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CD4 blockade directly inhibits mouse and human CD4$^+$ T cell functions independent of Foxp3$^+$ Tregs

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Abstract

$CD4^+$ helper T cells orchestrate protective immunity against pathogens, yet can also induce undesired pathologies including allergies, transplant rejection and autoimmunity. Non-depleting CD4-specific antibodies such as clone YTS177.9 were found to promote long-lasting T cell tolerance in animal models. Thus, CD4 blockade could represent a promising therapeutic approach for human autoimmune diseases. However, the mechanisms underlying anti-CD4-induced tolerance are incompletely resolved. Particularly, multiple immune cells express CD4 including $Foxp3^+$ regulatory T cells (Tregs) and dendritic cells (DCs), both controlling the activation of $CD4^+Foxp3^-$ helper T cells. Utilizing mixed leucocyte reactions (MLRs) reflecting physiological interactions between T cells and DCs, we report that anti-CD4 treatment inhibits $CD4^+Foxp3^-$ T cell proliferation in an IL-2-independent fashion. Notably, YTS177.9 binding induces a rapid internalization of CD4 on both $CD4^+Foxp3^-$ T cells and $Foxp3^+$ Tregs. However, no expansion or activation of immunosuppressive $CD4^+Foxp3^+$ Tregs was observed following anti-CD4 treatment. Additionally, cytokine production, maturation and T cell priming capacity of DCs are not affected by anti-CD4 exposure. In line with these data, the selective ablation of $Foxp3^+$ Tregs from MLRs by the use of diphtheria toxin (DT)-treated bacterial artificial chromosome (BAC)-transgenic DEREG mice completely fails to abrogate the suppressive activity of multiple anti-CD4 antibodies. Instead, tolerization is associated with the defective expression of various co-stimulatory receptors including OX40 and CD30, suggesting altered signaling through the TCR complex. Consistent with our findings in mice, anti-CD4 treatment renders human $CD4^+$ T cells tolerant in the absence of Tregs. Thus, our results establish that anti-CD4 antibodies can directly

tolerize pathogenic $CD4^+Foxp3^-$ helper T cells. This has important implications for the treatment of human inflammatory diseases.

Keywords: anti-CD4, YTS177.9, GK1.5, Foxp3, MLR, tolerance, autoimmunity, DC, T cells, DEREG

1. Introduction

Dendritic cells (DCs) are specialized antigen presenting cells essential for the activation of $CD4^+$ T cells and can be divided into the two major subsets of conventional DCs (cDCs) and plasmacytoid DCs (pDCs). After their migration into lymph nodes, activated DCs not only present specific antigenic peptides to the T cell receptor (TCR), but they also shape T cell activation, differentiation and survival by various co-stimulatory ligands and DC-derived cytokines . Co-stimulatory receptors on T cells are broadly categorized into immunoglobulin superfamily members (two prominent members of which are CD28 and ICOS) and tumour necrosis factor receptor superfamily (TNFRSF) members. The latter type includes OX40 and CD30, both of which are induced upon T cell activation and have been implied in autoimmunity by promoting the differentiation and survival of $CD4^+$ T cells . Thus, autoreactive $CD4^+$ helper T cells are dangerous immune cells that need to be controlled by various layers of immune tolerance in order to prevent autoimmunity .

$Foxp3^+$ regulatory T cells (Tregs) prevent self- and tumor-specific immunity by dominantly suppressing the activity of both DCs and T cells . Accordingly, the selective deletion of $Foxp3^+$ Tregs in genetically engineered neonatal DEREG mice is sufficient to induce systemic autoimmunity . Moreover, Foxp3 expression is highly restricted to $CD4^+$ T cells and Foxp3 mutations result in autoimmunity in both mice and humans . However, additional layers of tolerance can directly act on autoreactive $CD4^+$ T cells. These recessive tolerance mechanisms cause a state of functional unresponsiveness (anergy) or induce T cell apoptosis . Both outcomes can be mediated by co-inhibitory pathways (e.g. CTLA-4), antagonizing the co-stimulation of T cells . Thus, the expansion/activation

of Foxp3$^+$ Tregs and/or the interference with co-stimulatory pathways may represent efficient ways to inhibit pathogenic CD4$^+$ T cells therapeutically.

In this regard, CD4-specific monoclonal antibodies were shown in the late 1980s to inhibit helper T cell functions . It was soon recognized that their tolerizing activity does not require CD4$^+$ T cell depletion and could have therapeutic potential. Indeed, non-depleting anti-CD4 antibodies including the well characterized mouse-specific rat IgG2a clone YTS177.9 were shown to suppress or even prevent allograft rejection, allergic reactions and autoimmune responses . It should be underlined that this type of anti-CD4-induced tolerance can be induced in various species and is not restricted to a particular antibody clone, suggesting a core mechanism of action. However, the exact mode of action of anti-CD4-induced tolerance remains incompletely defined. This is surprising given that anti-CD4 antibodies are currently entering various clinical trials . Particularly, it is unclear whether anti-CD4 treatment acts via Foxp3$^+$ Tregs or if it directly inhibits pathogenic CD4$^+$ T cells, given that anti-CD4-mediated tolerance was described at a time before Foxp3$^+$ Tregs were defined. Additionally, subsets of cDCs and pDCs express CD4 . However, it is not known whether anti-CD4 binding to DCs modulates their function during T cell priming.

Interestingly, Foxp3$^+$ Tregs were recently suggested to be the central underlying mechanism of anti-CD4-induced tolerance in both mice and humans . T cell priming by DCs can be mimicked in vitro by the co-culture of DCs with allogeneic CD4$^+$ T cells (mixed leucocyte reaction; MLR). This interaction also contributes to allograft rejection in transplanted patients and can serve as a model for pathogenic CD4$^+$ T cell activation. We show here that anti-CD4 exposure efficiently inhibits CD4$^+$ T cell activation during

MLRs by an IL-2-independent mechanism. By using bacterial artificial chromosome (BAC)-transgenic DEREG mice, we specifically eliminated Foxp3$^+$ Tregs from MLRs. Strikingly, the inhibitory activity of anti-CD4 does not require Foxp3$^+$ Tregs. Additionally, anti-CD4 binding to DCs does not alter their functional capacities. Interestingly, a CD4 blockade results in the defective upregulation of co-stimulatory receptors including OX40 and CD30 on CD4$^+$ T cells. In line with our findings with mice, we show that human CD4$^+$ T cells can be efficiently tolerized by anti-CD4 antibody in the absence of Foxp3$^+$ Tregs. Thus, we discovered a previously unknown mechanism of anti-CD4-induced tolerance that is Foxp3$^+$ Treg-independent but directly acts on CD4$^+$ helper T cells. This has important implications for the clinical use of anti-CD4 antibodies and may highlight an unexpected synergy with treatments targeting Foxp3$^+$ Tregs.

2. Material and Methods

2.1. Mice

DEREG , OTI (all C57Bl/6 background), C57Bl/6 and Balb/c mice were maintained at the animal facilities of Twincore (Hanover, Germany) or the Johannes Gutenberg-University (Mainz, Germany) under specific pathogen-free conditions. Where indicated, WT and DEREG mice were injected i.p. with 1µg DT (Merck) or sterile PBS on two consecutive days and mice were sacrificed one day later for T cell isolation. All experiments were performed in accordance with institutional, state, and federal guidelines.

2.2. Anti-CD4 antibodies and flow cytometry

Functional grade αCD4 (clone YTS177.9) and isotype control antibody were purchased from AbD Serotec or BioXcell. Functional grade αCD4 clones RM4-4 and GK1.5 were kindly provided by Dr. Natalia Zietara and Dr. Guenther Bernhard (Hannover Medical School, Hannover). Fc receptors were blocked using αCD16/32 (2.4G2, produced in house). The following antibodies and secondary reagents were purchased from eBioscience: αCD4 (RM4-5 and GK1.5), αCD8α (53-6.7), αCD8β (H35-17.2), αCD25 (PC61.5), αCD30 (mCD30.1), αCD45R/B220 (RA3-6B2), αCD62L (MEL-14), αCD69 (H1.2F3), αCD86 (GL1), αFoxp3 (FJK-16s), αI-A/I-E (2G9), αICOS (7E.17G9), αIFN-γ (XMG1.2), αNK1.1 (PK136), αOX40 (OX-86), αTer119 (TER-119) and appropriate isotype controls. αCTLA-4 (UC10-4B9) was purchased from Biolegend. αCD4 (RmCD4-2) was kindly provided by Dr. Guenther Bernhard (Hanover Medical School, Hannover) and Dr. Elisabeth Kremmer (Helmholtz Center, Munich). αCD4 clones CT-CD4 and YTS191.1.2 were a kind gift from Dr. Ulrich Kalinke (Twincore, Hannover). CTLA-4- and Foxp3 stainings were carried out using the Foxp3 fixation/permeabilization kit

(eBioscience). IFN-γ staining was performed after fixation with 2% paraformaldehyde and permeabilization with 0.5% saponin. For human MLR stainings, αCD3ε (SK7) and αCD4 (SK3) were used. Dead cells were labeled by ethidium monoazide photolysis, propidium iodide or DAPI (all from Sigma). Cells were acquired using LSRII (BD) or Cyan (Beckman Coulter) and data were analyzed with FlowJo (Tristar). Live cells were gated and aggregates were routinely excluded by SSC pulse width. Cell sorting was carried out at the Cell Sorting Core Facility of the Hannover Medical School using FACSAria (BD), XDP or MoFlo (both Beckman Coulter) machines.

2.3. Mouse T cell isolation and labeling

$CD4^+$ T cells and $CD8^+$ T cells were negatively selected from pooled spleens and lymph nodes using the Dynal Mouse CD4 Cell Isolation Kit and Dynal Mouse CD8 Cell Isolation Kit (Invitrogen). In the case of DT-treated DEREG mice the antibody cocktail was supplemented with αCD25 (PC61.5) to deplete potentially activated T cells. The purity of enriched T cells was typically >80%. For Treg induction assays, the enriched $CD4^+$ T cells from DT-treated DEREG mice were additionally FACS-sorted as $CD8\alpha^-$ $B220^-NK1.1^-Ter119^-CD25^-$ cells. T cells were labeled with the CellTraceViolet Cell Proliferation Kit (Invitrogen), where indicated, according to the manufacturer's instructions. For Treg suppression assays, splenic $CD8^+$ OTI T cells were positively selected using CD8α-Microbeads, and splenic $CD4^+CD25^+$ Balb/c Tregs were enriched using αCD25-PE (7D4) and PE-Microbeads (all Miltenyi Biotech). $CD8^+$ T cells were additionally depleted from contaminating cells using anti-CD4 Dynabeads, B220 (CD45R)- and Mac-1 (CD11b/CD18)-coupled Dynabeads. The purity of the resulting $CD8^+$ T cells was typically 95-98%. Enriched $CD4^+CD25^+$ T cells were additionally

depleted from contaminating cells using anti-CD8 Dynabeads, B220 (CD45R)- and Mac-1 (CD11b/CD18)-coupled Dynabeads. The purity of the resulting $CD4^+CD25^+$ Tregs was typically > 95%.

2.4. Mouse MLR and restimulation

$5x10^4$ CellTraceViolet-labeled $CD4^+$ T cells (C57Bl/6 background) were cultured with 0.5-$1x10^4$ Balb/c GMCSF-driven bone marrow-derived DCs (BMDCs; GMCSF supernatant produced in house). BMDCs were activated over night with 0.5µM phosphorothioate-stabilized CpG-ODN 1826 (TCCATGACGTTCCTGACGTT; TIB MolBiol) prior to the MLR. For $CD8^+$ T cell MLRs, $5x10^4$ CellTraceViolet-labeled $CD8^+$ T cells (Balb/c background) were co-cultured with $2.5x10^4$ gradient-enriched splenic DCs from B16-FLT3L-treated C57Bl/6 mice in absence or presence of 1µM phosphorothioate-stabilized CpG-ODN 2216 or CpG-ODN 1826 (both TIB MolBiol). DT was added at 100ng/ml where indicated. YTS177.9 was added at 10µg/ml if not indicated otherwise. For some experiments, cultures were supplemented with 200U/ml rhIL-2 (Roche) or 5µg/ml αIL-2 (JES6-1A12; eBioscience). $CD8^+$ T cell MLRs were restimulated with 100ng/ml PMA and 1µg/ml ionomycin (both from Sigma) in presence of 1 x brefeldin A (eBioscience) for 4.5h at 37°C followed by flow cytometry.

2.5. Human MLR

PBMCs of healthy donors were isolated from buffy-coats (German Red Cross blood collection) by Ficoll density gradient centrifugation. Monocytes were purified from PBMCs by positive selection using CD14 Dynabeads (Invitrogen) according to the manufacturer's instructions. Total $CD4^+$ T cells were purified from allogeneic PBMCs

using the Dynabeads Untouched Human $CD4^+$ T Cell Kit (Invitrogen). Additionally, effector T cells were isolated using the Dynabeads Regulatory $CD4^+CD25^+$ T Cell Kit. Total T cells and effector T cells were labeled with PKH26 using the PKH26 Red Fluorescent Cell Linker Kit (Sigma Aldrich) according to the manufacturer's instructions. $1.5x10^5$ labeled $CD4^+$ T cells were co-cultured with $3.75x10^4$ monocytes in 200µl X-VIVO 15 medium using 96-well round bottom plates. Increasing concentrations of αCD4 (RPA-T4, BD) were added to the wells. After 7 days of culture at 37°C, cells were analyzed by flow cytometry to determine the number of proliferated $CD4^+CD3^+$ T cells.

2.6. Treg induction

$2.5x10^4$ $CD4^+Foxp3^-$ CellTraceViolet-labeled T cells negatively isolated from Treg-depleted DEREG mice were cultured with $1x10^4$ syngenic BMDCs in presence of 1µg/ml αCD3ε (145-2C11, eBioscience) and combinations of 2ng/ml rhTGF-β1 (Peprotech) or 10nM all-trans retinoic acid (RA; Sigma). Isotype antibody or YTS177.9 was added at 10µg/ml. Foxp3 induction was assessed by flow cytometry on day 5.

2.7. Treg suppression assay

$CD8^+$ OTI T cells ($2x10^4$) were stimulated in the presence or absence of $2x10^4$ freshly isolated Balb/c $CD4^+CD25^+$ Tregs. Mitomycin C-treated (60 µg/ml/10^7 cells, 30 min) A20 accessory cells ($2x10^3$/well) and SIINFEKL peptide (20ng/ml) were used as stimulus in combination with αCD3 (145-2C11, 3µg/ml) or αCD4 (YTS177.9, 5 µg/ml). After 96h, ^{3}H-thymidine was added to the cultures (0.5µCi/well) and ^{3}H-thymidine uptake was assessed by β-scintillation counting after additional 18h.

2.8. Splenic DC isolation and stimulation

DCs were enriched from collagenase D/DNaseI-digested spleens of B16-FLT3L-treated mice by optiprep gradient centrifugation followed by FACS sorting of $CD11c^+B220^+CD4^+$ plasmacytoid DCs (pDCs) and $CD11c^+B220^-CD4^+$ conventional DCs (cDCs) using non-competing αCD4 (RmCD4-2). $CD4^+$ DCs were plated at $1.5x10^4$ cells per well in complete RPMI1640, pre-incubated for 2h at 37°C with 50µg/ml YTS177.9 or isotype control followed by stimulation with 1µM phosphorothioate-stabilized CpG-ODN 2216 or CpG-ODN 1826 (both TIB MolBiol) for 24h. Alternatively, untouched gradient-enriched splenic DCs were plated at $5x10^4$ cells per well for stimulations as described for sorted DCs.

2.9. ELISA

IFNα contents in culture supernatants were quantified by sandwich ELISA using rat-αIFNα (RMMA-1), rmIFNα, polyclonal rabbit-α-mIFNα (all from PBL Biomedical Laboratories) and HRP-conjugated goat-α-rabbit IgG (Dianova). IL-12/IL-23p40 contents were determined by using the mouse IL-12/IL-23p40 DuoSet kit (R&D Systems) according to the manufacturer's instructions. HRP was revealed using the OptEIA TMB substrate reagent set (BD). After acidification, the optical density was measured at 450nm against 570nm as reference. Standard curves were generated using non-linear regression (R^2>0.99) and unknown sample values were interpolated using Prism. IFNα levels are expressed as U/ml per $1x10^6$ pDCs.

2.10. Statistical analysis

Statistical analysis was performed by Prism software using two-tailed Mann Whitney tests. P values <0.05 were considered statistically significant.

3. Results

3.1. Anti-CD4 binding induces CD4 internalization on both Foxp3$^+$ Tregs and CD4$^+$ helper T cells

To study the mechanisms of anti-CD4-induced tolerance, we here primarily employed the mouse-specific anti-CD4 clone YTS177.9 as a prototypic non-depleting anti-CD4 antibody with well documented tolerizing activity . YTS177.9 was previously reported to downmodulate CD4 expression on T cells . The functional relevance of this phenomenon for tolerance induction remains elusive. Furthermore, it is unknown whether Foxp3$^+$ Tregs and CD4$^+$ helper T cells respond differentially to anti-CD4 exposure. In order to study CD4 downmodulation and to faithfully detect anti-CD4-bound T cells, we identified clone RmCD4-2 to be non-competing with clone YTS177.9 for CD4 binding, in contrast to the clones RM4-5, GK1.5, CT-CD4 and YTS191.1.2 (Fig.1a). This is well in line with previous data showing that the epitopes of RmCD4-2 and YTA3.12 overlap at the membrane-proximal CD4 domains D3/D4, whereas YTS177.9, GK1.5 and YTS191.1 bind to domain D1 . Our data suggest that RM4-5 and CT-CD4 also bind near domain D1 (Fig.1a). Fluorescently labeled RmCD4-2 was therefore routinely used for all subsequent analyses to avoid any steric hindrance with YTS177.9. Of note, YTS177.9 exposure reduced CD4 expression levels on both Foxp3$^-$ helper T cells (Teff) and Foxp3$^+$ Tregs and within 6 hours (Fig.1b,c). This anti-CD4-induced CD4 downregulation was more pronounced at 24 hours (Fig.S1), yet both Teff and Tregs remained CD4-positive and their frequencies did not change (Fig.1c). Thus, both Tregs and helper T cells partially downmodulate CD4 expression after the binding of YTS177.9.

3.2. YTS177.9 inhibits CD4$^+$ helper T cell activation in an IL-2-independent fashion

We next utilized mixed leucocyte reactions (MLRs) as a suitable *in vitro* test system for pathogenic $CD4^+$ T cell activation. This is based on physiological interactions between the T cell recptor (TCR) and MHC-class-II molecules on allogeneic DCs. MLRs can be inhibited by the anti-CD4 clone GK1.5 independent of impairing physical DC - T cell interactions *in vitro* . Since YTS177.9 recognizes a closely related epitope (Fig.1a) we suspected that this prototypic tolerizing anti-CD4 clone acts in a similar way. Indeed, the early clustering of $CD4^+$ T cells around allogeneic BMDCs was not impaired by YTS177.9 (Fig.S2), whereas the proliferation of $CD4^+Foxp3^-$ T cells was inhibited in a dose-dependent manner (Fig.2a; left). Notably, $CD4^+Foxp3^+$ Tregs were more resistant to the inhibition of proliferation (Fig.S3), yet the total number of $CD4^+Foxp3^+$ Tregs was not increased by YTS177.9 treatment (Fig.2a, right) which could be expected given the implication of YTS177.9 in the *de novo* induction of $Foxp3^+$ Tregs .

The anti-CD4 clone GK1.5 was shown to inhibit IL-2 production by $CD4^+$ T cells . Since IL-2 has important roles in the differentiation of T cells, we assessed whether the inhibition of IL-2 production may be causative for the tolerogenic activity of YTS177.9. Interestingly, the addition of rhIL-2 to MLR cultures could not reverse the inhibition of $CD4^+Foxp3^-$ T cell proliferation induced by YTS177.9 (Fig.2b,c). rhIL-2 was functional because it enhanced the expression of CD25, a direct target of STAT5 downstream of the IL-2 receptor complex , on anti-CD3ε-stimulated $CD4^+$ T cells and $CD8^+$ T cells (Fig.S4a,b). Moreover, 200U/ml rhIL-2 exerted full biological activity because 20-fold higher concentrations led to no substantial further increase in CD25 expression (Fig.S4). Conversely, addition of neutralizing IL-2 antibody inhibited isotype-treated MLRs, but to a lower degree than did YTS177.9 in untreated control MLRs (Fig.2b,c). Moreover, anti-IL-2 synergized with YTS177.9 to further inhibit the MLR (Fig.2b,c). Collectively, these

results suggest that the suppression of IL-2 production is not the cause but rather a consequence of the inhibitory effect of YTS177.9 *in vitro*.

3.3. CD4 blockade does not prominently induce or activate Foxp3$^+$ Tregs

Although YTS177.9 did not expand Foxp3$^+$ Treg numbers, their relative proportion increased because CD4$^+$ helper T cell proliferation was inhibited to a much greater extent (Fig.2a,b). However, it was not possible to distinguish between *de novo* induced Tregs and pre-existing thymus-derived Tregs in these experiments. We thus isolated CD4$^+$Foxp3$^-$ T cells from DT-treated DEREG mice and performed polyclonal Treg induction assays in order to independently study the specific effects of YTS177.9 on the *de novo* generation of Tregs. YTS177.9 slightly enhanced Foxp3$^+$ Treg frequencies and numbers in the absence of TGF-β when compared to isotype-treated conditions (Fig.3a,b). However, YTS177.9 treatment failed to enhance or actually inhibited CD4$^+$Foxp3$^+$ Treg induction under Treg-polarizing conditions in the presence of TGF-β alone or combined with retinoic acid (RA) (Fig.3a,b).

Another possibility is that anti-CD4 stimulation activates the suppressive function of Foxp3$^+$ Tregs without necessarily changing their numbers. This point was addressed with antigen-specific Treg suppression assays where the stimulation of responder T cells is uncoupled from experimental Treg activation. By the use of CD8$^+$ OTI cells as responder T cells any direct effects of anti-CD4 were excluded. Tregs without stimulation (Treg) failed to suppress T cell proliferation, whereas anti-CD3α treatment rendered Tregs highly suppressive, as expected (Fig.3c). Importantly, YTS177.9 alone failed to activate the suppressive function of Tregs (Fig.3c). In summary, YTS177.9 treatment does not prominently induce, expand or activate Foxp3$^+$ Tregs *in vitro*.

3.4. Anti-CD4 tolerizes CD4$^+$ T cells in the complete absence of Foxp3$^+$ Tregs

To definitely address whether Foxp3$^+$ Tregs are required to suppress CD4$^+$ T cell activation following anti-CD4 treatment, we isolated CD4$^+$ T cells from DT-treated DEREG mice which are devoid of Foxp3$^+$ Tregs (Fig.4a). Moreover, MLRs were supplemented with DT to exclude any *de novo* generation of Foxp3$^+$ Tregs during the assay. Thus, MLRs remained completely depleted of Foxp3$^+$ Tregs (Fig.4b). DT-treated WT mice served as a control. Strikingly, the inhibitory action of YTS177.9 was completely independent from the presence of Foxp3$^+$ Tregs (Fig.4c). Similarly, YTS177.9 inhibited the expression of CD25 and CD69 as well as the downregulation of CD62L independent of Foxp3$^+$ Tregs at an earlier time point (Fig.4d). These results also rule out the possibility that anti-CD4 treatment renders Tregs only suppressive in conjunction with TCR signals, which was formally not tested in the suppression assay (Fig.3c). To address whether the Treg-independent mechanism is specific to the clone YTS177.9 or of general relevance, we also included clone GK1.5 (recognizing an overlapping epitope; Fig.1a) and clone RM4-4 (recognizing a distinct epitope;) in comparative analyses. Rat IgG2 control antibody had no effect on T cell proliferation (Fig.4e). Importantly, GK1.5 and RM4-4 potently tolerized conventional T cells in the absence of Foxp3$^+$ Tregs (Fig.4e). All together, our results suggest that multiple anti-CD4 clones act in a Foxp3$^+$ Treg-independent manner.

3.5. DC functions are not modulated by anti-CD4 treatment

DCs are important for the priming of autoreactive $CD4^+$ T cells and both plasmacytoid DCs (pDCs) and conventional DCs (cDCs) can themselves express CD4 . Particularly, once activated through TLR7/9 ligation by self nucleic acid-containing protein complexes, pDCs secrete large amounts of type I interferon and are thereby implicated in the pathogenesis of autoimmune diseases including psoriasis and systemic lupus erythematosus. Moreover, the activation of autoreactive $CD4^+$ T cells requires DCs . Whether anti-CD4 antibodies could indirectly inhibit $CD4^+$ T cell responses via effects on DCs has not been explored yet. One way to trigger DC activation is their exposure to bacterial DNA or synthetic CpG-oligodeoxynucleotides (CpG-ODN) as ligands for TLR9. Interestingly, YTS177.9 did not impair IFNα production of sorted splenic $CD4^+$ pDCs following stimulation with A-type CpG-ODN 2216 (Fig.5a). Similarly, $CD4^+$ pDCs displayed unchanged secretion of IL-12/23p40 upon B-type CpG-ODN 1826 stimulation in the presence of YTS177.9 (Fig.5b). Moreover, the upregulation of MHC-class-II and the co-stimulatory molecule CD86 on pDCs were not impaired (Fig.5c). When $CD4^+$ cDCs were stimulated with B-type CpG-ODN 1826, the production of IL-12/23p40 was also not altered by YTS177.9 (Fig.5d). To rule out that RmCD4-2 used for $CD4^+$ DC sorting had covered a functional effect, we next performed experiments with untouched gradient-enriched splenic DCs. These consisted of >90% $CD11c^+$ DCs including both $CD4^+$ cDCs and $CD4^+$ pDCs (Fig.S5a). In addition to YTS177.9 we also included GK1.5 in these experiments. Interestingly, both YTS177.9 and GK1.5 did not modify the capacity of pDCs to produce IFNα upon stimulation with A-type CpG-ODN 2216 (Fig.5e) and the upregulation of MHC-class-II and CD86 was not impaired (Fig.5f and Fig.S5c). A similar picture was seen when assessing IL-12/23p40 production and maturation of DCs

upon stimulation with B-type CpG-ODN 1826 (Fig.S5b,d,e). Interestingly, YTS177.9-exposure downmodulated CD4 expression on DCs (Fig.S5f) as on T cells (Fig.1b,c).

A final possibility is that anti-CD4 binding to DCs modulates their T cell priming function. To investigate this point, we established MLRs with ex vivo enriched splenic DCs and allogeneic $CD8^+$ T cells in presence of isotype antibody or YTS177.9 in absence or presence of TLR9 stimulation. Notably, both $CD8^+$ T cell proliferation and IFN-γ effector cytokine production were not altered by anti-CD4 treatment in all conditions tested and the absolute number of proliferated $CD8^+IFN\text{-}\gamma^+$ did not change (Fig.5g,h).

Therefore, cytokine production, maturation and T cell priming functions of DCs are not altered by anti-CD4 treatment. This is in line with the strong inhibitory action of YTS177.9 on $CD4^+$ T cells *in vitro* in the absence of CD4-expressing DCs (Fig.2 and Fig.4).

3.6. Anti-CD4 exposure impairs co-stimulatory receptor expression on $CD4^+Foxp3^-$ T cells

Since signals through the co-stimulatory TNF receptor family members OX40 and CD30 expressed on $CD4^+$ T cells are specifically important to induce autoimmunity , we hypothesized that anti-CD4-induced tolerance could attenuate signals from one or both receptors. Indeed, both OX40 and CD30 were prominently induced on proliferating $CD4^+$ T cells during MLRs, whereas the expression of both receptors was substantially impaired by anti-CD4 treatment (Fig.6a). Accordingly, the absolute numbers of $OX40^+$ and $CD30^+$ T cells were drastically reduced following anti-CD4 tolererization (Fig.6b). However, OX40- and CD30 double-deficient T cells could still be efficiently tolerized by YTS177.9 on a Treg-deficient background (Fig.S6a,b), ruling out a mechanistic

contribution of the two receptors in vitro. This does not contradict previous data showing that OX40/CD30 are not required for T cell proliferation but govern T cell survival in vivo . The stronger proliferation of Foxp3-deficient T cells is likely explained by their pre-activation since Foxp3-mutant scurfy mice suffer from lethal autoimmunity whereas Foxp3KO x OX40 KO x CD30 KO mice are protected . In this regard, the efficient tolerizing activity of YTS177.9 even on pre-activated autoimmune T cells (Fig.S6) is impressive and suggests that anti-CD4 therapy could be effective in Treg-deficient settings such as the human Immunodysregulation, Polyendrocrinopathy, Enteropathy, X-linked (IPEX) syndrome .

We next investigated various additional receptors and also included the anti-CD4 clones GK1.5 and RM4-4 in parallel to YTS177.9. As seen for OX40 and CD30, the frequencies of proliferated $CD4^+Foxp3^-$ T cells expressing the CD28 family co-stimulatory receptors ICOS and CD28 were reduced upon exposure to YTS177.9, GK1.5 and RM4-4 (Fig.6c and Fig.S7). The same was also seen for the inhibitory receptor CTLA-4 (Fig.6c) which is induced upon T cell activation. In summary, anti-CD4 binding to $CD4^+Foxp3^-$ T cells causes a general activation defect resulting in reduced T cell proliferation and in the reduced expression of various molecules associated with T cell activation.

3.7. Anti-CD4 treatment inhibits human $CD4^+$ T cell proliferation independent of $Foxp3^+$ Tregs

To further substantiate our findings, we finally explored whether human $CD4^+$ T cells can also be tolerized by anti-CD4 treatment independent of $Foxp3^+$ Tregs. Since YTS177.9 recognizes an epitope within the membrane-distal domain 1 of mouse CD4 , we chose the clone RPA-T4 which binds to the same domain of human CD4 . Notably, RPA-T4

inhibited the proliferation of human $CD4^+$ T cells in a dose-dependant manner (Fig.7). Importantly, $CD4^+$ helper T cells that were depleted of Tregs could still be substantially inhibited by RPA-T4 (Fig.7 and Fig.S8). These results suggest that, similar to the mouse setting, human anti-CD4 antibodies can have tolerizing activity independent of $Foxp3^+$ Tregs. Thus, the Treg-independent mechanism described here appears to be of general relevance because it is observed independent of the CD4 binding site and in both mouse and human systems of T cell activation.

4. Discussion

Our study proves, for the first time, that anti-CD4 treatment directly tolerizes $CD4^+$ helper T cells without the need for $Foxp3^+$ Tregs or $CD4^+$ DCs. These results are unexpected given the recent implication of $Foxp3^+$ Tregs in anti-CD4-mediated transplantation tolerance . However, these scenarios are not mutually exclusive. In fact, we anticipate that both mechanisms may operate simultaneously in Foxp3-sufficient settings. Besides, different mechanisms may be utilized upon combination of YTS177.9 with additional agents such as αCD8 and αCD40L which were required to induce allograft tolerance by Kendal et al. . Additionally, $Foxp3^+$ Treg depletion might cause graft rejection by mechanisms unrelated to and dominant over anti-CD4 induced tolerance. As examples for the scenario of allograft tolerance, Treg depletion accelerates transplant rejection of untreated mice , and the potency of Tregs at prolonging allograft survival is well established . Interestingly, a recent study reported that anti-CD4 antibody treatment induces tolerance to factor VIII independent of Foxp3 , supporting our findings.

The molecular events underlying Treg-independent anti-CD4-dependent tolerance await further analysis. We showed here that YTS177.9 causes CD4 internalization on both $Foxp3^+$ Tregs and $CD4^+Foxp3^-$ helper T cells. This phenomenon did not require accessory cross-linking cells (e.g. via FcγR) because purified $CD4^+$ T cells were sufficient to observe CD4 downregulation. Moreover, CD4 internalization does not require a functional TCR complex because it is also observed on splenic DCs lacking TCR and CD3ε expression. Yet, it appears that CD4 internalization is not a pre-requisite for anti-CD4-induced tolerance given that the KT6 clone can inhibit T cell proliferation without CD4 down-regulation . However, reduced CD4 expression may have additive impact given that CD4/TCR clustering classically promotes T cell functions . Obviously,

one explanation for the action of anti-CD4 antibodies is the direct disruption of CD4 co-receptor function and thereby the attenuation/alteration of concomitant TCR signals. Indeed, $CD4^+$ DO11.10 TCR-transgenic T cells pre-treated with YTS177.9 displayed drastically reduced ZAP-70- and LAT phosphorylation, two proximal signaling events downstream of the TCR complex, following stimulation with OVA-pulsed APCs . In the same line, we observed massively reduced CD25 expression on $CD4^+$ T cells after addition of YTS177.9 to MLRs. CD25 is induced by TCR signaling but can also be induced by signaling through the IL-2 receptor complex in a positive feedback loop . However, IL-2 was not involved in the tolerizing process because its supplementation failed to induce T cell proliferation after exposure to YTS177.9. This contrasts with a previous report showing that IL-2 restored T cell proliferation in the presence of GK1.5 . This is surprising given that GK1.5 and YTS177.9 bind to an overlapping epitope. However, one should note that IL-2 and GK1.5 were added only after T cell activation in that study. Thus, the T cells had presumably upregulated CD25 and were more responsive to IL-2 compared to our setting where CD25 expression is inhibited by YTS177.9.

In addition to CD25 we observed a defective expression of various molecules normally induced upon T cell activation. These include CD69, OX40, CD30, ICOS and CTLA-4. This picture is in agreement with a general defect in TCR-mediated T cell activation upon anti-CD4 binding. Although the reduced expression of OX40/CD30 was dispensable for primary T cell proliferation in vitro, it may explain in part a possible Foxp3-independent mechanism of action of anti-CD4 antibodies in vivo due to defective T cell survival .

One should stress that the CD4 co-receptor function may not exclusively be restricted to MHC-class-II binding. EL-4 lymphoma cells pre-incubated with YTS177.9 were shown to exhibit impaired Erk2-, NFAT- and AP1 activation following APC-free polyclonal stimulation . In accordance with that result, CD4 was suggested to physically associate with the TCR complex, presumably via the membrane-proximal domains 3/4, in order to modulate TCR signaling . Besides the inhibition of co-receptor function, CD4 ligation could also induce specific signals that interfere with TCR signaling. In this line, anti-CD4 stimulation was shown to transmit signals in the absence of TCR ligation, resulting in Erk2- and NFκB activation . Of note, most of the previous literature employed total $CD4^+$ T cell populations that implicitly contained both $Foxp3^+$ Tregs and $Foxp3^-$ T cells. Thus, future studies should aim to dissect the Treg-dependent and –independent signaling pathways of anti-CD4-induced tolerance. Moreover, it should be systematically dissected which binding properties of a given anti-CD4 antibody govern a particular tolerance mechanism. As we show here, the Treg-independent inhibition of T cell activation by anti-CD4 antibodies does not seem to depend on the CD4 binding site.

It is tempting to speculate that CD4-binding pathogens such as HIV may exploit similar tolerance mechanisms as induced by anti-CD4 antibodies to evade protective immunity. Indeed, HIV gp120 was shown to activate human Tregs and Tregs of humanized mice as well as to induce naïve T cell anergy . Moreover, the tolerizing anti-human CD4 antibody RPA-T4 competes with gp120 for CD4 binding , suggesting the recognition of a closely related epitope. In this regard, one concern is that pathogen-specific tolerance may be induced if an anti-CD4 antibody is administered. However, it was previously shown that anti-CD4-induced tolerance is antigen-specific and thus should only address

$CD4^+$ T cells that experience simultaneous activation . Whether this is also true for the novel Treg-independent component of anti-CD4-induced tolerance described in this paper remains to be explored. Moreover, the scenario of anti-CD4 administration during an acute or chronic infection, during which pathogen-derived antigens are highly abundant, deserves careful future investigation. Interestingly, a previous study reported that anti-CD4-mediated tolerance is abrogated when CD28-dependent co-stimulatory signals are provided . Moreover, infections can disrupt tolerance at additional layers .

5. Conclusion

Current dogma established that $Foxp3^+$ Tregs mediate anti-CD4-induced tolerance. In this paper, we prove the existence of a novel tolerance mechanism which does not require $Foxp3^+$ Tregs or $CD4^+$ DCs. Instead, we show that anti-CD4 directly tolerizes mouse and human $CD4^+Foxp3^-$ helper T cells that can mediate various pathologies

including autoimmunity. Considering that tolerizing anti-human CD4 antibodies are in clinical development, our study provides valuable novel insights into the mechanism of action of anti-CD4 antibodies and suggests that anti-CD4 therapies might potentiate therapies augmenting the number and/or suppressive function of Foxp3$^+$ Tregs. The latter has been implicated for the immunosuppressive drug rapamycin . Thus, anti-CD4 antibodies could represent an unexpectedly potent therapy for autoimmune diseases which are characterized by a dysfunction of Foxp3$^+$ Tregs.

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Conflict of interest

The authors have no conflicting financial interests.

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Figure legends

Figure 1. Anti-CD4 binding induces CD4 internalization on conventional T cells and Tregs

(a) Lymph node cells were incubated with 10µg/ml YTS177.9 on ice or were left untreated. After washing, cells were stained with the indicated fluorochrome-labeled anti-CD4 clones and were analyzed by flow cytometry. The control CD4 stainings (no YTS177.9) are shown as grey histograms. Samples treated with YTS177.9 are overlaid as black lines. Results are representative for two independent experiments. **(b, c)** $CD4^+$ T cells were negatively selected from spleens and lymph nodes of DEREG mice and were incubated with increasing concentrations of YTS177.9 at 37°C. After 6h, CD4 expression was analyzed by flow cytometry using the non-competing fluorochrome-labeled anti-CD4 clone RmCD4-2. **(b)** The MFIs of the $CD4^+$ population were calculated and the decrease relative to the untreated control is shown as mean + SD from three independent experiments. **(c)** Representative plots display CD4 vs. Foxp3-eGFP expression of an untreated sample (Ctrl) and of $CD4^+$ T cells treated with 10µg/ml YTS177.9. Frequencies of $CD4^+eGFP^-$ Teff and $CD4^+eGFP^+$ Tregs are indicated.

Figure 2. YTS177.9 inhibits $CD4^+$ T cell proliferation independent of IL-2

(a) YTS177.9 was added at indicated concentrations to MLRs. Mean counts per well + SD (quadruplicate well measurements) are shown for proliferated $CD4^+CellTraceViolet^-GFP^-$ conventional T cells (left) and for total $CD4^+GFP^+$ Tregs (right); NS: not statistically significant, * $p<0.05$ (two-tailed Mann Whitney test). **(b,c)** MLRs were left untreated (Ctrl) or were supplemented with rhIL-2 or anti-IL-2. Isotype control antibody or YTS177.9 was added. **(b)** Representative plots display CellTraceViolet dilution versus Foxp3

expression among $CD4^+$ cells. **(c)** Mean numbers per well + SD (quadruplicate wells) of proliferated $CD4^+$CellTraceViolet$^-$$Foxp3^-$ cells; NS: not statistically significant, * $p<0.05$ (two-tailed Mann Whitney test). Results are representative for three **(a)** or two **(b,c)** independent experiments. All MLRs were analyzed after 5 days of culture.

Figure 3. YTS177.9 treatment does not prominently induce or activate $Foxp3^+$ Tregs

(a,b) $CD4^+$ T cells negatively isolated from DT-treated DEREG mice were labeled with CellTraceViolet. T cells were stimulated for 5 days with anti-CD3ε in the presence of the indicated supplements and either isotype- or YTS177.9 antibody. **(a)** Representative plots from one out of two independent experiments display CellTraceViolet dilution vs. Foxp3 expression of live $CD4^+$ T cells. **(b)** Mean total cell numbers + SD (n=6, pool of two independent experiments) of $CD4^+Foxp3^+$ iTregs per well (** $p<0.01$, two-tailed Mann Whitney test compared to isotype). **(c)** $CD8^+$ OTI T cells were activated by SIINFEKL peptide in the presence of Balb/c Tregs without further additions (Treg), and after addition of αCD3 (Treg + anti-CD3) or YTS177.9 (Treg + YTS177.9). Wells containing Tregs and αCD3 but lacking OTI cells served as negative control (Neg). Proliferation was measured by ^{3}H-thymidine uptake. Data from triplicate measurements (±SD) are shown. Results are representative for three independent experiments.

Figure 4. Foxp3$^+$ Tregs are dispensable for the tolerizing activity of multiple anti-CD4 antibodies

WT and DEREG mice were injected with DT on two consecutive days and were sacrificed the following day. **(a)** Flow cytometric analysis after T cell purification displays CD4 vs. Foxp3 expression among CD4$^+$ cells and is representative for at least three independent experiments. **(b)** MLRs were established in presence of DT and were analyzed by flow cytometry after 5 days. CD4$^+$ T cells were also cultured in the absence of DT as control. YTS177.9 was added where indicated. The graph shows the absolute numbers of CD4$^+$Foxp3$^+$ Tregs per well. **(c)** YTS177.9 was added to the MLR cultures either in presence (DT) or absence (Medium) of DT. Cell numbers of proliferated CD4$^+$CellTraceViolet$^-$Foxp3$^-$ responder T cells were determined by flow cytometry and the mean relative decrease of cell numbers + SD (triplicate wells) induced by the YTS177.9 treatment compared to control treatment is displayed; NS, not statistically significant (two-tailed Mann Whitney test). **(d)** CD25, CD69 and CD62L expression among CD4$^+$CD8α$^-$ responder T cells was analyzed after two days (Ctrl stain: control staining; Medium: specific staining of untreated MLRs; YTS177.9: specific staining of YTS177.9-treated MLRs). **(e)** MLRs devoid of Foxp3$^+$ Tregs were established as described above in presence of DT and with increasing concentrations of rat IgG2 antibody or anti-CD4 clones YTS177.9, GK1.5 and RM4-4. Proliferation was quantified by flow cytometry 5 days later. The graph shows the absolute number of CD4$^+$CellTraceViolet$^-$ proliferated T cells per well (mean ± SD). Results are representative for two **(a, b, e)** or three to four **(c,d)** independent experiments.

Figure 5. CD4$^+$ DC functions are unimpaired by anti-CD4 treatment

(a-d) FACS-sorted CD4$^+$ pDCs and CD4$^+$ cDCs were treated with isotype control antibody or YTS177.9 followed by stimulation with A-type CpG-2216 or B-type CpG-1826 for 24h. Wells without CpG-ODN served as controls (Unstim). **(a)** IFNα contents in pDC supernatants are shown for three individual mice. **(b)** IL-12/IL-23p40 contents in pDC supernatants are shown for three individual mice. **(c)** pDCs were analyzed by flow cytometry after culture for up-regulation of I-A/E and CD86. Specific staining of isotype-treated (Rat IgG2a, grey line) and YTS177.9-treated (black line) pDCs as well as unstained controls (Ctrl stain, light grey filled histogram) are shown for unstimulated (Medium, upper row) and CpG-2216-activated (CpG-2216, lower row) pDCs. **(d)** IL-12/IL-23p40 contents in cDC supernatants are shown for three individual mice. **(e, f)** Untouched gradient-enriched splenic DCs (total DCs) were pre-treated with rat IgG2 control antibody, YTS177.9 or GK1.5 followed by stimulation with A-type CpG-2216 for 24h. Unstimulated wells served as controls (Unstim). **(e)** IFNα contents in DC supernatants are shown as mean of duplicate or triplicate wells. **(f)** DCs were analyzed by flow cytometry after culture for expression of I-A/E and CD86. Specific staining of isotype-treated (Rat IgG2a, grey line) and YTS177.9-treated (black line) DCs as well as unstained controls (Ctrl stain, light grey filled histogram) are shown for unstimulated (Medium, upper row) and CpG-2216-activated (CpG-2216, lower row) DCs. **(g, h)** Untouched gradient-enriched splenic DCs were co-cultured with CellTraceViolet-labeled CD8$^+$ allogeneic T cells in absence of stimuli (Medium) or in presence of A-type CpG-2216 or B-type CpG-1826. T cells cultured without DCs served as controls (No DCs). Wells were supplemented with 10µg/ml isotype antibody (Iso) or YTS177.9 antibody (YTS). After 5 days, T cells were restimulated with PMA/ionomycin in presence of

brefeldin A. IFN-γ production and proliferation were then assessed by flow cytometry. **(g)** Representative plots show CellTraceViolet dilution and IFN-γ production of live $CD8\beta^+$ T cells. **(h)** Absolute numbers of proliferated live $CD8\beta^+IFN\text{-}\gamma^+$ T cells per well are shown as mean + SD (triplicate wells). All results are representative for two to three independent experiments (NS: not statistically significant, two-tailed Mann Whitney test).

Figure 6. CD4 blockade impairs co-stimulatory receptor expression by $CD4^+$ T cells

(a) DEREG $CD4^+$ T cells were cultured for 5 days with allogeneic DCs in presence of isotype control (Iso) or YTS177.9 (YTS) and were then analyzed by flow cytometry. Representative plots display OX40-, CD30- or control stainings vs. CellTraceViolet dilution among live gated $CD4^+eGFP^-$ conventional T cells. **(b)** Absolute numbers of proliferated $CD4^+eGFP^-CellTraceViolet^-OX40^+$ (left graph) and $CD4^+eGFP^-CellTraceViolet^-CD30^+$ (right graph) cells per well. Results are representative for two independent experiments each containing 3-4 mice per group. **(c)** DEREG mice were injected with DT on two consecutive days and were sacrificed the following day. CellTraceViolet-labeled $CD4^+$ T cells were co-cultured with allogeneic DCs in presence of DT. Wells were supplemented with 10µg/ml rat IgG2 isotype antibody, YTS177.9, GK1.5 or RM4-4. After 5 days, cells were analyzed by flow cytometry. Representative plots display CellTraceViolet dilution and CTLA4- or ICOS expression among live $CD4^+CD8\alpha^-$ T cells. One out of two independent experiments is shown.

Figure 7. Anti-CD4 treatment inhibits human $CD4^+$ T cell proliferation in the absence of $Foxp3^+$ Tregs

PKH26-labeled human $CD4^+$ T cells (Total $CD4^+$) or Treg-depleted $CD4^+$ effector T cells (Teff) were stimulated with allogeneic monocytes in presence of increasing concentrations of RPA-T4. Proliferation was quantified by flow cytometry 7 days after culture. Graphs indicate the numbers of proliferated T cells as mean ± SD of triplicate wells. Two independent experiments are shown.