD28 antibodies, all $CD4^+$ T cells, including naïve ones, can be activated by engagement of CD28 with a so-called CD28 superagonist, a monoclonal antibody specific for rat CD28 and recognising a different epitope of CD28 molecules compared to conventional anti-CD28 mAbs (Fig. 1,(57)). In vitro, proliferation of T cells activated with CD28 superagonist is shown to be comparable with that of costimulated T cells (anti-TCR plus anti-CD28, (58)). Furthermore, upregulation of Bcl-X_L, an important counter player in apoptosis, is more pronounced in CD28 superagonist-stimulated T cells than costimulated T cells (59). Thus, signals transduced by of CD28 alone without apparent TCR ligation and proximal TCR-signalling (60, 61) can promote activation and reduce the sensitivity of stimulated T cells to apoptosis.

In vivo, a single injection of CD28 superagonist induces massive lymphocyte proliferation, and results in enlargement of secondary lymphoid organs and increased cell numbers (58). Unlike superantigens, which are polyclonal T-cell activators acting through the TCR (62), the systemic and antigen-unspecific T cell activation induced by CD28 superagonist is not accompanied by apparent discomfort and autoimmune diseases. Furthermore, lymphocyte cell number in CD28 superagonist stimulated rats returns to baseline slowly after reaching the maximal cell number. In addition, anti-inflammatory cytokines, such as IL-4 and IL-10, are profoundly expressed by $CD4^+$ splenocytes from CD28 superagonist injected rats (63). These findings indicate that T cell activation and inflammatory responses are under control in CD28 superagonist treated rats, in contrast to superantigen injected rats.

Interestingly, although all $CD4^+$ T cells are activated to proliferate by injection of CD28 superagonist in vivo, not all $CD4^+$ T cells express CD25, an activation marker upregulated in all T cells activated *in vitro* (58). However, pronounced increase of the absolute cell number and the percentage of $CD25^+CD4^+$ T cells are observed. Whether these $CD25^+CD4^+$ T cells are composed of activated $CD25^-CD4^+$ T cells, or are all derived from $CD25^+CD4^+$ regulatory T cells which are preferentially expanded upon CD28 superagonist stimulation, needs investigation.

1.5 Aim of this work

Although rats represent an important animal model in autoimmune diseases and organ transplantation, knowledge about the negative regulators of T cell responses, CTLA-4 and $CD25^+CD4^+$ regulatory T cells, is limited. This is partly due to the lack of

anti-rat CTLA-4 monoclonal antibodies, which are an important marker for $CD25^+CD4^+$ regulatory T cells and essential for investigation of rat CTLA-4. Therefore, in the present work, monoclonal anti-CTLA-4 antibodies were generated to characterise rat CTLA-4, and importantly rat $CD25^+CD4^+$ T cells in detail. Also CTLA-4 and $CD25^+CD4^+$ T cell functions were examined in *in vitro* readout systems. Furthermore, since $CD25^+CD4^+$ T cell numbers and percentage increase in CD28 superagonist treated rats, it is possible that these $CD25^+CD4^+$ T cells originate from naïve $CD25^+CD4^+$ T cells. Thus, the phenotype and functions of $CD25^+CD4^+$ T cells versus $CD25^-CD4^+$ T cells from CD28 superagonist injected rats were characterised. Finally, the possibility to activate and expand $CD25^+CD4^+$ regulatory T cells with CD28 superagonist in vitro was also investigated here.

CHAPTER 2 MATERIALS AND METHODS

2.1 Animals:

LEW rats (6-8 week old) and BALB/c mice (6-8 week old) were kept under conventional conditions in the animal facilities in Institute for Virology and Immunobiology, University Würzburg.

2.2 Antibodies:

Anti-rat monoclonal antibodies used in the present work were as followed: HRL1 (anti-CD62L, (64)), JJ316 (superagonistic anti-CD28, (58)), JJ319 (conventional anti-CD28, (65)), OX8 (anti-CD8, (66)), OX22 (anti-CD45RC, (67)), OX26 (recognizing transferrin receptors, which are expressed on proliferating cells, (68)), OX35 (anti-CD4, (69)), R73 (anti-TCR$\alpha\beta$, (70)) from BD PharMingen (Heidelberg, Germany). OX39 (anti-IL-2Rα, (71)) was purchased from Serotec (Oxford, U.K.). 3H5 (anti-CD80, (72)) was kindly provided by Dr. Maeda. WKH1 and WKH203 (anti-rat CTLA-4) were generated in the present work.

2.3 Construction of rat CTLA-4hIg fusion proteins expressing vector pHßAPr-1-gpt-rCTLA-4-Hr1

Rat CTLA-4hIg fusion proteins (rCTLA-4hIg, Fig. 2A), containing rat CTLA-4 extracellular domain and human IgG1 Fc region, were used for immunization to

generate anti-rat CTLA-4 mAbs. For construction of pHßAPr-1-gpt-rCTLA-4-Hr1 (Fig. 2B), an expression vector of rCTLA-4hIg fusion proteins, rat CTLA-4 cDNA was cloned from Con A-stimulated rat splenocytes by RT-PCR (C.-H. Lin, unpublished data), and was used as template to clone the CTLA-4 extracellular region. Extracellular regions of rat CTLA-4 cDNA were amplified by PCR with primers contained appropriate restriction sites (5'-GGGGGAGCTCACTATGGCTTGTCTTGGACT-3' and 5'-GGCAAGCTTACTTACCTGAA-TCTGGGCATGGTTCT-3'), and cloned into the SstI-HindIII sites of the human IgG1 expression vector pHßAPr-1-gpt-Hr1 (73). All constructs were verified by automated DNA sequencing. Rat CTLA-4hIg fusion proteins were produced by J558L cells transfected with pHßAPr-1-gpt-rCTLA-4-Hr1 using protoplasm fusion method (65). The production of rCTLA-4hIg fusion proteins were examined by incubation of rat B7-1 (CD80) expressing L929 cells with supernatants from transfected J558L cells followed by incubation with PE-conjugated secondary anti-human antibodies (Data not shown, rat B7-1 expression vector was kindly provided by Dr. Maeda (72).

2.4 Immunization and cell fusion

Anti-rat CTLA-4 mAbs WKH1 and WKH203 were generated by immunizing BALB/c mice with purified rat CTLA-4hIg fusion proteins as described below. Six to-eight-wk-old mice were immunized i.p. with purified rCTLA-4hIg fusion proteins (10µg/mouse) and inactivated *B. pertusis* (1x 10^{10}/mouse) in adjuvant aluminum hydroxide weekly for 6 weeks follwed by two week pause without immunization. Three days before fusion, mice were injected i.v. with rCTLA-4hIg fusion proteins in PBS (10 µg/mouse).

Fusion of myeloma cell line X63-Ag8.653 and splenocytes from immunized mice to generate monoclonal antibody-secreting hybridoma cells was performed 3 days after challenging. Briefly, splenocytes and myeloma cells X63-Ag8.653 were washed and resuspended in BSS/BSA. Splenocytes and myeloma cells were then mixed at the ratio of 5:1-10:1 in RPMI 1640, and centrifuged for 10 min at 1600 rpm at RT. Cells were then carefully resuspended with preheated PEG (37°C) and gently mixed for 1 min to allow the fusion to be accomplished. Five milliliter RPMI medium (1ml/min) and subsequently 10 ml RPMI (2mi/min) were added into cell suspension drop-wise. Subsequently, 30 ml RPMI supplemented with 5% FCS was added and cells were left at 37°C for 30 min. After centrifugation for 10 min at 1600 rpm, cell pellet was gently suspended in 100 ml RPMI containing 5% FCS and 100U/ml IL-6, and cells were plated on 96 well flat bottomed-plates (100µl/well). After 48 hour incubation, 100µl/well of RPMI containing 5% FCS, 100U/ml IL-6 and 4% azaserin/hypoxanthine was added as selection medium to inhibit the growth of the myeloma cells. Unlike hybridomas from fusion of splenocyte and myeloma cell, myeloma cells have no hypoxanthine-guanine phosphoribosyl transferase (HPRT), and can not use salvage pathway to synthesize nucleotides. Thus, myeloma cells are unable to synthesize purines in the presence of azaserin, inhibitor of the de novo nucleotide synthesis pathway, and are unable to grow. Seven to ten days after fusion, supernatants from growing hybridomas were examined for the presence and specificities of monoclonal antibodies.

2.5 Construction of a CTLA-4-GFP retroviral Vector and retroviral transduction

Rat CTLA-4 cDNA (Lin, unpublished data) was amplified with primers (5'-CCGAATTCCCACCATGGCTTGTCTTGGACTCCA-3' and 5'-GGGGAATTCATGGCCTTTCAGTTGATTGGAATA-3') and inserted into the EcoRI site of a modified MMLV-based retroviral vector (pczCFG5 IEGZ), which contains cDNA of green fluorescent protein (GFP) under the control of an internal ribosomal entry site (IRES) (kindly provided by Dr. I. Berberich (74)). Virus was produced by the transient transfection of the human embryonic kidney epithelial cell line 293T with retroviral vectors and packaging plasmids (pHIT-60 and pVSVG) using calcium phosphate transfection method as described below. At first day, $2x10^6$ 293T cells in 5 ml DMEM (supplemented with 10% FCS, 100U/ml penicillin/streptomycin, sodium pyruvate, and HEPES) were plated into one 6cm-culture dish. Twenty-four hours later, medium was aspirated, and fresh HEPES-free DMEM medium was added into the petra dish. Fifteen micrograms of sterile plasmid mixture (pczCFG5 IEGZ, pHIT-60 and pVSVG: each 5ug pro transfection) were solved in 438µl H_2O, and 62µl 2M $CaCl_2$ solution was added into plasmid mixture subsequently. After vortex, 500µl 2x HBS was added into plasmid solution by bubbling. The DNA transfection solution was then immediately added drop-wise into the culture dish containing 293 T cells.

After 7-10 hour incubation, HEPES-free medium was replaced by normal DMEM medium, and cells were incubated for further 12 hours (overnight). Cells were then incubated with fresh 3 ml DMEM medium containing 10mM Na-Butyrate (Sigma-Aldrich, Taufkirchen, Germany) to activate CMV promotors. After 8-12 hour culture, Na-Butyrate

containing medium was aspirated. Cells were washed once with DMEM, and incubated with 3 ml DMEM medium supplemented with FCS. After incubation overnight, cell supernatant containing virus particles was collected, and sterilized through a 0.45 um syringe filter into a 15 ml tube containing 30ul polybrene (800 ug/ml).

To transduce BW cells to express rat CTLA-4 and GFP or GFP proteins alone, BW cells were incubated with the collected retroviral supernatants. Retroviral supernatants were placed into 12-well culture plate, and $1x10^5$ BW cells were added drop-wise into supernatant. Cells were centrifuged for 90 min at 2000 rpm at RT. After further culture in incubator for one hour, the retroviral containing supernatants were replaced by 3 ml normal medium.

2.6 ELISAs

An ELISA for examination of the specificity of monoclonal antibodies produced by hybridomas was established here (section 2.4). Rat CTLA-4hIg fusion proteins or commercial human Ig Fc (Dianova, Hamburg, Germany) in PBS (1 μg/ml) were immobilized on 96-well flat-plates by overnight incubation at 4°C. After washing with PBS, plates were incubated for 1 hour at RT with PBS plus 10%FCS to reduce the unspecific binding. Collected supernatants were then added into fusion proteins or human Ig Fc-coated wells for 2 hours at RT. Bound mAbs were detected by incubation with biotinylated donkey anti-mouse Ig and subsequent incubation with AP (alkaline phosphatase)-coupled streptavidin and with colorimetric alkaline phosphatase substrate (Sigma-Aldrich, 1 mg/ml in 10% diethanolamine buffer). Quantitation was performed on

a spectrophotometer. Monoclonal antibodies, which recognized only fusion proteins but not human Ig Fc, were selected for further characterization (Fig. 3).

For determination of cytokine (IL-2, IL-4, IL-10 and IFN-γ) amounts produced by cells, supernatants were collected from cells ($5x10^4$cells/well) at the indicated time points. Capture ELISAs (OptEIA™ ELISA kits, BD PharMingen) were then used to detect the cytokine levels according to the manufacturer's instructions. Data were analyzed using SoftMax Pro software (Molecular Devices, Sunnyvale, CA). The limits of detection were 15.6 pg/ml for all cytokines.

2.7 Western blotting

Rat CTLA-4hIg fusion proteins, commercial human Ig Fc (Dianova) and protein standards were denatured by boiling for 5 min in reducing sample buffer, and subjected to analyze by SDS-PAGE using 12% gel. Proteins were then transferred to Hybond C membranes (Amersham) for overnight at 40 V. The position of protein standards was determined by staining membranes with Ponceau S solution. Membranes were then incubated for one hour at RT in blocking solution (5% skimmed milk powder in PBS), and subsequently probed with indicated primary Abs (WKH1 or WKH203) in blocking buffer overnight at 4°C. Blots were washed three times in 0.1% Tween20/PBS. The binding of monoclonal antibodies to the rCTLA-4-hIg or human Ig was detected by incubation of the membranes with peroxidase-conjugated goat anti-mouse Ig in blocking buffer for 1 hour. Blots were then washed three times with 0.1% Tween-20 in PBS and developed with enhanced chemiluminescence detection (ECL, Amersham).

2.8 Cell purification and cell culture

Single cell suspensions were prepared from pooled rat peripheral and mesenteric lymph nodes for isolation of T cell subpopulations. All T cell subsets ($CD4^+$, $CD4^+CD25^-$ or $CD4^+CD25^+$ cells) were purified by magnetic separation using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. $CD4^+$ T cells were purified by removing B- and $CD8^+$ cells. Briefly, cells were incubated with biotinylated anti-CD8 (OX8) and biotinylated mouse anti-rat Ig and subsequently with streptavidin microbeads. B- and $CD8^+$ Cells, which were coated with antibodies and streptavidin microbeads ($CD8^+$- and B cells), were removed by the magnetic field of a MACS separator. To separate $CD25^+$ cells from $CD25^-$ cells, lymphocytes were at first incubated with FITC-coupled anti-CD25 mAbs and anti-FITC microbeads. Cells coated with microbeads were retained in the columns, and were eluted from the columns after removing the magnetic field ($CD25^+$ cells). Unlabeled $CD25^-$ cells were then negative selected to remove $CD8^+$ and B cells as described above. $CD25^+$ $CD4^+$ cells were isolated with average purities of 85%. Purities of $CD25^-$ $CD4^+$ cells were 93-97%.

All cells were cultured at 37 °C in a 6% CO_2 and H_2O-saturated atmosphere. Unless indicated, cell lines were maintained in RPMI 1640 (Life Technologies, Karlsruhe, Germany) supplemented with 5% heat-inactivated fetal calf serum (Sigma-Aldrich), 2 mM L-Glutamine (Gibco), 100U/ml penicillin/streptomycin (Gibco), 50μM ß-mercaptoethanol (Roth, Karlsruhe, Germany), and non-essential amino acid. For culture of primary cells, X-VIVO-15 medium (BioWhittaker, Vervier, Belgium) supplemented with

5% FCS, 100 U/ml penicillin/streptomycin and 2mM L-glutamine (hereafter referred to as complete medium) was used.

2.9 Immobilization of mAbs on latex beads

Latex beads were purchased from Polysciences Europe GmbH (Eppelheim, Germany). Immobilization of mAbs on latex beads was performed according to the manufacturer's instructions. In brief, $1x10^7$ beads were washed twice with PBS, and then incubated with10 μg mAbs in 1 ml PBS for 1 hour at RT. Beads were then washed twice with PBS to move the unbound antibodies.

2.10 Immobilization of mAbs on Dynabeads

To investigate the suppressive effects of $CD25^+CD4^+$ cells under costimulation condition, cells ($CD25^-CD4^+$ indicator cells and $CD25^+CD4^+$ cells) were stimulated with anti-TCR plus anti-CD28 immobilized on Dynabeads (Dynal, Hamburg, Germany). Attachment of monoclonal antibodies on Dynabeads was performed following manufacturer's instructions. Briefly, $1x\ 10^7$ beads in 1 ml BSS/BSA were incubated with 2μg anti-TCR- and 1μg anti-CD28 antibodies at 4°C for at least one hour. Unbound antibodies were removed by washing with BSS/BSA for three times. The ratio of beads to cells in assays was 5:1.

2.11 Proliferation assays and Suppression assays

Proliferation of cells was determined by ^{3}H-thymidine incorporation. Stimulation was performed by incubation of purified T cell subpopulations with antibodies immobilized on latex beads (section 2.9), Dynabeads (section 2.10), or on 96-well plates (see below). To immobilize mAbs on 96-well F-bottomed plates, plates were first coated overnight with capture antibodies (Sheep anti-mouse Ig, ShaMIg) in coating buffer (0.1 M sodium carbonate, pH 9.5) and washed 4 times with BSS/0.2% BSA. For costimulation, ShaMIg-coated plates were incubated with anti-TCR mAbs (R73, 2μg/ml) in BSS/0.2% BSA for two hours at RT (hereafter referred to as plate-bound anti-TCR). Soluble, conventional anti-rat CD28 mAbs (JJ319, 0.5μg/ml) were then added into wells containing plated-bound anti-TCR. For in vitro superagonistic anti-CD28 stimulation, 5 μg/ml superagonistic anti-CD28 mAbs (JJ316, BD PharMingen) were added directly into wells coated with ShaMIg to cross-link CD28 superagonist. Cells were plated into wells in a final volume of 200 μl of medium with or without 300U/ml recombinant human IL-2 (Strathmann Biotec, Hamburg).

For *in vitro* suppression assays, indicator cells (naive $CD25^{-}$ or $CD25^{-}$ cells activated by superagonistic anti-CD28) were incubated toghether with $CD25^{+}CD4^{+}$ cells as indicated. Cells were cocultured in a final volume of 200 μl complete medium in 96-well Flat-plates. For suppressive effect under costimulation condition, either cells were stimulated with plate-bound anti-TCR plus soluble anti-CD28, or with anti-TCR and anti-CD28 attached on Dynabeads as described previously (section 2.10). Wells were pulsed with [^{3}H]-thymidine (0.25 μCi/well; Amersham Pharmacia Biotech) for the last 16 hours of incubation. Cells were harvested at the indicated time points and ^{3}H-incorporation

was determined using a scintillation counter. Data are presented as the mean of triplicate wells.

2.12 Flow cytometry

For extracellular staining, cells were firstly incubated with normal mouse immunoglobulin (10 μg/ml) to reduce the unspecific binding (4°C, 10 min), and then incubated with mAbs for 20 min at 4 °C in PBS (0.1% BSA, 0.02% NaN_3). After washing, cells were resuspended in PBS and analyzed by flow cytometry. Forwards and side light scatter gates were used to exclude dead cells. Data were analyzed by using CellQuest™ software (BD Biosciences). To determine CTLA-4 expression, cells were surface stained with indicated mAbs, fixed with Cytofix/Cytoperm™ (BD PharMingen), and subsequently permeabilized by incubation in saponin buffer (PBS, 0.1% BSA, and 0.5% saponin; Sigma Aldrich) at RT for 10 min. Cells were then incubated with PE-conjugated anti-CTLA-4 in 0.5% saponin buffer, washed twice in saponin buffer, and once in PBS (0.1% BSA, 0.02% NaN_3) before analyzed by flow cytometry. To determine cell divisions undergone by individual cells, carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution method was used. In brief, cells were resuspended in protein free RPMI 1640 (1x 10^7 cells/ml), and incubated for 5 min at RT with 2.5 μM CFSE (MoBiTec GmbH, Göttingen). Cells were washed twice with RPMI supplemented with 10% FCS before use.

2.13 Analysis of DNA content

7AAD (Sigma Aldrich) was used to determine the DNA content of individual cells. In brief, cells were firstly surface-stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD25, washed with PBS (0.1% BSA, 0.02% NaN_3), and then incubated with 3.3 μg/ml 7AAD in 0.1% saponin for 30 min at 4°C before analyzed by flow cytometry.

2.14 *In vivo* expansion of T cells by CD28 superagonist

In vivo expansion of rat T cells induced by one single injection of CD28 superagonist has been described previously (58). In brief, six to eight week-old LEW rats were injected i.v. with one milligram CD28 superatgonist (in 1 ml PBS). At the indicated time points, phenotype of pooled lymph node cells (peripheral and mesenteric LN) was analyzed.

2.15 Transfer of CFSE labelled $CD25^-CD4^+$ T cells

To investigate whether $CD25^-CD4^+$ T cells express CD25 upon CD28 superagonist stimulation *in vivo*, $CD25^-CD4^+$ T cells from pooled lymph nodes (peripheral and mesenteric lymph nodes) were isolated, labelled with CFSE, and then injected i. v. into rats ($1x10^7$cells/rat). Eight hours later, rats received one milligram CD28 superagonist. Two or four days after injection of CD28 superagonist, CD25 expression on endogenous $CD4^+$ T cells and CFSE-labelled $CD25^-CD4^+$ T cells were analyzed.

CHAPTER 3 MODULATION OF T CELL RESPONSES WITH CTLA-4 SPECIFIC ANTIBODIES

3.1 Introduction

Like its homolog CD28, cytotoxic T lymphocyte antigen-4 (CTLA-4 or CD152) belongs to the immunoglobulin superfamily (26). CTLA-4 is a highly conserved molecule: the rat CTLA-4 polypeptide shows 90% and 76% identity with the mouse and human CTLA-4 amino acid sequences, respectively (75-77). Furthermore, intracellular amino acid sequences are identical among these three species, implicating the conservation and importance of CTLA-4 signalling in the immune system. The extracellular motif MYPPPY, which is responsible for the interaction of CTLA-4/CD28 with their ligands CD80/CD86, is present in CD28 and CTLA-4 of all species examined (78). The conservation of this motif in CD28 and CTLA-4 predicts that the interaction of CD28 and CTLA-4 with their natural ligands CD80 (B7-1) and CD86 (B7-2) is species non-specific. Indeed, rat CTLA-4 is able to bind to mouse B7 (data not shown).

Since CTLA-4 and CD28 bind the same ligands (23, 79, 80), it is difficult to dissect CTLA-4 functions in vitro and in vivo from those of CD28 by examining the interaction of these two receptors with their ligands directly. Present knowledge about the expression and functions of CTLA-4 was obtained mostly from CTLA-4 deficient mice (81-83) and with the help of monoclonal antibodies specific for CTLA-4 (25).

In mice and humans, constitutive CTLA-4 expression is restricted to a subpopulation of thymocytes and naive T cells: this population co-expresses CD4 and CD25 (28, 46), and is identified as regulatory T cells which can modulate responses of T and B cells (45, 54). Furthermore, all T cells are induced to express CTLA-4 upon activation (29, 84), and TCR signal strength may influence the level of CTLA-4 expression (32). In addition, CTLA-4 is predominantly an intracellular protein coupled to the endocytosis machinery via its intracellular domain (30, 85, 86). Upon stimulation, the CTLA-4 intracellular domain is phosphorylated, resulting in uncoupling of CTLA-4 from the endocytosis machinery (30, 87). Thus, not only the total CTLA-4 protein level, but also surface expression of CTLA-4 increases in activated T cells.

Parallel to the phenotype of CTLA-4 deficient mice, which show a lymphoproliferative disorder and multi-organ autoimmune diseases (35, 81), *in vitro* cross-linking of CTLA-4 using monoclonal antibodies also shows that CTLA-4 functions as a negative regulator of the T cell response (33)(88). Furthermore, delivery of blocking anti-CTLA-4 monoclonal antibodies, which interfere with the binding of CTLA-4 to B7, is found to enhance anti-tumour immunity in mice (89) and humans (37).

To examine rat CTLA-4 expression and function, monoclonal antibodies specific for rat CTLA-4 were generated here. In addition to establishing an *in vitro* read out system for CTLA-4 function, these monoclonal antibodies can also be used to examine CTLA-4 functions in rat disease models by delivery of antibody into animals to modulate the outcome of immune the response.

3.2 Characterization of rat CTLA-4 by monoclonal antibodies

3.2.1 Generation of rat CTLA-4 specific monoclonal antibodies

To generate monoclonal antibodies specific for rat CTLA-4, BALB/c mice were immunised with a rat CTLA-4hIg fusion protein (rCTLA-4hIg) containing the extracellular region of rat CTLA4 and the Fc part of human immunoglobulin IgG1 (Fig. 2A). Details about the generation of rat CTLA-4hIg fusion proteins and immunisation are described in Materials and Methods (Chapter 2). Monoclonal antibody-secreting hybridomas were generated by fusion of splenocytes from immunized mice and myeloma cells X63-Ag8.653. Supernatants from hybridomas were collected and examined for their specificity in ELISA. WKH1 and WKH203, two monoclonal antibodies that recognized only rat CTLA-4hIg fusion proteins, but not commercial human IgG1 Fc, were selected (Fig 3A). Further examination in western blot confirmed that these two monoclonal antibodies recognized only rat CTLA-4hIg fusion proteins (Fig. 3B). Because WKH1 and WKH203 competed with each other for binding of rCTLA-4hIg and thus are likely to bind to the same or to related epitopes (data not shown), only WKH203 was purified and used for further characterisation of rat CTLA-4.

Next, WKH203 was examined for its ability to bind native rat CTLA-4 molecules. For this purpose, BW cells were transduced to express rat CTLA-4 and green fluorescent protein (BW-CTLA-4-GFP). To exclude unspecific binding of WKH203 to cellular proteins other than CTLA-4, BW cells expressing green fluorescent protein alone (BW-GFP) were used as negative control. As shown in Fig. 4, WKH203 but not control antibody (mouse IgG1) stained CTLA-4 expressing BW cells. The binding was specific,

since BW cells expressing only GFP were not stained by WKH203. Interestingly, no difference in the fluorescence intensity was observed between CTLA-4 expressing BW cells surface stained with WKH203 and the same cells incubated with control antibody (data not shown), although chimeric proteins, composed of the rat CTLA-4 extracellular domain and the CD28 intracellular part, were readily detected at the BW cell surface by WKH203 (K. Dennehy, personal communication). This result indicates that rat CTLA-4 expressed in BW cells is localised mainly in intracellular compartments, and is consistent with the finding that CTLA-4 is predominantly an intracellular protein in mouse and human T cells (30, 31, 85). In conclusion, WKH203 recognizes not only denatured CTLA-4, as shown by its reactivity with CTLA-4hIg fusion proteins in ELISA and western blot, but also binds native CTLA-4 proteins.

3.2.2 Expression of CTLA-4 in naive rat $CD4^+$ T cells

In mice and humans, only a subpopulation of $CD4^+$ T cells, which co-expresses CD25, was shown to express CTLA-4 constitutively. No serologically detectable CTLA-4 expression is found in naive $CD25^-CD4^+$ cells (28) (46). Thus, CTLA-4 expression in thymocytes and in lymph node cells freshly isolated from unimmunized rats was first examined here.

Consistent with earlier observations in rats (90), about 5% of rat CD4-single positive thymocytes expressed CD25 (Fig. 5A). Furthermore, these $CD25^+CD4^+$ thymocytes were CTLA-4 positive, since a clear shift of fluorescence intensity was observed when this population was stained with PE-conjugated WKH203 (solid line), compared with cells blocked with unconjugated WKH203 before incubation with PE-conjugated

WKH203 (dotted line). In contrast, there was no detectable CTLA-4 expression in $CD25^-$ $CD4^+$ thymocytes. A similar expression pattern was observed in peripheral $CD4^+$ lymph node T cells: $CD25^+CD4^+$ T cells (about 5% of $CD4^+$ T cells) expressed CTLA-4, while $CD25^-$ T cells were not stained by anti-rat CTLA-4 antibody (Fig. 5B). Thus, only $CD25^+CD4^+$ cells from naive rats had detectable CTLA-4 expression.

3.2.3 Upregulation of CTLA-4 in stimulated rat T cells

While only *ex vivo* $CD25^+CD4^+$ T cells constitutively express CTLA-4, CTLA-4 expression is induced in initially CTLA-4 negative mouse T cells by anti-TCR stimulation (29, 84). Moreover, T cells further increase CTLA-4 expression upon anti-TCR plus anti-CD28 stimulation (costimulation). Is rat CTLA-4 regulated in the same way? To answer this question, CTLA-4 expression in rat T cells upon anti-TCR- and costimulation was investigated. In addition, CTLA-4 expression in T cells activated by CD28 superagonist (Fig. 1) was also examined. Since CD28 superagonist activates T cells without engagement of TCR (58), CD28 superagonist provides the possibility to test whether signals transduced from engagement of TCR is obligatory for the upregulation of CTLA-4 in activated T cells.

T cells isolated from lymph nodes were either stimulated with anti-TCR (R73) alone, with anti-TCR plus anti-CD28, or with CD28 superagonist. Naïve rat T cells stimulated with anti-TCR alone became blasts transiently, and most cells stimulated in this way died during the first two days of stimulation (data not shown). In contrast, rat T cells were fully activated to proliferate by both costimulation and CD28 superagonist stimulation. Following TCR-stimulation, rat T cells were found to express CTLA-4 after

24 hours, although the expression level was very low (data not shown), and no further upregulation of CTLA-4 was observed at later time points. In contrast, rat T cells stimulated by costimulation expressed high levels of CTLA-4 (Fig. 6). The kinetics of CTLA-4 upregulation in costimulated rat $CD4^+$ T cells was similar to the kinetics observed in mice (29): expression of CTLA-4 reached the maximum after 48 hour stimulation. Interestingly, CTLA-4 was also upregulated in CD28 superagonist-stimulated rat T cells, although $CD4^+$ T cells stimulated in this way had delayed kinetics in CTLA-4 upregulation as compared to costimulated cells. Thus, CTLA-4 expression increases in activated $CD4^+$ T cells, and TCR-ligation was not obligatory for CTLA-4 upregulation.

Whether CTLA-4 is important in negatively regulating both $CD4^+$ and $CD8^+$ T cell responses is still unclear. Interestingly, CTLA-4 expression in activated $CD8^+$ T cells was and remained low upon stimulation (Fig. 6). $CD4^+$ T cells had much higher CTLA-4 expression (an up to four fold difference) than $CD8^+$ T cells, although both $CD8^+$ and $CD4^+$ T cells upregulated CD25 upon costimulation (Fig. 6). Possibly, CTLA-4 is more important for deactivation of $CD4^+$ T cells than $CD8^+$ T cells.

3.3 Regulation of rat T cell responses by CTLA-4

It has been shown that CTLA-4 functions as a negative regulator of the T cell response, since cross-linking of CTLA-4 with monoclonal antibody leads to decreased proliferation of costimulated T cells (33, 88). The in vitro inhibitory effect of CTLA-4 is demonstrated by using latex beads coated with anti-TCR, anti-CD28 and anti-CTLA-4

monoclonal antibodies as 'artificial antigen presenting cells' (91). In this system, the ratio of immobilised anti-CTLA-4 mAb to anti-TCR and anti-CD28 is very high (anti-CTLA-4: anti-TCR: anti-CD28 = 9:1:1) in order to achieve strong cross-linking of CTLA-4 molecules.

To investigate the effect of CTLA-4 ligation on rat T cells upon stimulation in vitro, the latex bead system was adopted. Although it would have been of interest to examine the effect of CTLA-4 on T cells stimulated by CD28 superagonist, this experiment was not feasible because rat T cells were unresponsive to CD28 superagonist immobilised on latex beads (data not shown). Therefore, only the effect of CTLA-4 on costimulated T cells was examined.

Costimulation was performed by using latex beads ($1x10^5$ beads/well) coated with anti-TCR, anti-CD28 mAbs, and with anti-CTLA4 or control mouse IgG1 (anti-TCR: anti-CD28: CTLA-4/control Ab at the ratio of 1:1:9) together with the same number of purified T cells. Upon co-stimulation, most T cells (94%) were induced to express the activation marker CD71 (transferrin receptor, (92)), while only forty-eight percent of T cells were CD71 positive in the presence of anti-CTLA-4 (Fig. 8A). Thus, T cell activation was dampened upon CTLA-4 ligation.

To investigate whether CTLA-4 can inhibit cell divisions, the responding T cells were labelled with the strong fluorescent dye CFSE, which is diluted into the two daughter cells during each cell division (93). Thus, by examining the fluorescence intensity of CFSE-labelled cells, cell divisions undergone by each individual cell can be followed. As

shown in Fig. 7B, most T cells stimulated with anti-TCR plus anti-CD28 (costimulation) underwent three to five cell divisions. Upon cross-linking of CTLA-4, the majority of T cells underwent less than three cell divisions. In addition, cells had on average a smaller cell size in the presence of anti-CTLA-4 (Fig. 8), regardless of the division numbers cells underwent. Thus, signalling mediated by CTLA-4 downregulated T cell activation, and influenced the cell size.

As expected from the anti-CTLA-4 effect on T cell divisions, costimulation-induced ^{3}H-thymidine incorporation was decreased by cross-linking CTLA-4 (Fig. 9A). Correlated to the expression kinetics of CTLA-4 upon costimulation, T cell proliferation affected by anti-CTLA-4 was more prominent at later time points, although inhibition was readily observed after one-day stimulation. Furthermore, production of IL-2, an important growth factor for T cells *in vitro*, was almost reduced to baseline level in the presence of anti-CTLA-4 after twenty-four hour incubation (Fig. 9B). Thus, consistent with observations in mice and humans (33, 94), rat CTLA-4 deactivated T cell responses upon costimulation.

3.4 Discussion

An optimal T cell response does not only require activation of T cells; after elimination of foreign invaders or abnormal cells, the immune response has to be down-regulated, and the size of expanded T-cell clones must be reduced. Although some activated T cells can be eliminated by activation induced cell death (AICD, (95)), other mechanisms, such as interaction of deactivating molecules of T cells with their ligands, also contribute to the down-regulation of T cell responses without directly causing T cell

death. The importance of 'deactivating molecules' is demonstrated by mice lacking these molecules. The phenotype of CTLA-4 deficient mice, which suffer lymphoproliferative disorder and die in 2-4 weeks after birth, shows that CTLA-4 functions as an important negative regulator of T cells and may be involved in maintenance of peripheral tolerance (96, 97).

The mechanisms by which CTLA-4 exerts its functions in T cells remain under intensive investigation (see review: (98)). Also, the signals transduced by CTLA-4 ligation are incompletely understood (99-101) and controversial. The binding of the same ligands with CD28, the principal costimulatory molecule in naive T cells, complicates the analysis of the CTLA-4 signalling pathway. Thus, monoclonal antibodies specific for CTLA-4 are a useful tool to examine the role of CTLA-4 in vivo and in vitro. In this study, monoclonal antibodies against rat CTLA-4 were generated. Using these antibodies, CTLA-4 expression and in vitro functions of rat T cells were examined for the first time. It is not the major subject here to use the anti-rat CTLA-4 mAbs to clarify the signalling transduced by CTLA-4, neither did this study provide more details about the underlying mechanisms by which CTLA-4 acts to downregulate T cell response. Since transgenic or knockout rats are still rare or unavailable, the possibilities for such analysis are quite limited. Rather, these monoclonal antibodies were used to establish an *in vitro* read out system for examining rat CTLA-4 function. Based on the knowledge from *in vitro* experiments, further investigation will concentrate on the role of CTLA-4 in rat autoimmune disease models, such as EAE, and examine the possibility of therapeutic use of this antibody.

The two anti-rat CTLA-4 monoclonal antibodies, WKH1 and WKH203, were generated by immunizing mice with rat CTLA-4hIg fusion proteins (Fig. 2). These two antibodies competed with each other for the binding to rat CTLA-4. Although this did not mean necessarily that both antibodies bind to the same epitope and have the same effect in vivo and in vitro, they had, however, shown similar *in vitro* inhibitory effects on activated T cells. Thus, only WKH203 was used for further characterisation of rat CTLA-4. Interestingly, WKH203 did not interfere with the interaction between CTLA-4 and the natural ligands B7, as binding of CTLA-4Ig fusion proteins to CD80 expressing cells was not affected by incubation of CTLA-4Ig with WKH203 before staining (data not shown). Whether this can induce different effects compared to effects induced by delivery of blocking anti-CTLA-4 monoclonal antibodies into animals, is unclear and worth of investigation.

As expected from the highly conserved amino acid sequences between rat, mouse, and to a less extent, human CTLA-4, the expression pattern of rat CTLA-4 is similar to the pattern found in mouse and human T cells (29, 102): only rat $CD25^+CD4^+$ T cells expressed serologically detectable CTLA-4 ex vivo. All $CD4^+$ T cells, however, upregulated CTLA-4 expression upon activation. Interestingly, rat $CD4^+$ T cells activated by CD28 superagonist without TCR engagement also expressed CTLA-4, although CTLA-4 upregulation in CD28 superagonist activated T cells had a delayed kinetics as compared to cells stimulated with anti-TCR plus anti-CD28. Whether the delayed upregulation of CTLA-4 in CD28 superagonist stimulated $CD4^+$ cells means that cells stimulated in this way are less sensitive to CTLA-4 mediated deactivation, needs further investigation.

Consistent with results obtained with mouse CTLA-4, cross-linking of rat CTLA-4 induced downregulation of an activation marker (CD71), cell proliferation, and IL-2 production of T cells (88) (91). Interestingly, although inhibition of proliferation was more pronounced at later time points (after 72 hours), IL-2 production was readily reduced to baseline in the presence of anti-CTLA-4 already after 24-hours costimulation, at the time point that little CTLA-4 was detected by monoclonal antibodies. This indicates that signals transduced through CTLA-4 could interfere with the signalling pathway required for IL-2 production, such as the CD28-signalling cascade. Since T cells used in the experimental system demonstrated here contained $CD25^{+}CD4^{+}$ regulatory T cells, which constitutively express CTLA-4 (see Chapter 4), another possibility is that the early reduction of IL-2 production in the presence of anti-CTLA-4 mAb might be due to the action of regulatory T cells.

In addition to the inhibition of CD71 expression, proliferation, and IL-2 production, engagement of CTLA-4 by mAb was found to reduce the cell size of costimulated T cells (Fig. 8). In line with this, Doyle et al. (42) have shown that CTLA-4 deficient T cells have an average larger cell size upon costimulation, regardless how many cell divisions these cells underwent. Thus, CTLA-4 signalling is also important for the regulation of cell size.

In this study, novel anti-rat CTLA-4 monoclonal antibodies were generated. The novel anti-rat CTLA-4 monoclonal antibodies allow the characterisation of an important cell population, namely $CD25^{+}CD4^{+}$ T cells in rats (Fig. 5 and see below), and the regulation of CTLA-4 in rat T cells. Evidence from mouse and human CTLA-4 together with these findings indicate that CTLA-4 is highly conserved both in its expression and

functions. Further investigation of CTLA-4 using these antibodies in modulating immune responses in rat disease models may give more insights about the possibility to manipulate immune reactions in humans.

CHAPTER 4 MODULATION OF T CELL RESPONSES BY RAT CD25+CD4+ T CELLS

4.1 Introduction

Recently, CD25+CD4+ regulatory T cells (Treg cells) have attracted intensive attention because of their regulatory effects on immune responses (see reviews: (103)). Depletion of CD25+CD4+ cells, either by thymectomy 3 days after birth or by delivery of anti-CD25 mAb caused organ-specific autoimmune disease, such as thyroiditis (104) or inflammatory bowel disease (47). Furthermore, co-transfer of CD25+CD4+ regulatory T cells together with naive CD25-CD4+ T cells into lymphopenic organisms prevents autoimmune diseases, which are induced by transferring of CD25-CD4+ T cells alone (105). Hence, the potentially harmful autoreactive T cells are controlled by CD25+CD4+ regulatory T cells under certain experimental conditions.

Although CD25+ CD4 single positive cells are found in the thymus (90, 106), it is still controversial whether CD25+CD4+ regulatory T cells represent a distinct lineage; thus, several studies have shown that CD25+CD4+ cells can arise in the periphery (107, 108) and may constitute a heterogeneous population of cells. Nevertheless, there is phenotypic and functional continuity between CD25+CD4+ thymocytes and CD25+CD4+ T cells found in secondary lymphoid organs: they are the only cells constitutively expressing CTLA-4, and both exhibit regulatory activity in controlling autoreactive T cell responses in vivo and in vitro.

About 10 % of peripheral $CD4^+$ T cells in mice and humans are CD25 positive (45, 46). Upon *in vitro* anti-TCR stimulation, $CD25^+CD4^+$ T cells proliferate poorly and do not produce IL-2 (45, 55). Furthermore, proliferation and IL-2 production of naive $CD25^-$ $CD4^+$ T cells upon stimulation can be suppressed by $CD25^+CD4^+$ regulatory T cells, when both populations are cultured together (46). The suppressor activity of 'naive' $CD25^+CD4^+$ T cells freshly isolated from untreated animals can be overcome by exogenous IL-2 or strong costimulatory stimuli (45, 104). Furthermore, the in vitro suppression effect of Treg cells is shown to be cell-cell contact dependent (104, 109, 110). However, the relevance of the *in vitro* suppressor activity of $CD25^+CD4^+$ regulatory T cells for their in vivo regulatory functions is still unclear. Nevertheless, because all mouse and human $CD25^+CD4^+$ regulatory T cells exhibit suppressive activity in vitro, this can be utilised as an in vitro read out system for their initial characterization.

In rats, a subpopulation of CD4 positive cells (about 5%) expresses CD25 in thymus and peripheral lymph nodes and these $CD25^+$ $CD4^+$ cells are able to control diabetes *in vivo* (90). Still, knowledge about phenotype and functions of these rat $CD25^+CD4^+$ cells is restricted. Using the anti-rat CTLA-4 monoclonal antibody WKH203 described in the previous chapter, rat $CD4^+CD25^+$ cells were characterised for the first time in detail. Also, the proliferative response and the cytokine profile of $CD25^+CD4^+$ versus $CD25^-$ $CD4^+$ T cells upon stimulation were examined here. Furthermore, an *in vitro* suppression assay was established in this study to investigate the suppressor activity of rat $CD25^+CD4^+$ T cells.

Another question addressed in this study was the responses of rat $CD25^+CD4^+$ T cells toward CD28 superagonist. The interest in a possible CD28 superagonist effect on Treg cells was based on the earlier findings that *in vivo* delivery of CD28 superagonist into rats causes massive lymphocyte proliferation (58), but no 'cytokine storm' and no signs of inflammatory disease are observed (63, 111), as one may expect from animals undergoing polyclonal T-cell activation, e.g. after injection of superantigens, which also have proliferating lymphocytes and suffer from autoimmune disease (112). At the same time, $CD25^+CD4^+$ T cells were increased both by percentage and absolute cell numbers. These phenomena suggested that $CD25^+CD4^+$ T cells might be preferentially expanded in CD28 superagonist injected rats, and these cells can inhibit the activation of potentially pathogenic T cells and prevent autoimmune disease. Thus, investigation was focused on the characterisation of $CD25^+CD4^+$ T cells in CD28 superagonist treated rats and the possibility to use CD28 superagonist to expand $CD25^+CD4^+$ T cells *in vitro* and *in vivo*.

4.2 Characterisation of $CD25^+CD4^+$ cells from untreated rats

In mice and humans, $CD25^+CD4^+$ regulatory T cells are found in thymus and in secondary lymphoid organs (46, 55, 106, 113). These cells constitutively express CTLA-4, are CD45R low, and express a high level of L-selectin (CD62L). Consistent with the findings in mice, Mason et al. (90) have shown that in rat thymus, about 5% of CD4 single positive thymocytes express CD25. Furthermore, these cells are able to prevent diabetes when injected together with autoreactive T cells into susceptible animals. This indicated the presence of $CD25^+CD4^+$ regulatory cells in the rat thymus. However, rat

CD25$^+$CD4$^+$ cells were incompletely characterised. Using the new anti-rat CTLA-4 mAb (see chapter 3), CD25$^+$CD4$^+$ thymocytes were found to express CTLA-4, while CD25$^-$ CD4$^+$ thymocytes were CTLA-4 negative (Fig. 5A). Also consistent with the phenotype of CD25$^+$CD4$^+$ regulatory T cells in mice, rat CD25$^+$CD4$^+$ T cells from peripheral lymph organs constitutively expressed CTLA-4 (Fig. 5B), were predominately CD45RC low (Fig. 10A), and expressed a high level of CD62L (Fig. 10B).

4.3 *In vitro* responses of rat CD25$^+$CD4$^+$ T cells

4.3.1 Effect of exogenous IL-2 on CD25$^+$CD4$^+$ cells

In mice, IL-2 signalling is required for the maintenance and functions of CD25$^+$CD4$^+$ regulatory T cells *in vivo* (114-116). Consistent with these findings, isolated rat CD25$^+$ CD4$^+$ T cells cultured in medium died spontaneously: up to 70% of cells were not in the 'live' gate of forward and sideward light scatter (data not shown). To confirm this, the DNA content of individual cells was analysed by flow cytometry after staining with 7AAD, which can enter into permeabilised cells and bind DNA. As shown in Fig. 11A, only about 38% of CD25$^+$CD4$^+$ T cells had intact, diploid nuclei after 48 hours cultured in medium. The majority of rat CD25$^+$CD4$^+$ T cells had undergone DNA degradation and showed a subdiploid phenotype. In contrast, most CD25$^-$CD4$^+$ cells (70%, as indicated in M1 of Fig. 11B) had intact nuclei and thus were live cells. In the presence of exogenous IL-2, CD25$^+$ cells were rescued from cell death (Fig. 11A), as now most CD25$^+$ cells had a full diploid DNA content (81%, as indicated in M1). As expected, there was no effect on survival of CD25$^-$ cells with exogenous IL-2. Interestingly, a few

$CD25^+CD4^+$ T cells began to cycle and to synthesize DNA, since about 7% of $CD25^+$ cells were in the S and G2 phases of the cell cycle. The increase in uptake of radioactive labelled thymidine by $CD25^+CD4^+$ cells in the presence of IL-2 also supports that IL-2 could induce $CD25^+CD4^+$ T cells to proliferate to some extent (data not shown). In addition, exogenous IL-2 increased the CD25 expression of rat $CD25^+CD4^+$ cells (5 fold increase, Fig. 12).

Taken together, exogenous IL-2 was not only important for the survival of $CD25^+CD4^+$ cells *in vitro*, but actually promoted the upregulation of CD25 and induced some cell proliferation. Whether in vivo IL-2 is also necessary for the maintenance of rat $CD25^+CD4^+$ cells remains to be examined. Furthermore, since CD25, one component of IL-2 receptor, is known not to transduce signals by itself, the ability of $CD25^+$ cells to respond to exogenous IL-2 indicated that they express the other chains of IL-2 receptor, IL-2 Rβ and IL-2Rγ, which are involved in IL-2 induced signalling pathway.

4.3.2 Costimulation versus CD28 superagonist stimulation

One characteristic of $CD25^+CD4^+$ regulatory T cells from mice and humans is that $CD25^+CD4^+$ regulatory T cells are unable to proliferate and do not produce IL-2 upon engagement of the T cell receptor complex alone (45, 55, 117). In the presence of ligands of the costimulatory molecule CD28 and/or IL-2, however, proliferation of $CD25^+CD4^+$ regulatory T cells can be enhanced (104). Since the $CD25^+CD4^+$ T cells identified in rats had a similar phenotype as mouse and human $CD25^+CD4^+$ regulatory T cells, and may function as regulatory T cells in rats, the responses of rat $CD25^+CD4^+$ T

cells to antibody-induced stimulation were examined. Stimulation was performed by using monoclonal antibodies for T cell receptor and CD28 without antigen presenting cells for two reasons. Firstly, stimulation of rat T cells with monoclonal antibodies is well established in our laboratory. Secondly, since only $CD25^+CD4^+$ and/or $CD25^-CD4^+$ T cells were used in experiments, any observed effects resulted from the response and the interaction of these two cell populations only.

To compare the response of rat $CD25^+CD4^+$ versus $CD25^-CD4^+$ T cells upon stimulation, $CD4^+$ (containing 10% of $CD25^+$ cells), $CD25^-CD4^+$, and $CD25^+CD4^+$ cells and were purified (Fig. 13, control staining). Because rat T cells did not respond to anti-TCR stimulation alone (data not shown), all experiments were performed under conditions of costimulation (anti-TCR plus anti-CD28) or CD28 superagonist stimulation with or without exogenous IL-2.

Upon costimulation or CD28 superagonist stimulation, all cells became blasts as they had shown increased forward scatter (FSC) in flow cytometry (data not shown). Although $CD4^+$ cells contained 10% $CD25^+CD4^+$ cells, i.e. the proposed frequency of Treg cells, at the beginning, proliferation of $CD4^+$ cells was comparable to proliferation of $CD25^-CD4^+$ cells after forty- and sixty-four hour stimulation (Fig. 14). Furthermore, exogenous IL-2 did not enhance the proliferation of $CD4^+$ cells and $CD25^-CD4^+$ cells at the examined time points, suggesting that costimulation or CD28 superagonist stimulation alone without exogenous IL-2 could induce the maximum cell expansion during the first three days. Although $CD25^+CD4^+$ cells showed comparable proliferation to $CD25^-CD4^+$ T-cells at first 40 hours, their proliferation was reduced to only one third of

$CD25^-CD4^+$ cell proliferation after 64 hour costimulation. This reduction could be totally overcome by the addition of high concentrations of exogenous IL-2 (300 U/ml). In contrast, $CD25^+CD4^+$ cells stimulated with CD28 superagonist proliferated as well as $CD4^+$- and $CD25^-CD4^+$ cells.

Unlike $CD25^-CD4^+$ T cells, $CD25^+CD4^+$ regulatory T cells described in mice and humans do not produce IL-2 upon costimulation (45, 110). Therefore, IL-2 secreted by $CD4^+$, $CD25^-CD4^+$, and $CD25^+CD4^+$ cells upon stimulation was examined by ELISA. Similar to the findings in mice and humans, high amounts of IL-2 were secreted by $CD4^+$ or $CD25^-CD4^+$ cells upon costimulation (Fig. 15). Even more IL-2 (a two fold increase) was found in supernatants of CD28 superagonist stimulated $CD4^+$ cells and $CD25^-CD4^+$ cells. In contrast, no IL-2 was detectable in supernatants of costimulated-$CD25^+CD4^+$ cells and only little IL-2 was found in supernatants of $CD25^+CD4^+$ cells upon CD28 superagonist stimulation after 24-hour stimulation, and then decreased to baseline afterwards.

An important cytokine involved in $CD25^+CD4^+$ regulatory T cell function *in vivo* and *in vitro* is IL-10 (52, 118). Therefore, IL-10 production by cells upon in vitro stimulation was determined. As shown in Fig. 15, $CD25^+CD4^+$ cells produced readily detectable amounts of IL-10 after one day of stimulation, and even more IL-10 was found in supernatants collected at 48- or 72-hours. In contrast, stimulated $CD25^-CD4^+$ cells produced little or no IL-10 at the first 48 hours. $CD4^+$ cells (containing $CD25^+CD4^+$ cells and $CD25^-CD4^+$ cells in natural proportion) produced higher amounts of IL-10 than $CD25^-CD4^+$ cells after 48 hour-stimulation, and further increased IL-10 production to the level observed in

$CD25^+CD4^+$ cells after three days of costimulation. The earlier kinetics and higher amounts of IL-10 production by unseparated $CD4^+$ cells compared to pure $CD25^-CD4^+$ cells probably was due to the presence of $CD25^+CD4^+$ cells in the $CD4^+$ cell population.

Interestingly, $CD4^+$ or $CD25^-CD4^+$ cells produced much less IL-10 upon CD28 superagonist stimulation than upon costimulation (2 to 10 fold difference), in contrast to higher IL-2 production of cells upon CD28 superagonist stimulation. Furthermore, since $CD4^+$ cells and $CD25^-CD4^+$ cells showed comparable proliferation, but produced different amounts of IL-10, this argues against a direct inhibition of T cell proliferation by IL-10.

In mice and humans, $CD25^-CD4^+$ cells can be induced to express CD25 *in vitro* upon stimulation by autocrine IL-2 (119). This is also true for rat $CD25^-CD4^+$ cells. As shown in Fig. 16, CD25 expression was induced on $CD25^-CD4^+$ cells upon costimulation and CD28 superagonist stimulation. Exogenous IL-2 had no additional effect on CD25 expression of $CD25^-CD4^+$ cells. Like $CD25^-CD4^+$ cells, rat $CD25^+CD4^+$ cells increased their CD25 expression upon costimulation and CD28 superagonist stimulation. Furthermore, costimulation induced CD25 upregulation in $CD25^+CD4^+$ cells was further increased in the presence of exogenous IL-2. Interestingly, although both $CD25^-CD4^+$ cells and $CD25^+CD4^+$ cells upregulated their CD25 expression upon stimulation, $CD25^+CD4^+$ cells expressed consistently more CD25 (up to four fold higher CD25 in $CD25^+CD4^+$ cells).

Taken together, although both $CD25^-CD4^+$ and $CD25^+CD4^+$ T cells became blasts and had similar cell size (data not shown) upon stimulation, they exhibited different

responses: while $CD25^-CD4^+$ cells proliferated vigorously and produced high amounts of IL-2, $CD25^+CD4^+$ cells had reduced proliferation upon costimulation and were unable to produce IL-2. Furthermore, CD28 superagonist greatly enhanced the proliferative capacity of $CD25^+CD4^+$ cells, with little or no effect on IL-2 production of this population. While IL-2 enhanced the responses of $CD25^+CD4^+$ cells upon costimulation, addition of IL-2 had no effect on $CD25^-CD4^+$ cells, probably because IL-2 was in excess. Although it is still controversial whether $CD25^+CD4^+$ cells are the major source of IL-10 and whether IL-10 can inhibit T cell proliferation directly (120-122), rat $CD25^+CD4^+$ T cells were found to produce high amounts of IL-10 upon costimulation and CD28 superagonist stimulation, while $CD25^-CD4^+$ T cells produced little IL-10 and only with delayed kinetics.

4.4 Interaction between $CD25^+CD4^+$ and $CD25^-CD4^+$ cells from naive LEW rats

In addition to the hyporesponsive states upon anti-TCR stimulation, mouse and human $CD25^+CD4^+$ regulatory T cells are shown to control the *in vitro* response of other T cells : in coculture, $CD25^+CD4^+$ regulatory T cells can suppress proliferation and IL-2 production of $CD25^-$ T cells upon anti-TCR stimulation (45, 117). Upon costimulation or in the presence of exogenous IL-2, suppression can be overcome. This *in vitro* suppressive effect by $CD25^+CD4^+$ regulatory T cells is used as a read out system of regulatory T cell activity. Furthermore, while $CD25^+CD4^+$ thymocytes and T cells are reported to control autoimmune disease in rats (90), *in vitro* interaction between $CD25^+CD4^+$ and $CD25^-CD4^+$ cells had not been investigated. Thus, the coculture system

was adopted here to examine the suppressive effect of rat $CD25^+CD4^+$ cells on $CD25^-CD4^+$ cells.

Purified $CD25^+CD4^+$ cells and $CD25^-CD4^+$ cells were cocultured at the ratio of 1:1, and examined for ^{3}H-thymidine incorporation upon costimulation and CD28 superagonist stimulation (Fig. 17A). Consistent with the observations described earlier (Fig. 14), costimulated $CD25^+CD4^+$ cells proliferated less well than costimulated $CD25^-CD4^+$ cells (here: a 2 fold difference), while no difference in $CD25^+CD4^+$ and $CD25^-CD4^+$ cell proliferation was observed upon CD28 superagonist stimulation. Furthermore, no suppressive effect of $CD25^+CD4^+$ cells based on proliferation was detected, since the proliferation of cells in coculture was comparable to the proliferation of $CD25^-CD4^+$ cells alone under conditions of costimulation and CD28 superagonist stimulation.

While ^{3}H-thymidine incorporation assays measure the bulk contribution of all proliferating cells in coculture, responses of individual cells in coculture cannot be examined. In order to distinguish $CD25^+CD4^+$ cells from the cocultured $CD25^-CD4^+$ cell population, and to follow the cell divisions undergone by $CD25^-CD4^+$ cells in the coculture $CD25^-CD4^+$ cells were labelled with CFSE.

As shown in Fig. 18A, the majority of $CD25^-CD4^+$ cells underwent 2 to 3 cell divisions after two days of costimulation. A slight reduction in cell divisions of cocultured $CD25^-CD4^+$ cells was observed, although there was no difference in ^{3}H-thymidine incorporation between $CD25^-CD4^+$ cells alone and in culture of $CD25^-/CD25^+CD4^+$ T cells (Fig. 17). Furthermore, CD25 expression of $CD25^-CD4^+$ cells in coculture was lower

than that of $CD25^-CD4^+$ cells simulated alone, while $CD25^+CD4^+$ cells in coculture had higher surface CD25 expression than $CD25^+CD4^+$ cells cultured alone. In contrast, there was no reduction in cell divisions of cocultured $CD25^-CD4^+$ cells upon CD28 superagonist stimulation. Under CD28 superagonist stimulation, all $CD25^-CD4^+$ cells, which now expressed a high level of CD25, maintained their level of CD25 expression in coculture.

The higher CD25 expression on $CD25^+CD4^+$ cells in coculture as compared to $CD25^+CD4^+$ cells cultured alone indicates that the response of $CD25^+CD4^+$ cells might also be modulated by $CD25^-CD4^+$ cells in coculture. Consistent with this, cycling of $CD25^+CD4^+$ cells (as indicated by the fraction of cells in S + G2/M phases) was increased in coculture with $CD25^-CD4^+$ cells, while slightly decreased cycling of $CD25^-CD4^+$ cells was observed at the same time (Fig. 19). Furthermore, in the presence of IL-2, both $CD25^-$ cells and $CD25^+CD4^+$ cells had an increased percentage of cycling cells (data not shown). Since CD25 expression (Fig. 16) and survival/proliferation of $CD25^+CD4^+$ cells were influenced by exogenous IL-2, $CD25^-CD4^+$ cells in coculture might modulate the $CD25^+CD4^+$ cell response by producing IL-2 and in a paracrine way, although some other unknown factors might also participate.

Thus, under costimulation or CD28 superagonist stimulation, coculture of $CD25^-CD4^+$ T cells with freshly isolated rat $CD25^+CD4^+$ T cells did not cause a reduction in proliferation as read out by bulk ^{3}H-thymidine incorporation. However, coculture of $CD25^-CD4^+$ T cells with $CD25^+CD4^+$T cells under costimulation led to modulation of responses in both populations: while $CD25^-CD4^+$ T-cells showed lower CD25

expression, reduced cycling, and underwent fewer cell divisions, $CD25^+CD4^+$ T cells were more activated in coculture since they had higher CD25 expression and more cycling cells. This is consistent with the observation of a suppressive effect exerted on $CD25^-$ T cells by $CD25^+CD4^+$ T cells as reported in mice and humans. Interestingly, although $CD25^+CD4^+$ T cells also had higher CD25 expression in coculture upon CD28 superagonist stimulation, activation of $CD25^-CD4^+$ T cells were not inhibited at the time point of examination.

4.5 Responses and suppressive effect of in vitro activated rat $CD25^+CD4^+$ cells

4.5.1 Proliferation of preactivated $CD25^+CD4^+$ T cells upon restimulation

As described above, rat $CD25^+CD4^+$ T cells proliferated vigorously upon CD28 superagonist stimulation, while they proliferated only transiently in response to anti-TCR plus anti-CD28 stimulation (Fig. 14). Since $CD25^+CD4^+$ regulatory T cells are rare, CD28 superagonist is an attractive tool for increasing their cell numbers. Furthermore, preactivated mouse $CD25^+CD4^+$ regulatory T cells are more suppressive than freshly isolated ones (104, 110). Therefore, the proliferative response and suppressive activity of rat $CD25^+CD4^+$ cells, which were precultured for three days with either exogenous IL-2, or additionally with anti-TCR plus anti-CD28 or with CD28 superagonist, were compared here.

Consistent with earlier findings, $CD25^+CD4^+$ cells upregulated surface CD25 expression after three day preculture with IL-2, costimulation plus IL-2, or CD28 superagonist plus IL-2 (data not shown). Furthermore, stimulated $CD25^+CD4^+$ cells were even more sensitive to cell death than freshly isolated $CD25^+CD4^+$ cells, since no more than 4% of activated $CD25^+CD4^+$ cells survived after 48 hours cultured in medium alone (data not shown). Again, exogenous IL-2 was able to rescue pre-activated $CD25^+CD4^+$ cells, since about 60% of $CD25^+CD4^+$ cells survived in the presence of exogenous IL-2 (data not shown). When the preactivated $CD25^+CD4^+$ cells were restimulated with anti-TCR plus anti-CD28, no proliferation was observed (Fig. 20). In contrast, preactivated $CD25^+CD4^+$ cells proliferated vigorously upon restimulation with the CD28 superagonist.

Thus, like mouse $CD25^+CD4^+$ regulatory T cells, preactivated rat $CD25^+CD4^+$ T cells proliferated poorly upon restimulation with anti-TCR plus anti-CD28. In contrast, preactivated $CD25^+CD4^+$ T cells responded well to CD28 superagonist restimulation.

4.5.2 Suppressive effect of preactivated $CD25^+CD4^+$ T cells

To investigate the suppressive potency of preactivated $CD25^+CD4^+$ cells, $CD25^+CD4^+$ cells were precultured with IL-2, or additionally with anti-TCR plus anti-CD28 or CD28 superagonist, and cocultured with naive $CD25^-CD4^+$ indicator cells at the ratio of 1:1. Compared to $CD25^-CD4^+$ cells cultured alone, coculture of $CD25^-CD4^+$ indicator cells with $CD25^+CD4^+$ cells preactivated with CD28 superagonist plus IL-2 led to a profound reduction in proliferation (up to 10 fold), while only a two fold decrease in proliferation was observed in coculture of indicator cells with $CD25^+CD4^+$ cells

preactivated with IL-2 or costimulation plus IL-2 (Fig. 21). In contrast to costimulation, no difference in ^{3}H-thymidine incorporation was observed between CD28 superagonist stimulated $CD25^-CD4^+$ cells cultured alone or cocultured with preactivated $CD25^+CD4^+$ cells.

Since in coculture, only half of the cells were $CD25^-CD4^+$ indicator cells compared to culture with $CD25^-CD4^+$ cells alone, and since preactivated $CD25^+CD4^+$ cells proliferated poorly upon costimulation, the reduction of ^{3}H-Thymidine incorporation might be merely due to the reduction of $CD25^-CD4^+$ cell numbers in coculture. But this is not the case. Comparison of cell divisions and CD25 expression of CFSE labelled $CD25^-CD4^+$ cells in coculture with $CD25^-CD4^+$ cells cultured alone showed that coculture of $CD25^-CD4^+$ cells with $CD25^+CD4^+$ cells led to a clear reduction of cell divisions and CD25 expression of $CD25^-CD4^+$ cells (data not shown). Thus, preactivated $CD25^+CD4^+$ cells could inhibit the activation of $CD25^-CD4^+$ indicator cells.

Taken together, the response of preactivated rat $CD25^+CD4^+$ cells re-iterated that found with $CD25^+CD4^+$ cells freshly isolated from unstimulated rats: they proliferated poorly upon costimulation, but were readily activated to proliferate under CD28 superagonist restimulation. Consistent with data on mouse and human $CD25^+CD4^+$ regulatory T cells, preactivated $CD25^+CD4^+$ cells were more potent than freshly isolated $CD25^+CD4^+$ cells in suppressing the activation of $CD25^-CD4^+$ cells, since reduction of proliferation, cell division and CD25 expression of indicator cells were observed in coculture with preactivated $CD25^+CD4^+$ cells. Interestingly, although $CD25^+CD4^+$ cells were unable to suppress the activation of $CD25^-CD4^+$ cells under CD28 superagonist

stimulation, CD28 superagonist preactivated CD25$^+$CD4$^+$ cells were highly potent in suppressing costimulated CD25$^-$CD4$^+$ indicator cells. Thus, CD28 superagonist stimulation did not only increase cell numbers of CD25$^+$CD4$^+$ cells by activating them to proliferate, it also increased the suppressive potency of CD25$^+$CD4$^+$ cells, and thus might be useful for treating autoimmune disease.

4.5.3 Suppressive capacity of CD28 superagonist activated CD25$^-$- versus CD25$^+$CD4$^+$ T cells

The potent suppressive effect of CD28 superagonist activated CD25$^+$CD4$^+$ cells on the indicator cell proliferation raised the question whether this suppressive activity was restricted to CD28 superagonist stimulated rat CD25$^+$CD4$^+$ T cells, or could be acquired by CD25$^-$CD4$^+$ cells, since CD25$^-$CD4$^+$ cells also upregulated CD25 expression and became CD25-positive upon *in vitro* CD28 superagonist stimulation. To examine this possibility, naïve CD25$^+$CD4$^+$ cells and CD25$^-$CD4$^+$ cells were isolated and stimulated with CD28 superagonist separately. Upon stimulation, both CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ cells upregulated CD25. Higher CD25 expression was observed in the initially CD25-positive cells (Fig. 16), while CD4 expression was comparable in both populations (data not shown). This was not due to different kinetics of CD25 expression of the two populations, because CD25 expression of CD25$^-$CD4$^+$ cells reached the plateau readily after 48 hour stimulation (Fig. 16).

Next, the proliferative capacity of both CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ cells after CD28 superagonist stimulation was examined: while preactivated CD25$^+$CD4$^+$ cells did not

proliferate upon costimulation, initially CD25-negative cells (which now also expressed CD25) proliferated vigorously upon restimulation with anti-TCR plus anti-CD28 (Fig. 22). Thus, *in vitro* induction of CD25 expression was not equal to the induction of a hyporesponsive state to costimulation.

As shown in Fig. 21, CD28 superagonist activated $CD25^+CD4^+$ cells inhibited the proliferation of cocultured naive $CD25^-CD4^+$ indicator cells. In contrast, coculture of CD28 superagonist activated $CD25^-CD4^+$ cells with indicator cells did not lead to a reduction in proliferation as read out by ^{3}H-thymidine incorporation (Fig. 23). Because CD28 superagonist activated $CD25^-CD4^+$ cells responded well to costimulation, one could argue that these cells did inhibit the naive indicator cells as potently as activated $CD25^+CD4^+$ cells. Thus, the observed ^{3}H-thymidine incorporation in coculture with activated $CD25^-CD4^+$ cells and naive $CD25^-CD4^+$ cells might be actually due to the vigorous proliferation of preactivated $CD25^-CD4^+$ cells. To answer this question, naive $CD25^-CD4^+$ indicator cells were labelled with CFSE to follow their cell divisions. Naive $CD25^-CD4^+$ indicator cells underwent more cell divisions in coculture with activated $CD25^-CD4^+$ cells than in coculture with the same number of activated $CD25^+CD4^+$ cells. Although cell divisions undergone by indicator cells were indeed decreased by incubating them with activated $CD25^-CD4^+$ cells, the extent of reduction was ten fold less than the reduction caused by coculture with CD28 superagonist activated $CD25^+CD4^+$ cells (Fig. 24).

Another feature shared by freshly isolated and CD28 superagonist activated $CD25^+CD4^+$ T cells was that both failed to suppress the activation of $CD25^-CD4^+$ cells in

response to CD28 superagonist stimulation (Fig. 18 and Fig. 21). Examining the CFSE labelled $CD25^-CD4^+$ indicator cells further supported this observation: there was no reduction in CD25 expression, the number of cell divisions (Fig. 25 A), and cycling of $CD25^-CD4^+$ indicator cells in coculture (Fig. 25B), although CD25 expression and cycling of cocultured $CD25^+CD4^+$ cells were apparently increased.

Taken together, regardless whether $CD25^+CD4^+$ cells were freshly isolated from unstimulated rats or preactivated *in vitro*, in the presence of $CD25^-CD4^+$ indicator cells or exogenous IL-2, $CD25^+CD4^+$ regulatory T cells became more activated: they expressed higher CD25, divided more often (data not shown), and had more cycling cells in coculture than when cultured alone, under both costimulation and CD28 superagonist stimulation. At the same time, costimulation induced activation of naive $CD25^-CD4^+$ indicator cells was inhibited by $CD25^+CD4^+$ cells, with the CD28 superagonist stimulated $CD25^+CD4^+$ cells being the most potent suppressors, while no deactivation effect on $CD25^-CD4^+$ indicator cells by $CD25^+CD4^+$ cells was observed upon CD28 superagonist stimulation.

4.6 Characterisation of CD25$^+$CD4$^+$ cells from CD28 superagonist injected rats

It was shown that CD28 superagonist stimulates T cells to proliferate without TCR engagement *in vitro* and *in vivo* (58). Furthermore, although one single injection of one milligram CD28 superagonist induces massive lymphocyte proliferation (about 5 fold increase in CD4$^+$ T cell number) of healthy rats, there are no signs of apparent discomfort or inflammatory autoimmune diseases of treated animals. Interestingly, although all CD4$^+$ T cells proliferate after 72 hour treatment of CD28 superagonist, not all CD4$^+$ T cells expressed CD25 (up to 20% of CD4$^+$ T cells, Fig. 26). Examination of the CD25 expression of CD4$^+$ cells showed that there was a transient increase of CD25$^+$CD4$^+$ cells (percentage and absolute cell number) during the first three days, and then returned to baseline six days after CD28 superagonist treatment.

Furthermore, analysis of cycling cells in CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ cells at different time points after CD28 superagonist treatment revealed that about one third of both CD25$^-$ and CD25$^+$CD4$^+$ cells were in S or G2/M phases (indicated in M2) 24 hours after CD28 superagonist treatment (Fig. 27). While the same percentage of CD25$^+$CD4$^+$ cells remained in cycle, most CD25$^-$CD4$^+$ cells stopped cycling 48 hours after injection. Thus, either CD28 superagonist only supported sustained proliferation of CD25$^+$CD4$^+$ cells *in vivo*, or alternatively, expansion of CD25$^-$CD4$^+$ cells by CD28 superagonist recruited them into CD25$^+$CD4$^+$ cell pool.

Since $CD25^-CD4^+$ T cells upregulated CD25 expression upon *in vitro* CD28 superagonist stimulation, accumulation of $CD25^+CD4^+$ T cells in CD28 superagonist treated rats might not be derived from naive $CD25^+CD4^+$ regulatory T cells, but from $CD25^-CD4^+$ T cells, which might express CD25 upon stimulation with CD28 superagonist. To test if CD25 was upregulated by $CD25^-CD4^+$ T cells in CD28 superagonist treated rats, CFSE-labelled naïve $CD25^-CD4^+$ T cells were introduced into rats, followed by i.v. injection of CD28 superagonist. Although injected CFSE-labelled $CD25^-CD4^+$ T cells were activated to undergo several rounds of cell division, no CD25 expression was induced (Fig. 28). In contrast, there was an increase of $CD25^+CD4^+$ cells in the endogenous $CD4^+$ cells. This is in contrast to the *in vitro* stimulation, where CD28 superagonist induced proliferation of $CD25^-CD4^+$ T cells results in CD25 expression. The most likely reason for this difference is the presence of free IL-2 in culture, which is known to upregulate the α-chain of its own receptor, i.e. CD25.

Next, phenotype and *in vitro* suppressive effect of the $CD25^+$ and $CD25^-$ subsets of $CD4^+$ cells from anti-CD28 treated rats were examined. Both $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells downregulated CD45RC expression as a consequence of in vivo activation by CD28 superagonist, in keeping with the loss of high molecular-weight isoforms of CD45 during T-cell activation (Fig. 29B). Furthermore, $CD25^+CD4^+$ cells expressed high levels of CTLA-4 upon injection of CD28 superagonist. As expected, $CD25^-CD4^+$ cells now expressed CTLA-4, since they were also activated by CD28 superagonist. The expression level of CTLA-4 however was four fold lower than that found in $CD25^+CD4^+$ cells (Fig. 29A). This is consistent with observations in mice and humans: although both

$CD25^+CD4^+$ cells and $CD25^-CD4^+$ cells can upregulate their CTLA-4 expression *in vivo* and *in vitro*, $CD25^+CD4^+$ regulatory T cells express higher amounts of CTLA-4 (46, 123).

Upon *in vitro* restimulation, $CD25^+CD4^+$ cells isolated from CD28 superagonist treated rats did not proliferate upon costimulation, while $CD25^-CD4^+$ cells from the same rats proliferated vigorously (Fig. 30). Unlike the results obtained with naive $CD25^+CD4^+$ cells, exogenous IL-2 could only partially enhance the proliferation of $CD25^+CD4^+$ cells from CD28 superagonist treated rats upon *in vitro* restimulation with anti-TCR plus anti-CD28. In contrast, $CD25^+CD4^+$ cells proliferated readily upon *in vitro* restimulation with CD28 superagonist, although the proliferation was one fourth of the proliferation of $CD25^-CD4^+$ cells from the same treated rats. Exogenous IL-2 was able to increase the CD28 superagonist induced proliferation of $CD25^+CD4^+$ cells to the extent that $CD25^-CD4^+$ cells exhibited. This indicated that in vivo CD28 superagonist activated $CD25^+CD4^+$ cells were more dependent on IL-2 than naïve ones.

Similar to results with naive $CD25^+CD4^+$ T cells, no IL-2 was detectable in supernatants of $CD25^+CD4^+$ cells from CD28 superagonist treated rats upon *in vitro* restimulation (Fig. 31). Supernatants of $CD25^-CD4^+$ cells from the same animals contained high levels of IL-2 upon costimulation during the first 48 hours, which declined to baseline at 72 hours, while even higher amounts of IL-2 were detected in supernatants of CD28-superagonist restimulated $CD25^-CD4^+$ cells during three-day stimulation, suggesting that higher amounts of IL-2 are consumed during costimulation. Alternatively, only CD28 superagonist restimulation induced sustained IL-2 production. Also consistent with the results obtained with naive $CD25^+CD4^+$ T-cells, activated

$CD25^+CD4^+$ cells produced high levels of IL-10 (Fig. 31). Although *in vivo* activated $CD25^-CD4^+$ cells now also secreted IL-10 upon restimulation, they produced this cytokine in a lower extent and with delayed kinetics. Since delivery of CD28 superagonist into rats did not cause inflammatory disease, the production of the pro-inflammatory cytokine IFN-γ and the anti-inflammatory cytokine IL-4 were also examined. There was no significant difference in IL-4 production between $CD25^-CD4^+$ cells and $CD25^+CD4^+$ cells. $CD25^-CD4^+$ cells produced high amounts of IFN-γ upon *in vitro* restimulation, while no or little IFN-γ was produced by $CD25^+CD4^+$ cells. Interestingly, activated $CD25^-CD4^+$ cells stimulated with anti-TCR plus anti-CD28 secreted two fold higher IFN-γ than cells stimulated with CD28 superagonist. This indicates that costimulation might preferentially induce TH1 cytokines (such as IFN-γ) compared to CD28 superagonist stimulation.

4.7 Suppressor activity of in vivo CD28 superagonist activated $CD25^+CD4^+$ cells

Since $CD25^+CD4^+$ cells from CD28 superagonist treated rats showed the phenotypic properties of regulatory T cells, suppressor functions in vitro was examined next. Unlike $CD25^+CD4^+$ cells from untreated rats, activated $CD25^+CD4^+$ cells clearly inhibited ^{3}H-thymidine incorporation of both naive $CD25^-CD4^+$ cells and $CD25^-CD4^+$ cells from CD28 superagonist treated rats: a three fold reduction in proliferation of coculture was observed compared to $CD25^-CD4^+$ cells cultured alone upon costimulation (Fig. 32). In addition, cell divisions undergone by naive $CD25^-CD4^+$ cells were also reduced in coculture (Fig. 33). In contrast, no reduction in proliferation and cell divisions of $CD25^-$

$CD4^+$ cells was observed in coculture upon in vitro restimulation with superagonistic anti-CD28.

Furthermore, while high amounts of IL-2 and IFN-γ were detected in supernatants from $CD25^-CD4^+$ T cells upon in vitro restimulation, reduced amounts of IL-2 and IFN-γ but further increased IL-10 production in coculture of $CD25^-CD4^+$ T cells with $CD25^+CD4^+$ T cells were observed (Fig. 31). Thus, not only the proliferation capacity of $CD25^-CD4^+$ T cells was reduced in the coculture, also the cytokine production (IL-2 and IFN-γ) was modulated in the presence of $CD25^+CD4^+$ T cells.

4.8 Discussion

The recent interest in $CD25^+CD4^+$ regulatory T cells is based on the apparent ability of these cells to deactivate the immune response and thus to prevent harmful autoimmune reactions. This makes them highly attractive in the treatment of autoimmune diseases, such as diabetes or rheumatoid arthritis. Although the existence of a regulatory cell population was first demonstrated in thymectomized rats (124), experiments were mostly performed with mouse or human $CD25^+CD4^+$ regulatory T cells. The phenotype and *in vitro* functions of rat $CD25^+CD4^+$ T cells were, however, incompletely examined. The present study thus focused firstly on the characterisation of rat $CD25^+CD4^+$ T cells. Using the anti-rat CTLA-4 monoclonal antibody generated here (see chapter 3), rat $CD25^+CD4^+$ T cells were shown to phenotypically correspond to $CD25^+CD4^+$ regulatory T cells found in mice and humans: they express constitutively CTLA-4, are predominantly CD45R low, and CD62L (L-selectin) high (28, 46, 125).

Furthermore, in vitro maintenance of rat $CD25^+CD4^+$ T cells depends on exogenous IL-2. In addition, they had reduced proliferative capacity upon costimulation, while CD28 superagonist could induce rat $CD25^+CD4^+$ T cells to proliferate as well as $CD25^-CD4^+$ T cells. Interestingly, these CD28 superagonist stimulated Treg cells could survive and proliferate in the absence of IL-2, since supernantants from CD28 superagonist stimulated $CD25^+CD4^+$ T cells contained little or no IL-2 (Fig. 15), and exogenous IL-2 did not further increase $CD25^+CD4^+$ T cell proliferation under this condition (data not shown). This indicates that naive $CD25^+CD4^+$ T cells were less dependent on IL-2 for their proliferation/survival upon CD28 superagonist stimulation as compared to conventional costimulation with anti-TCR plus anti-CD28 mAbs. Thus, by bypassing the TCR signaling, rat $CD25^+CD4^+$ T cells might be less IL-2 independent upon cross-linking of CD28. Since both CD28- and IL-2 signals lead to activation of the common anti-apoptotic pathway through serine/threonine kinase AKT (126, 127), the IL-2 independency observed in rat $CD25^+CD4^+$ T cells under CD28 superagonist stimulation suggests that some extent of redundancy in CD28- and IL-2 signalling may exist.

Although IL-10 plays an important role in $CD25^+CD4^+$ regulatory T cell mediated inhibition of autoimmune disease (52, 128), whether $CD25^+CD4^+$ regulatory T cells is the unique source of IL-10 is still under debate (56, 106, 109, 129). Some evidence from the human system suggests that $CD25^+CD4^+$ regulatory cells do not produce higher amounts of IL-10 than $CD25^-CD4^+$ T cells upon stimulation (106). By examining the kinetics of IL-10 production in culture, however, rat $CD25^+CD4^+$ T cells were shown to produce more IL-10 and with faster kinetics upon both costimulation and CD28 superagonist stimulation (Fig. 15). Whether the discrepancy in the ability of $CD25^+CD4^+$

T cells to produce IL-10 between rats and humans is due to different stimulation conditions requires further investigation. Nevertheless, proliferation of rat $CD4^+$ T cells seemed to be unaffected by IL-10, since $CD25^-CD4^+$ T cells and $CD25^+CD4^+$ T cells had the same ability to proliferate, despite different levels of IL-10 detected in culture (Fig. 14 and Fig. 15). This is consistent with the findings that not T cells but antigen presenting cells (APC) are the major target cells of IL-10 mediated inhibition (120, 121).

In this study, the *in vitro* suppressor activity of rat $CD25^+CD4^+$ T cells on $CD25^-CD4^+$ T cells was examined by incubating $CD25^+CD4^+$ T cells with $CD25^-CD4^+$ T cells without antigen presenting cells and stimulated either with anti-TCR plus anti-CD28 (costimulation) or with plate-bound CD28 superagonist (Fig. 17A). Although no reduction in ^{3}H-thymidine incorporation of coculture was observed, $CD25^+CD4^+$ T cells could inhibit the activation of costimulated $CD25^-CD4^+$ T cells, as $CD25^-CD4^+$ T cells in coculture had lower CD25 expression and underwent fewer cell divisions than $CD25^-CD4^+$ T cells stimulated alone (Fig. 18). Thus, $CD25^+CD4^+$ T cells suppressed the activation of $CD25^-CD4^+$ T cells directly without APCs. Interestingly, $CD25^+CD4^+$ T cells were found more 'activated' in coculture, as higher CD25 expression (Fig. 18) and more cycling $CD25^+CD4^+$ T cells (Fig. 19) were found in the presence of indicator cells. This indicates that some factors, for example IL-2, produced by stimulated $CD25^-CD4^+$ T cells, might support the growth/survival of $CD25^+CD4^+$ T cells. Whether suppression exerted by $CD25^+CD4^+$ T cells was through the competition of these factors with $CD25^-CD4^+$ T cells, or $CD25^+CD4^+$ T cells just became more activated in the presence of $CD25^-CD4^+$ T cells but exhibited suppressor activity through other mechanisms, needs further investigation.

In contrast to costimulation, no inhibition of proliferation, cell division and CD25 expression of $CD25^-CD4^+$ T cells was observed in coculture with $CD25^+CD4^+$ T cells upon CD28 superagonist stimulation. This might argue for the dependency of TCR-signalling for the suppressive effect of $CD25^+CD4^+$ T cell (130). However, the stimulation conditions used in suppression assays were not fully comparable for costimulation (using monoclonal antibodies coated on Dynabeads) and for CD28 superagonist stimulation (using plate-coated CD28 superagonist). Thus, to conclude that $CD25^+CD4^+$ T cells were generally unable to suppress $CD25^-CD4^+$ T cells upon CD28 superagonist stimulation might be misleading. In fact, IL-2 and INF-γ production by $CD25^-CD4^+$ T cells are affected in the presence of $CD25^+CD4^+$ T cells (Fig. 31). Furthermore, evidence from rats injected with CD28 superagonist showed that $CD25^+CD4^+$ T cells might interfere with the proliferation of $CD25^-CD4^+$ T cells in the same animals (see below). Thus, the suppressive effect of $CD25^+CD4^+$ T cells upon costimulation versus CD28 superagonist might only differ in quantity.

One major barrier to the therapeutic application of $CD25^+CD4^+$ regulatory T cells is that these cells are rare in numbers and difficult to expand *in vitro* and *in vivo*. Here, rat $CD25^+CD4^+$ T cells were not only stimulated to proliferate by CD28 superagonist, but $CD25^+CD4^+$ T cells activated in this way were more potent in suppressing $CD25^-CD4^+$ T cell proliferation than $CD25^+CD4^+$ T cells activated by costimulation (Fig. 21). Although these cells became more dependent on exogenous IL-2 (data not shown), they still responded to CD28 superagonist restimulation. By combination of CD28 superagonist

and IL-2, one could expand this cells in culture for at least 3 weeks (data not shown), and increase the number and potency of $CD25^+CD4^+$ T cells.

Furthermore, experiments showed that the strong suppressive effect was inherent in the $CD25^+CD4^+$ T cells, and was not acquired by $CD25^-CD4^+$ T cells stimulated with CD28 superagonist, although both $CD25^+CD4^+$ T cells and $CD25^-CD4^+$ T cells upregulated CD25 and CTLA-4 expression upon *in vitro* stimulation. Also, $CD25^-CD4^+$ T cells were shown to contain regulatory T cells, which could be activated by CD28 superaonist (90, 131). In contrast to $CD25^+CD4^+$ T cells, however, CD28 superagonist preactivated $CD25^-CD4^+$ T cells still responded to anti-TCR plus anti-CD28 restimulation. Furthermore, although the cell divisions (Fig. 24) undergone by naive $CD25^-CD4^+$ indicator cells were indeed reduced in coculture with activated $CD25^-CD4^+$ T cells, the suppressive potency was ten fold less than that of activated $CD25^+CD4^+$ T cells. Thus, functional and potent $CD25^+CD4^+$ regulatory T cells can only be generated from $CD25^+CD4^+$ T cells by *in vitro* CD28 superagonist stimulation.

In addition to the *in vitro* studies, the influence of CD28 superagonist on rat $CD25^+CD4^+$ T cells was also examined *in vivo*. Based on the observation that despite the potential danger caused by polyclonal T cell expansion, massive lymphocyte proliferation induced by CD28 superagonist is not associated with inflammatory autoimmune diseases, we proposed that $CD25^+CD4^+$ T cells might play a role in controlling potential harmful autoreactive $CD25^-CD4^+$ T cells which were also activated *in vivo* by CD28 superagonist, as shown by their downregulation of CD45RC and upregulation of CTLA-4 (Fig. 29). Upon CD28 superagonist injection, there was

sustained cycling of $CD25^+CD4^+$ T cells (up to 38% in S +G2/M) during the three day stimulation (Fig. 27), while cycling $CD25^-CD4^+$ T cells were reduced to less than five percent 48 hours after injection. This resulted in an increased percentage and absolute cell number of $CD25^+CD4^+$ T cells in the treated rats (Fig. 27). Although the present study did not demonstrate directly that $CD25^+CD4^+$ regulatory T cells indeed proliferated upon injection of CD28 superagonist, the possibility that increased $CD25^+CD4^+$ T cells were recruited from $CD25^-CD4^+$ T cells was excluded, since injected CFSE-labelled $CD25^-CD4^+$ T cells failed to upregulate CD25 expression upon in vivo CD28 superagonist stimulation, although these cells were induced to undergo several rounds of cell division (Fig. 28). Thus, CD28 superagonist induced activation and accumulation of $CD25^+CD4^+$ regulatory T cells in vivo.

Further characteristics of these $CD25^+CD4^+$ T cells were that $CD25^+CD4^+$ T cells had high CTLA-4 expression, while $CD25^-CD4^+$ T cells were also induced to express CTLA-4 but to a much lower level (about four fold lower). Unlike $CD25^-CD4^+$ T cells, $CD25^+CD4^+$ T cells did not proliferate vigorously upon costimulation, and no IL-2 but high amounts of IL-10 were produced upon *in vitro* restimulation. Furthermore, $CD25^+CD4^+$ T cells from CD28 superagonist treated rats were suppressive. They could inhibit not only the proliferation of naive $CD25^-CD4^+$ indicator T cells, but also suppressed the $CD25^-CD4^+$ T cells from the same CD28 superagonist treated rats. In addition, production of the pro-inflammatory cytokines IL-2 and IFN-γ by $CD25^-CD4^+$ T cells was also affected by in vivo activated $CD25^+CD4^+$ T cells, since less IL-2 and IFN-γ were found in supernatants in coculture. Whether the reduction of IL-2 and IFN-γ in coculture was caused by modulation of $CD25^-CD4^+$ T cell responses by $CD25^+CD4^+$ T cells, or was solely a

consequence of $CD25^+CD4^+$ T cell-mediated suppression of $CD25^-CD4^+$ T cell proliferation, requires further investigation.

Upon *in vivo* CD28 superagonist stimulation, $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells both underwent cell expansion and cell contraction phases (Fig. 26 and data not shown). Interestingly, during the expansion phase, $CD25^+CD4^+$ T cells appear to proliferate more intensively than $CD25^-CD4^+$ cells, leading to an increase in the percentage of $CD25^+CD4^+$ T cells in $CD4^+$ T cell population (Fig. 27). Since proliferation of $CD25^+CD4^+$ regulatory T cells was comparable to proliferation of $CD25^-CD4^+$ T cells upon in vitro CD28 superagonist stimulation, the preferential expansion of $CD25^+CD4^+$ cells might be due to the generation of required growth /survival factors for $CD25^+CD4^+$ cells by CD28 superagonist. Alternatively, $CD25^+CD4^+$ T cells might interfere with the proliferation of $CD25^-CD4^+$ T cells in the CD28 superagonist treated rats, leading to the less sustained proliferation of $CD25^-CD4^+$ cells.

CHAPTER 5 GENERAL DISCUSSION

T cell immunity is controlled by positive and negative regulatory mechanisms to maintain self-tolerance and at the same time to allow protective immune responses to occur. Although factors regulating T cell activation are well known, mechanisms which negatively regulate T cell responses are beginning to emerge. In this context, CTLA-4 at the molecular level (38, 132) and $CD4^+CD25^+$ regulatory T cells (103, 133) attract special attention recently due to their critical role in inhibiting and downregulating T cell responses. Understanding regulatory functions mediated by CTLA-4 and regulatory T cells would facilitate the manipulation of immune responses and the delicate balance between immunity and self-tolerance. For example, anti-tumor immune responses are enhanced by blocking CTLA-4 and regulatory T cell functions (37, 98, 134), while introduction of $CD4^+CD25^+$ regulatory T cells inhibits autoimmune diseases such as colitis induced by $CD25^-CD4^+$ T cells in susceptible mice (51, 105).

Using rat anti-CTLA-4 monoclonal antibodies generated in the present work, the expression pattern and in vitro functions of rat CTLA-4 were examined for the first time. CTLA-4 expression is upregulated in all $CD4^+$ T cells upon costimulation and CD28 superagonist stimulation (Fig.6). This suggests that TCR signalling is not obligatory for upregulation of CTLA-4, since the CTLA-4 expression level increased under stimulation conditions without apparent engagement of TCR and proximal TCR signalling (58, 61).

Upon costimulation, CTLA-4 expression is much lower in $CD8^+$ T cells than in $CD4^+$ T cells, while comparable CD25 expression is observed in $CD4^+$ and $CD8^+$ T cells (Fig. 7). The low CTLA-4 expression in activated $CD8^+$ T cells implies that CTLA-4 may not be the central player in deactivating rat $CD8^+$ T cell responses, and $CD4^+$ and $CD8^+$ are subject to different regulatory mechanisms.

Consistent with the observation in mice and humans, only $CD25^+CD4^+$ regulatory T cells from unprimed rats express CTLA-4. The constitutive expression of CTLA-4 in $CD25^+CD4^+$ Treg cells indicates that CTLA-4 may play a role in maintaining regulatory activities of Treg cells. Because cross-linking CTLA-4 with monoclonal antibodies inhibits production of IL-2 and downregulation of T cell responses ((91) and Fig. 7-9), it is plausible that a defect in producing IL-2 and the observed hyporesponsiveness of $CD25^+CD4^+$ regulatory T cells upon costimulation may be partly due to the constitutive signalling transduced by CTLA-4. In addition, since activated $CD25^-CD4^+$ T cells also express CTLA-4, CTLA-4 mediated deactivation can also act directly on the non-regulatory T cells.

Interestingly, rat $CD25^+CD4^+$ cells proliferate poorly upon costimulation but proliferate as vigorously as $CD25^-CD4^+$ cells when stimulated with CD28 superagonist (Fig. 14). Since delayed kinetics of CTLA-4 upregulation is observed in bulk $CD4^+$ T cells (Fig. 6), it is possible that CD28 superagonist stimulated Treg cells may also have delayed kinetics in upregulating CTLA-4, and are subject to less inhibitory signalling mediated by CTLA-4 than costimulated Treg cells.

The cytokine IL-2 is known to influence the activity/survival of $CD25^+CD4^+$ regulatory T cells. *In vitro*, rat $CD25^+CD4^+$ cells cultured in medium alone are prone to cell death (Fig. 11). Exogenous IL-2 rescues Treg cells from cell death, indicating that $CD25^+CD4^+$ cells are not only unable to produce IL-2, but actually need extrinsic factors such as IL-2 for their survival. This is in line with the findings that IL-2 deficient mice are defect in maintenance of Treg cells and suffer from multi-organ specific autoimmune diseases.

The inability of $CD25^+CD4^+$ regulatory T cells to produce IL-2, which is necessary for Treg cell survival, raises the question whether $CD25^+CD4^+$ regulatory T cells may control activation of other T cells *in vitro* simply by consuming IL-2. In line with this, cocultured $CD25^-CD4^+$ cells are found to express lower level of CD25, compared to $CD25^-CD4^+$ cells cultured alone (Fig. 18), suggesting that less IL-2 is available for $CD25^-CD4^+$ cells in coculture. However, since the requirement of IL-2 for the activation/survival of other T cells in vivo is unclear, as illustrated by the intact proliferative responses of $CD4^+$ T-cells in IL-2-/- mice (136, 137), the relevance of competition for IL-2 and the *in vivo* regulatory functions of Treg cells needs to be established.

Consistent with studies with mouse and human $CD25^+CD4^+$ regulatory T cells, no IL-2 and IFNγ are produced by *in vitro* costimulated or CD28 superagonist-stimulated rat $CD25^+CD4^+$ regulatory T cells (Fig. 15 and Fig. 31). $CD25^+CD4^+$ cells actually inhibit IL-2 and IFNγ produced by stimulated $CD25^-CD4^+$ T cells in coculture. In addition, $CD25^+CD4^+$ T cells produce large amounts of IL-10 upon stimulation. Taken together, these data support the role of rat $CD25^+CD4^+$ regulatory T cells in inhibiting inflammatory immune responses, as recently demonstrated in mice (135).

In mice and humans, preactivated $CD25^+CD4^+$ regulatory T cells are more potent in suppressing the proliferation of indicator T cells (110). Consistent with this, preactivated rat $CD25^+CD4^+$ cells, either by costimulation or by CD28 superagonist, are more suppressive. Importantly, $CD25^+CD4^+$ cells activated in vitro with CD28 superagonist are the most potent suppressor cells (Fig. 21). Furthermore, the strong suppressive activity was restricted to the initial $CD25^+CD4^+$ cells, but not acquired by $CD25^-CD4^+$ cells, which become CD25 positive after stimulation with CD28 superagonist (Fig. 23-24). Since naïve $CD25^+CD4^+$ cells proliferated vigorously in vitro upon CD28 superagonist stimulation, and become very potent suppressor cells, efficient expansion of potent $CD25^+CD4^+$ regulatory T cells for therapeutic purposes can be achieved with CD28 superagonist stimulation.

In addition to expansion and activation of $CD25^+CD4^+$ regulatory T cells *in vitro*, CD28 superagonist induces a transient accumulation of $CD25^+CD4^+$ regulatory T cells *in vivo*. Furthermore, *in vivo* CD28 superagonist expanded $CD25^+CD4^+$ T cells have the phenotype of 'activated' regulatory T cells: they express higher levels of CD25 (Fig 26) and CTLA-4 (Fig. 29A) than naïve $CD25^+CD4^+$ regulatory T cells, and are more suppressive than $CD25^+CD4^+$ regulatory T cells freshly isolated from naïve rats. $CD25^-CD4^+$ T cells are also activated upon CD28 superagonist injection, as they downregulate

CD45RC and express CTLA-4 (Fig. 29). However, these $CD25^-CD4^+$ T cells are not hyporesponsive to *in vitro* restimulation and less suppressive than $CD25^+CD4^+$ T cells (Fig. 30).

Several mechanisms may involve in the selective increase of $CD25^+CD4^+$ regulatory T cells upon CD28 superagonist stimulation *in vivo*. Firstly, CD28 superagonist stimulation may induce growth factors necessary for the proliferation of $CD25^+CD4^+$ cells, which otherwise are absent in the presence of TCR signalling. Secondly, CD28 superagonist stimulation is found to inhibit apoptosis, such as by upregulation of Bcl-XL (59). This may contribute to slow down the turnover rate of $CD25^+CD4^+$ cells by inhibiting cell death. Furthermore, the disproportional increase of $CD25^+CD4^+$ cell population upon injection of CD28 superagonist may also be due to the suppressive effects by $CD25^+CD4^+$ T cells on the proliferation of $CD25^-CD4^+$ cells. Thus, while $CD25^+CD4^+$ T cells continue to proliferate, proliferation of $CD25^-CD4^+$ cells is inhibited, resulting in the increase of the percentage of $CD25^+CD4^+$ cells.

Both $CD25^-CD4^+$ and $CD25^+CD4^+$ T cells undergo expansion and contraction phases, as the cell number of both $CD25^+CD4^+$ T cells and $CD25^-CD4^+$ T cells increases in three days and then returns to the level found in naïve rats 10 days after CD28 superagonist injection (Fig. 26 and data not shown). Furthermore, the CD25 expression level of $CD25^+CD4^+$ T cells also returns to the level observed on 'naïve' $CD25^+CD4^+$ T cells. Thus, accumulation and activation of $CD25^+CD4^+$ T cells induced by in vivo CD28 superagonist stimulation are fast (reach the maximum in three days)

and transient (return to baseline in ten days). Also, $CD25^{-}CD4^{+}$ T cells from CD28 superagonist treated rats respond vigorously to in vitro restimulation (Fig. 30). This suggests that no sustained immune suppression due to increase in $CD25^{+}CD4^{+}$ T cells is caused by CD28 superagonist.

Since CD28 superagonist promotes fast and transient accumulation of $CD25^{+}CD4^{+}$ regulatory T cells and induces a massive production of anti-inflammatory cytokines (63), it is attractive to use CD28 superagonist as a novel therapy for treating inflammatory autoimmune diseases by tipping autoaggressive T immune responses toward self-tolerance through increase of activated $CD25^{+}CD4^{+}$ regulatory T cells and possibly through modulation of pathogenic T cell responses. Further investigations of the effect of CD28 superagonist on autoimmune disease models, such as EAE or adjuvant arthritis would facilitate the development of strategies to use CD28 superagonist in treating autoimmune diseases.

APPENDICES

Abbreviations

References

Summary

Zusammenfassung

Lebenslauf

Publikationsliste

Abbreviations:

7AAD	7-amino-actinomycin D
CFSE	Carboxyfluorescein diacetate Succinimidyl Ester
CTLA-4	Cytotoxic T lymphocyte Antigen-4
EAE	Experimental Autoimmune Encephalomyelitis
FCS	Fetal Calf Serum
FITC	Fluoresecin isothiocyanate
GFP	Green Fluorescent Protein
Ig	Immunoglobulin
IL-2	Interleukin-2
i. p.	intra peritoneal
i. v.	intra venous
mAb	monoclonal antibody
MHC	Major Histocompatibility Complex
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
RT	Room Temperature
SP	Single Posotive
TCR	T Cell Receptor

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Summary:

In the present work, two important negative regulators of T cell responses in rats were examined. At the molecular level, rat CTLA-4, a receptor important for deactivating T cell responses, was examined for the expression pattern and in vitro functions. For this purpose, anti-rat CTLA-4 mAbs were generated. Consistent with the studies in mice and humans, rat CTLA-4 was detectable only in $CD25^+CD4^+$ regulatory T cells in unstimulated rats, and was upregulated in all activated T cells. Cross-linking rat CTLA-4 led to the deactivation of anti-TCR- and anti-CD28 stimulated (costimulation) T cell responses such as reduction in activation marker expression, proliferation, and cytokine IL-2 production. Although T cells stimulated with the superagonistic anti-CD28 antibody alone without TCR engagement also increased their CTLA-4 expression, a delayed kinetics of CTLA-4 upregulation was found in cells stimulated in this way. The physiological relevance of this finding needs further investigation.

At the cellular level, rat $CD25^+CD4^+$ regulatory T cells were examined here in detail. Using rat anti-CTLA-4 mAbs, the phenotype of $CD25^+CD4^+$ regulatory T cells was investigated. Identical to the mouse and human Treg phenotype, rat $CD25^+CD4^+$ T cells constitutively expressed CTLA-4, were predominantly CD45RC low, and expressed high level of CD62L (L-selectin). $CD25^+CD4^+$ cells proliferated poorly and were unable to produce IL-2 upon engagement of the TCR and CD28. Furthermore, rat $CD25^+CD4^+$ cells produced high amounts of anti-inflammatory cytokine IL-10 upon stimulation. Importantly, freshly isolated $CD25^+CD4^+$ T cells from naïve rats exhibited suppressor activities in the in vitro suppressor assays.

In vitro, $CD25^+CD4^+$ regulatory T cells proliferated vigorously upon superagonistic anti-CD28 stimulation and became very potent suppressor cells. *In vivo*, a single injection of CD28 superagonist into rats induced transient accumulation and activation of $CD25^+CD4^+$ regulatory T cells. These findings suggest firstly that efficient expansion of $CD25^+CD4^+$ cells without losing their suppressive effects (even enhance their suppressive activities) can be achieved with the superagonistic anti-CD28 antibody in vitro. Secondly, the induction of disproportional expansion of $CD25^+CD4^+$ cells by a single injection of superagonistic anti-CD28 antibody in vivo implies that superagonistic anti-CD28 antibody may be a promising candidate in treating autoimmune diseases by causing a transient increase of activated $CD25^+CD4^+$ T cells and thus tipping ongoing autoimmune responses toward self-tolerance.

Zusammenfassung:

In der vorliegenden Arbeit wurden zwei wichtige negative Regulatoren von T-Zellantworten in der Ratte untersucht.

Auf molekularer Ebene wurde Ratten-CTLA-4, ein für die Deaktivierung von T-Zellimmunantworten wichtiger Rezeptor, hinsichtlich seines Expressionsmusters und seiner *in vitro* Funktionen untersucht. Zu diesem Zweck wurden spezifische monoklonale Antikörper gegen Ratten-CTLA-4 erzeugt. In Übereinstimmung mit anderen Untersuchungen in Mensch und Maus war CTLA-4 konstitutiv nur in $CD25^+CD4^+$ regulatorischen T-Zellen exprimiert, nach Stimulierung war es in allen aktivierten T-Zellen nachweisbar. Kreuzvernetzung von CTLA-4 führte zur Inhibition von mit anti-TCR- und anti-CD28-Antikörpern stimulierten (Kostimulation) T-Zellantworten, insbesondere zur Verminderung der Expression von Aktivierungsmarkern, der Proliferation und der Produktion von IL-2. Obwohl T-Zellen nach Stimulation mit superagonistischem Anti-CD28-Antikörper ohne T-Zellrezeptorstimulation ihre CTLA-4-Expression ebenfalls steigerten, wurde eine verzögerte Kinetik für die Hochregulation von CTLA-4 in diesen Zellen festgestellt. Die physiologische Bedeutung dieses Befundes bedarf noch weiterer Untersuchungen.

Auf zellulärer Ebene wurden $CD25^+CD4^+$ regulatorische T-Zellen detailliert analysiert. Der Phänotyp $CD25^+CD4^+$ regulatorischen T-Zellen wurde charakterisiert. Wie in Mensch und Maus exprimierten die regulatorischen T-Zellen der Ratte konstitutiv CTLA-4, in größerem Umfang CD62L (L-Selectin), aber nur wenig CD45RC. $CD25^+CD4^+$ Zellen proliferierten nach Stimulation von CD28 und T-

Zellrezeptor nur schwach und produzierten kein IL-2, im Gegensatz dazu aber große Mengen an IL-10. Hervorzuheben ist, dass für frisch isolierte $CD25^+CD4^+$ T-Zellen aus naiven Ratten Suppressoraktivität durch *in vitro*-Suppressionsassays nachgewiesen werden konnte.

In vitro proliferierten $CD25^+CD4^+$ regulatorische T-Zellen nach superagonistischer anti-CD28-Stimulation äußerst stark und wurden zu potenten Suppressorzellen. *E*ine einzelne Injektion von superagonistischem Anti-CD28-Antikörper resultierte in einer transienten Akkumulation von $CD25^+CD4^+$ regulatorischen T-Zellen *in vivo* und induzierte deren Aktivierung. Diese Ergebnisse weisen zum einen darauf hin, dass eine effiziente Expansion von $CD25^+CD4^+$ Zellen ohne Verlust ihrer supprimierenden Effekte durch superagonistischen Anti-CD28-Antikörper *in vitro* erzielt werden kann. Zum anderen läßt die Induktion einer disproportionalen Expansion von $CD25^+CD4^+$ Zellen durch eine einzige Injektion des CD28-Superagonisten den Schluss zu, dass dieser Antikörper ein vielversprechender Kandidat zur Behandlung von Autoimmunkrankheiten sein könnte, der über eine transiente Zunahme von aktivierten $CD25^+CD4^+$ T-Zellen eine bestehende Autoimmunantwort hin zur Selbsttoleranz lenkt.

Lebenslauf

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Publikationsliste

1. Originalartikel in Fachzeitschriften

- Bischof, A., T. Hara, **C.-H. Lin,** A. D. Beyers, T. Hünig. 2000. Autonomous induction of proliferation, JNK and NF-alphaB activation in primary resting T cells by mobilized CD28. European Journal of Immunology **30**: 876-882

- **Lin, C.-H.** and T. Hünig. 2003. Efficient expansion of regulatory T cells in vitro and in vivo with a CD28 superagonist. European Journal of Immunology **33**: 626-638

- Esther N.M. Nolte-'t Hoen, Josée P.A. Wagenaar-Hilbers, Elmieke P.J. Boot[,a], **Chia-Huey Lin,** Ger J.A. Arkesteijn, Willem van Eden, Leonie S. Taams, Marca H.M. Wauben. Identification of a $CD4^+CD25^+$ T cell subset committed *in vivo* to suppress antigen-specific T cell responses without additional stimulation. In submission.

2. Kongress- bzw. Tagungspublikation

- Bischof, A., **C.-H. Lin**, and T. Hünig. **(1999).** TCR triggered mobilization of signalling-competent CD28: A two-step model of costimulation. *Immunobiology* 200, No. 3-5: 355.

- Lin, C.-H., N. Torres-Nagel, and T. Hünig. (2001). Detection of CTLA-4 in the rat by monoclonal antibodies. *Immunobiology* 204, No. 1-2: 88.

- **Lin, C.-H.,** and T. Hünig. **(2002).** Rat $CD25^+CD4^+$ regulatory T cells: characterisation, efficient expansion, and modulation of their response by $CD25^-$ cells. *Immunobiology :* 206, No. 1-3: 151.

- **Lin, C.-H.**; J. P. A. Wagenaar-Hilbers, M. H. Wauben, M.; T. Hünig, T. Hanke. 2003. Prophylactic and therapeutic effects of anti-CD28 superagonic antibody on rat adjuvant arthritis. *Immunobiology* 208, No. 1-3: 143. (Jahrestagung der Gesellschaft für Immunologie, 24.-27. 09. 2003 in Berlin)

- Bayersdorf, N**.**, **C.-H. Lin**, K. Balbach, S. Gaupp, R. Gold, T. Hanke, T. Kerkau, and T. Hünig. (2003). Expansion of functional rat CD4+CD25+ regulatory T-cells by superagonistic CD28 specific monoclonal antibodies. *Immunobiology* 208, No. 1-3: 75.

- E. N.M. Nolte-'t Hoen, J. P.A. Wagenaar-Hilbers, E. P.J. Boot, **C.-H. Lin**, G. J.A. Arkesteijn, W. van Eden, L. S. Taams, and M. H.M. Wauben. 2003. Identification of a $CD4^+CD25^+$ T cell subset committed *in vivo* to suppress antigen-specific T cell responses without additional

stimulation. Winter meeting of the Dutch Society for Immunology, 18.-19. 12. 2003.