



## Modulation of CD4 T cell function *via* CD6-targeting

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### ABSTRACT

In recent years molecules involved on the immune synapse became successful targets for therapeutic immune modulation. CD6 has been extensively studied, yet, results regarding CD6 biology have been controversial, in spite of the ubiquitous presence of this molecule on virtually all CD4 T cells. We investigated the outcome of murine and human antibodies targeting CD6 domain 1. We found that CD6-targeting had a major impact on the functional specialization of CD4 cells, both human and murine. Differentiation of CD4 T cells towards a Foxp3<sup>+</sup> Treg fate was prevented with increasing doses of anti-CD6, while Th1 polarization was favoured. No impact was observed on Th2 or Th17 specialization. These *in vitro* results provided an explanation for the dose-dependent outcome of *in vivo* anti-CD6 administration where the anti-inflammatory action is lost at the highest doses. Our data show that therapeutic targeting of the immune synapse may lead to paradoxical dose-dependent effects due to modification of T cell fate.

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### 1. Introduction

CD6 is a transmembrane glycoprotein (105/130 kDa) expressed mostly on mature T cells, but also in thymocytes, B1a lymphocytes and CD56<sup>+</sup> NK cells. Its structure includes three extracellular scavenger receptor cysteine-rich (SRCR) domains and a cytoplasmic tail (244 amino acids) without catalytic activity but with several sites for phosphorylation and recruitment of signal transduction proteins [1–3]. So far, two CD6 ligands have been described: CD318, that binds CD6 domain 1 (d1), being expressed mostly on synovial tissues by epithelial cells but also in some tumors; and the activated leukocyte cell adhesion molecule (ALCAM) or CD166, that binds CD6 d3 and is expressed mainly by monocyte-derived cells and endothelial cells [4–7]. From the two ligands, ALCAM is the best characterized, being established that its ligation to CD6 allows for stable T cell-antigen presenting cell (APC) interactions, essential for maturation of immunological synapse (IS) and consequent optimal T cell proliferation [8–12].

The high degree of conservation of CD6 and ALCAM binding regions suggests an evolutionary relevance for this specific interaction [13–16]. Because CD6 is a SRCR family member, present at the immune synapse during activation, a putative role for CD6 in the pathogenesis of autoimmunity has been investigated [17,18]. Indeed, it was reported that CD6-deficient mice have altered susceptibility to autoimmunity. However, while in experimental autoimmune encephalomyelitis (EAE) and imiquimod-induced psoriasis CD6-deficient mice had disease protection or attenuation, in collagen-induced arthritis (CIA) CD6-deficient mice had more severe disease [19–21]. Different genetic backgrounds, different knockout strategies, and particularities intrinsic to the pathogenesis of each disease model might justify the differences.

In humans, CD6 was also implicated in the pathogenesis of several autoimmune diseases, including rheumatoid arthritis (RA), Sjögren's syndrome and psoriasis [22–25]. Furthermore, genome wide association studies (GWAS) and gene-specific candidate-driven studies also identified *CD6* as a major susceptibility locus for multiple sclerosis (MS), psoriasis and Behcet's disease [26–29].

Given the involvement of CD6 in autoimmunity, there has been an effort to develop therapeutic strategies based on CD6-targeting [30,31].

One of these strategies relates to Itolizumab, a humanized non-depleting mAb targeting CD6 d1, that was shown effective and safe for

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### Research in context

#### Evidence before the study

CD6 has been linked to autoimmunity, and CD6-targeting antibodies have been proposed as a promising autoimmune therapy. A key issue regarding the biology of CD6-targeting has remained unaddressed: its impact on the functional polarization of CD4 T cells.

#### Added value of this study

We found a surprising dose-response effect of anti-CD6 monoclonal antibodies on the functional specialization of murine and human CD4 T cells: High doses of anti-CD6 inhibited polarization towards Foxp3+ Treg cells while favouring Th1 polarization. Th2 and Th17 polarization remained unaffected. The impact of CD6-targeting on T cell specialization was observed in the absence of any major effect on T cell survival or proliferation.

#### Implications

Our data show that therapeutic antibodies targeting the immune synapse, namely anti-CD6, may lead to paradoxical dose-dependent effects due to modification of T cell fate.

the treatment of psoriasis [32,33]. Clinical trials in RA also showed clinical benefits, with lower doses providing the highest and long-lasting improvements [34,35]. Thus, we investigated how different dosages of CD6 d1-targeting would impact on murine neuroinflammatory disease. We found that high doses of anti-CD6 were not protective and could even promote inflammation. In order to find the mechanism for such high-dose exacerbation of disease, we addressed the impact of CD6 d1-targeting on the functional specialization of activated CD4 T cells. Here we show that CD4 T cells exposed to higher doses of anti-CD6 were prevented from acquiring a regulatory T (Treg) cell phenotype, while preferentially differentiating towards Th1. Our findings were observed with murine and human cells.

## 2. Materials and methods

### 2.1. Ethics and in vivo experiments

C57BL/6 and OVA-specific TCR-transgenic mice (OT-II *Rag2*<sup>-/-</sup>) were bred and maintained under specific pathogen-free conditions. Sex-matched mice, between 8 and 10 weeks of age were used in the experiments. All experimental protocols were approved by the Local Ethics Committee and are in compliance with European Union guidelines. EAE was induced in C57BL/6 mice by s.c. immunization with 125 µg MOG<sub>35–55</sub> (MEVGWYRSPFSRVVHLYRNGK) (AnaSpec, Inc.) emulsified in complete Freund's Adjuvant (CFA) suspension (4 mg/ml mycobacteria in IFA), and i.v. injection of 200 ng pertussis toxin (List Biological Laboratories) on days 0 and 2 following immunization. Disease severity was scored daily: 1, tail atony; 2, hind limb weakness; 3, hind limb paralysis; 3.5- flattening of hind quarters with complete paralysis; 4, quadriplegia; 5, moribund.

### 2.2. Histopathology

Mice were deeply anesthetized for transcardiac perfusion with PBS, followed with 4% paraformaldehyde. After perfusion, head and spinal cord were further immersed into neutral buffered formalin for 48 h. Brain and spinal cord were then removed from the bone, trimmed and routinely processed for paraffin embedding. Sections with 4 µm were stained with hematoxylin-eosin and Luxol fast blue, and screened by a

pathologist blinded to experimental groups, in a Leica DM2500 microscope coupled to a Leica MC170 HD microscope camera. Semi-quantification of inflammation and demyelination were performed using a 5-tier system with 0–4 grading scale: 0, absent; 1, minimal; 2, mild; 3, moderate; 4, marked.

### 2.3. mAbs and flow cytometry

Anti-mouse CD6 d1 (10F12) and anti-human CD6 d1 (Itolizumab), as well as isotype controls were produced at the CIM (Havana, Cuba). Anti-IL-4 (11B11) and anti-IFNγ mAbs were produced at IMM (Lisbon, Portugal) using Integra CL1000 flasks (IBS Integra Biosciences, Chur, Switzerland), purified by 50% (w/v) ammonium sulphate precipitation, dialyzed against PBS, and purity was checked by native and SDS gel electrophoresis. Murine single cell suspensions were stained with CD4 PE (GK1.5); CD4 APC-eFluor® 780 (RM4–5); TCRβ APC-eFluor® 780 (H57–597); CD25 PE-Cy7 (PC61.5); IFNγ FITC (XMG1.2); IL-13 PE (eBio13A); IL-17 PE (ebio17B7); Foxp3 APC (FJK-16 s), CD6 PE (BX222 Biologend), anti-rat IgG Biotin and Streptavidin PE (all from eBioscience). Human single cells suspensions were stained with CD4 PE (RPA-T4); CD4 FITC (OKT4); CD3 PE (OKT3); CD25 PE-Cy7 (BC96); CD45RA APC-eFluor® 780 (HI100); IFNγ PerCP-Cy5.5 (4S. B3); IL-13 PerCP-Cy5.5 (JES10-5A2); IL-17 APC (eBio64DEC17); Foxp3 APC (PCH101), CD6 FITC (BL-CD6) and anti-human IgG APC-Cy7. Cell viability was detected with Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) and Annexin V Apoptosis Detection Kit (eBioscience). CellTrace™ Violet Cell Proliferation Kit was used for cell proliferation assessment according to the manufacturer's protocol (ThermoFisher). In some studies, cytokine production was assessed following 4 h stimulation with 50 ng/ml PMA, 500 ng/ml ionomycin, 10 µg/ml brefeldin (all from Sigma Aldrich) and 0.66/ml Golgistop™ (BD Biosciences). Cells were permeabilized with eBioscience kit (# A25866A).

### 2.4. Recombinant mouse extracellular CD6 protein

Murine soluble CD6 (Gly17-Thr398) was provided by INVIGATE GmbH, Jena, Germany ([www.invigate.com](http://www.invigate.com)). The recombinant protein is derived from HEK 293 cells and comprises C-terminally fused HA-Tag (YPYDVPDYA), BirA-Tag (GLNDIFEAQKIEWH) and His-Tag (HHHHHH).

### 2.5. T-cell activation and polarization (murine cells)

OVA-specific CD4<sup>+</sup> T cells were magnetically sorted with CD4 (L3 T4) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from OTII-*Rag2*<sup>-/-</sup> mice, with a purity >90%. T cells were cultured for 4 days and activated with bone marrow-derived dendritic cells (bmDCs) (at 2:1 ratio) [44] and 10 µM OVA<sub>323–339</sub> (Eurogentec) or with 3 µg/ml plate bound anti-CD3 and 2 µg/ml soluble anti-CD28 (OKT3 and 37.51, eBioscience). For Th1 polarization the medium was supplemented with 5 ng/ml IL-2 and 10 ng/ml IL-12 (both from Peprotech), and 0.5 mg/ml anti-IL-4 (11B11). For Treg polarization, we added 5 ng/ml IL-2 and 5 ng/ml TGF-β (R&D). For Th17 polarization, the medium included 10 ng/ml IL-1β and 20 ng/ml IL-6 (both from Peprotech), 1 ng/ml TGF-β (R&D), and 0.5 mg/ml anti-IFNγ (R46A2). Finally, for Th2 polarization we added 5 ng/ml IL-2, 10 ng/ml IL-4 (Peprotech), and 0.5 mg/ml anti-IFNγ.

### 2.6. T-cell activation and polarization (human cells)

Peripheral blood mononuclear cells (PBMCs) were isolated from blood or buffy-coats from healthy volunteers provided by Instituto Português do Sangue e Transplantação (IPST), following informed consent, by Ficoll gradient (Sigma Aldrich) using SepMate™ (STEMCELL Technologies). Naïve CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD3<sup>+</sup>CD25<sup>-</sup>CD45RA<sup>+</sup>) were

then sorted with FACS Aria III (BD Biosciences). Irradiated (25 Gy) PBMCs were used as APCs (iPBMCs). CD4<sup>+</sup> cells were cultured with iPBMCs (at 1:2 ratio) and 1 µg/ml of soluble anti-CD3 (OKT3); or with 3 µg/ml plate bound anti-CD3 (OKT3) and 2 µg/ml soluble anti-CD28 (CD28.2). For Th1 polarization the medium was supplemented with 10 ng/ml IL-2, 2.5 ng/ml IL-12 (both from Peprotech), and 5 µg/ml anti-IL-4 (11B11) mAb. For Treg polarization we added 10 ng/ml IL-2 and 10 ng/ml TGF-β (R&D).

### 2.7. Statistical analysis

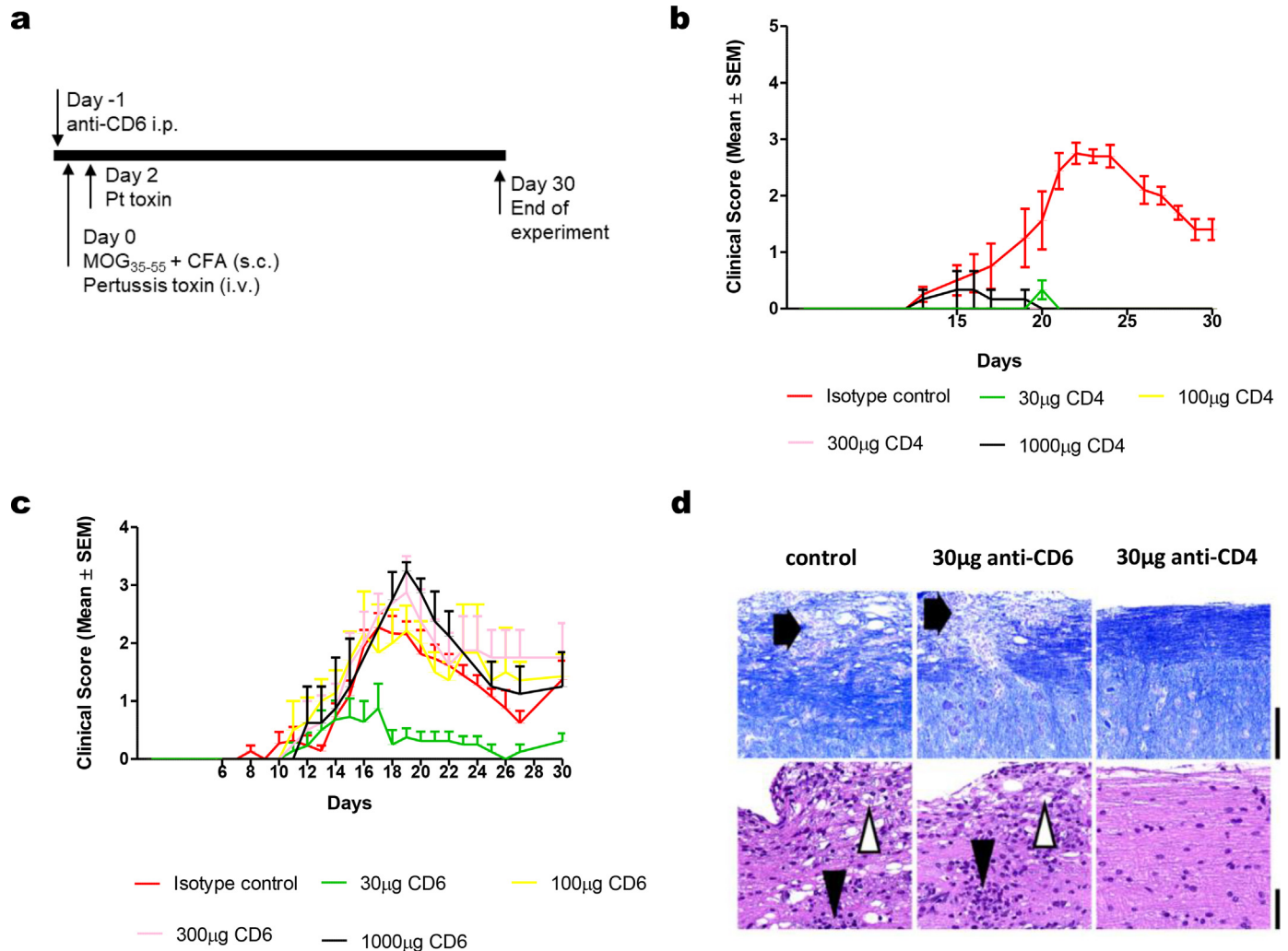
Statistical significance was calculated using nonparametric Mann-Whitney *U* test, and Kruskal-Wallis one-way analysis of variance, *p* values of <0.05 were considered significant (\**p* < .05, \*\**p* < .01, \*\*\**p* < .001). Results are presented as mean ± SEM.

## 3. Results

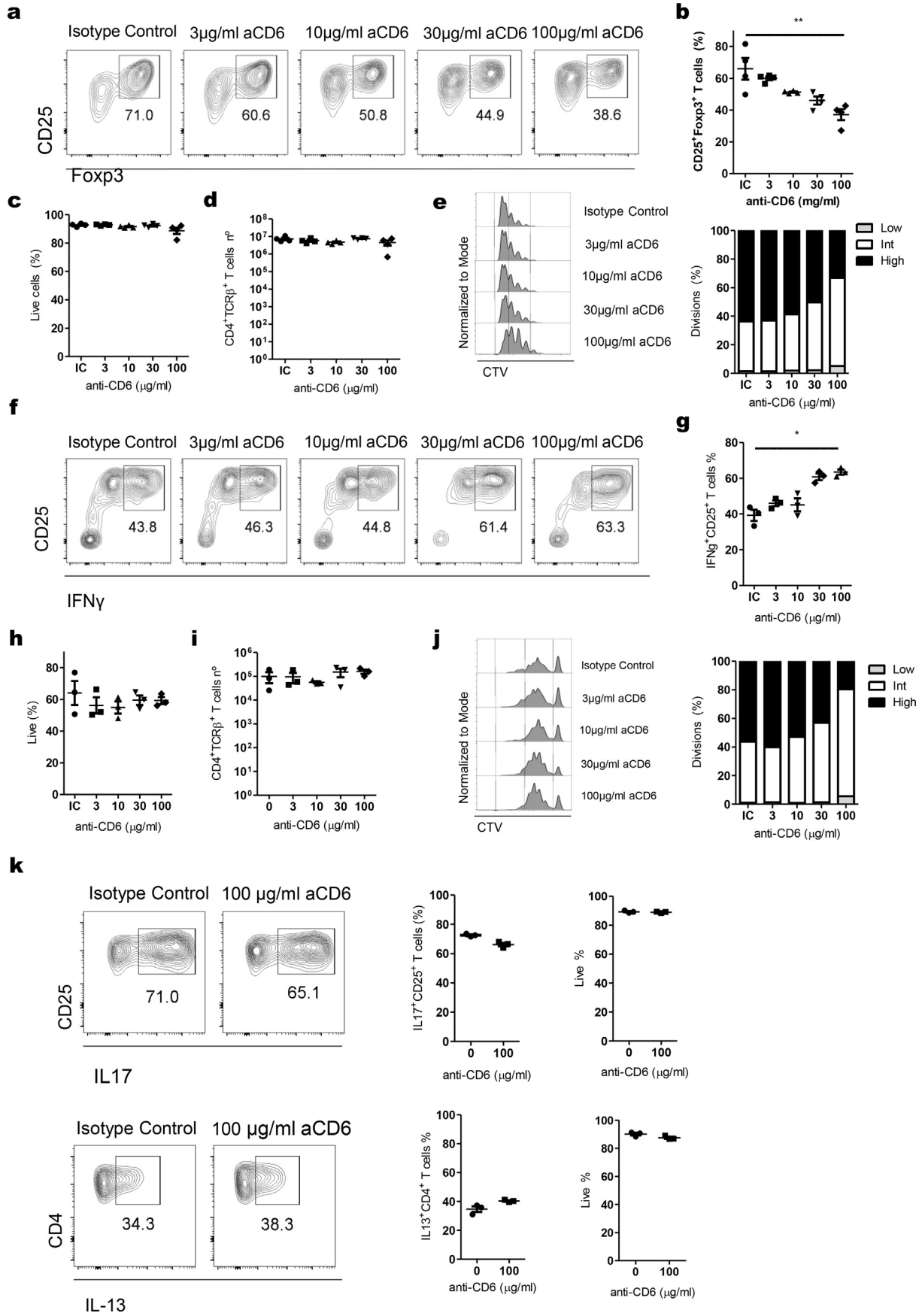
### 3.1. Targeting CD6 d1 leads to anti-inflammatory effects exclusively at low doses

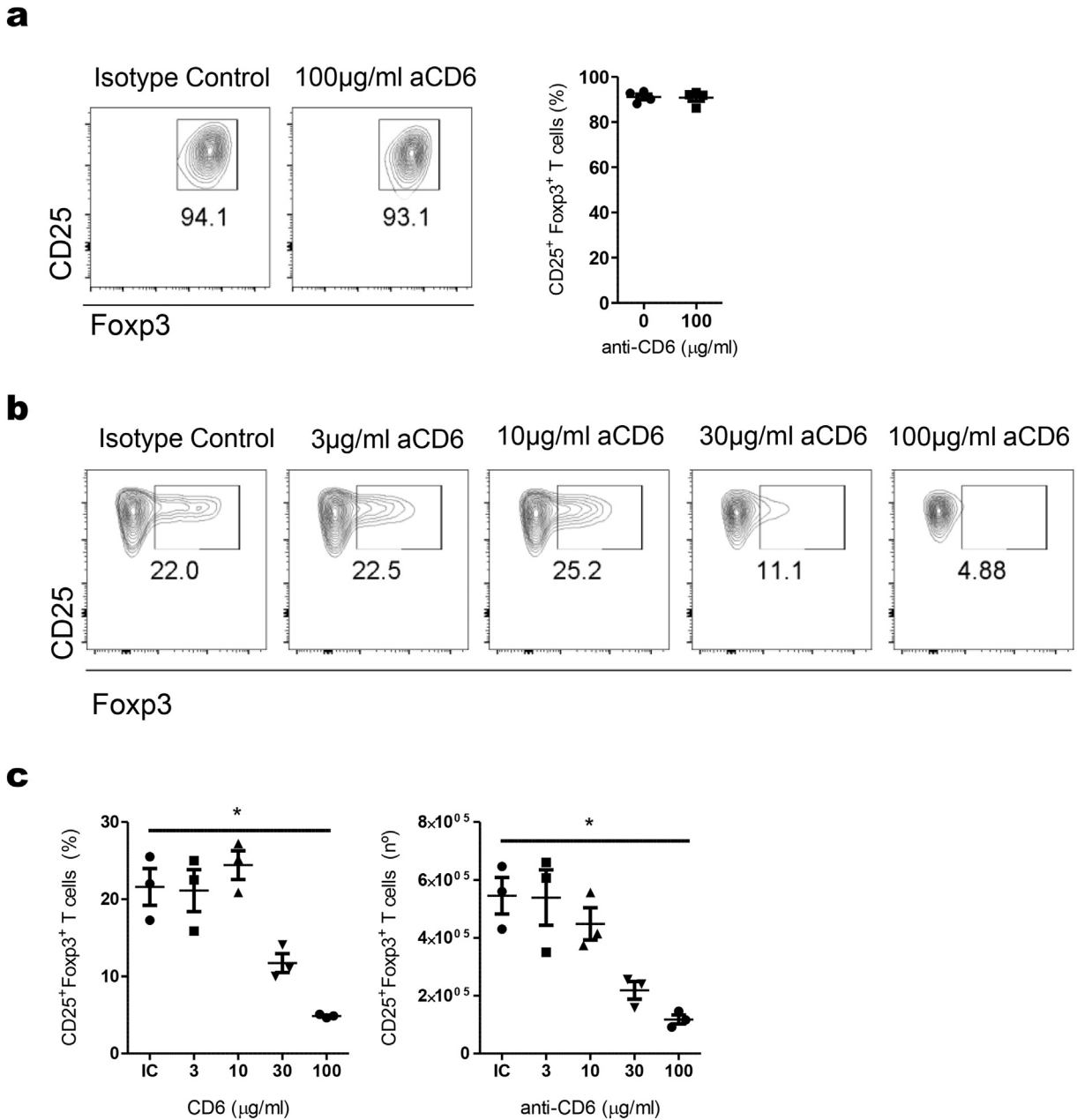
Given prior reports on the importance of CD6 alleles for MS susceptibility [26,27], and studies showing CD6-deficient mice resist induction of EAE [19], we investigated whether antibodies targeting CD6 d1 can prevent neuroinflammation.

We used an established model of EAE, induced following MOG-CFA and pertussis toxin administration to C57BL/6 mice (Fig. 1a). It is possible to prevent the onset of EAE in this experimental system using antibodies that promote peripheral induction of Treg cells, such as neutralizing anti-CD4 antibodies that do not induce cell lysis [36]. We used YTS177 (a non-depleting pro-tolerogenic anti-CD4) as positive control (Fig. 1b and Supplementary Table 1).



**Fig. 1.** Low dose of anti-CD6 mAb (10F12) prevents the onset of EAE. (a) C57BL/6 mice were immunized with MOG and treated with different doses of anti-CD6, or an isotype control at day 0. (b) Clinical score of mice treated with different doses of non-depleting anti-CD4 (YTS177), on the day before MOG<sub>35-55</sub> immunization. All mice treated with anti-CD4 were protected from EAE (*n* = 5 per group). (c) Clinical score for each concentration of anti-CD6 and control group of mice immunized with MOG<sub>35-55</sub> peptide are shown as mean values ± SEM, pooled data from three independent experiments. Mice treated with 30 µg anti-CD6 (*n* = 11) were protected from EAE. However, mice treated with 100 µg anti-CD6 (*n* = 8), or greater doses (*n* = 4 per group), developed EAE with disease severity and incidence similar to the control group (*n* = 15). (d) Longitudinal sections of spinal cord from mice 22 days after MOG<sub>35-55</sub> immunization. In the Luxol fast blue stained section (upper panel), mice treated with anti-CD6 but not anti-CD4 show demyelination of the peripheral spinal cord white matter (black arrow), similar to control mice (original magnification 10×, bar 250 µm). In the hematoxylin-eosin-stained section (lower panel), mice treated with anti-CD6 but not anti-CD4 also show an intense mononuclear inflammatory infiltration of the peripheral white matter, with macrophage-rich areas that include numerous myelin-containing phagocytes (white arrowhead), and with fewer lymphocytes (black arrowhead), similar to control (original magnification 40×, bar 50 µm).





**Fig. 3.** CD4<sup>+</sup>T cell polarization is dependent on CD166 binding. (a) CD25<sup>+</sup>Foxp3<sup>+</sup> T cells within CD4<sup>+</sup>TCRβ<sup>+</sup> T cells at the end of 4-days culture of OVA-specific TCR-transgenic OT-II.Rag<sup>-/-</sup> CD4 T cells with plate-bound anti-CD3 and anti-CD28 under Treg polarizing conditions. Anti-CD6 did not alter the frequency of induced Foxp3<sup>+</sup> T cells. (b) OT-II.Rag<sup>-/-</sup> CD4 T cells were cultured in a 2:1 ratio with BMDC for 4 days under Treg polarizing conditions with increasing concentrations of soluble CD6. Representative dot plots and (c) graphs showing the frequency and number of CD25<sup>+</sup>Foxp3<sup>+</sup> T cells within CD4<sup>+</sup>TCRβ<sup>+</sup> T cells. Data are representative of two independent experiments (n = 4).

In mice treated with mAbs targeting CD6 d1 we could only find a partial protection from EAE with the lowest dose (Fig. 1c and Supplementary Table 2). On the contrary, the highest doses of anti-CD6 d1 did not show any beneficial effect (Fig. 1c).

The partial suppression afforded by low-dose anti-CD6 was not sufficient to completely abrogate inflammatory changes observed by histopathology, or to completely abolish infiltration of the CNS with lymphocytes (Fig. 1d and Supplementary Table 3).

### 3.2. CD6 d1-targeting favours Th1 differentiation while suppressing Treg cell induction

Given our previous results showing that protection from EAE following CD4-blockade was due to peripheral expansion of Treg cells at the expense of effector T cell (Th1 and Th17) polarization [36], we investigated how CD6 d1 targeting impacted on T cell polarization.

**Fig. 2.** CD6-targeting increases Th1 polarization while inhibiting Treg differentiation. OVA-specific TCR-transgenic OT-II.Rag<sup>-/-</sup> CD4 T cells were cultured for 4 days in a 2:1 ratio with bone marrow derived dendritic cells (BMDC) in Th1 and Treg polarizing conditions. (a, b) Representative flow cytometry dot plots and scatter plots showing the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> T cells within CD4<sup>+</sup>TCRβ<sup>+</sup> T cells at the end of Treg polarizing cultures with different doses of anti-CD6 (10F12) or 100 µg/ml isotype control (IC). (c) Survival of CD4 T cells at the end of culture. (d) Number of CD4 T cells recovered at the end of the culture. (e) Representative histograms showing CTV dilution of T cells following culture and bar graph displaying the frequency of cells within gates representing low, intermediate and high proliferation as displayed in the histograms. (f, g) Representative flow cytometry dot plots and scatter plots showing the percentage of CD25<sup>+</sup>IFNγ<sup>+</sup> T cells within CD4<sup>+</sup>TCRβ<sup>+</sup> T cells in Th1-polarizing cultures. (h) Viability of CD4 T cells under Th1 polarizing conditions. (i) Number of CD4 cells recovered at the end of culture. (j) T cell proliferation under Th1 polarizing conditions. (k) Representative dot plots and scatter plots showing the percentage of T cells producing IL-17 (top) or IL-13 (bottom) following culture under, respectively, Th17 and Th2 polarizing conditions as well as their viability (right). Statistical tests: Kruskal-Wallis and Mann-Whitney. Data are representative of three independent experiments, each with n = 3. \*p < .05 \*\*p < .01.

To do that, we sorted CD4<sup>+</sup> OVA-specific TCR-transgenic cells from OT-II.Rag<sup>-/-</sup> mice. Because virtually all murine CD4 T cells constitutively bear CD6 on their surface, it was unnecessary to sort subsets of CD4 cells based on CD6 levels (Supplementary Fig. 1). The sorted OVA-specific CD4 T cells were then stimulated with OVA-loaded DCs under the appropriate cytokine environment to promote functional polarization of uncommitted CD4 cells towards Treg, Th1, Th2, or Th17 effector phenotype. We found that CD6 d1 targeting showed a dose-dependent suppression of Treg polarization, as assessed by the decrease of Foxp3 expression (Fig. 2a,b), without an impact on T cell viability (Fig. 2c). Cell proliferation also remained largely unaffected as the number of cells retrieved at the end of the culture remained unchanged (Fig. 2d), although a trend in slower progression through intermediate classes of cell division was observed at the highest dose of anti-CD6 (Fig. 2e). We confirmed that the number of Treg cells were consistently decreased, and CD6 was not downmodulated/internalized during *in vitro* cultures or *in vivo*, in mice treated with anti-CD6 (Supplementary Fig. 2).

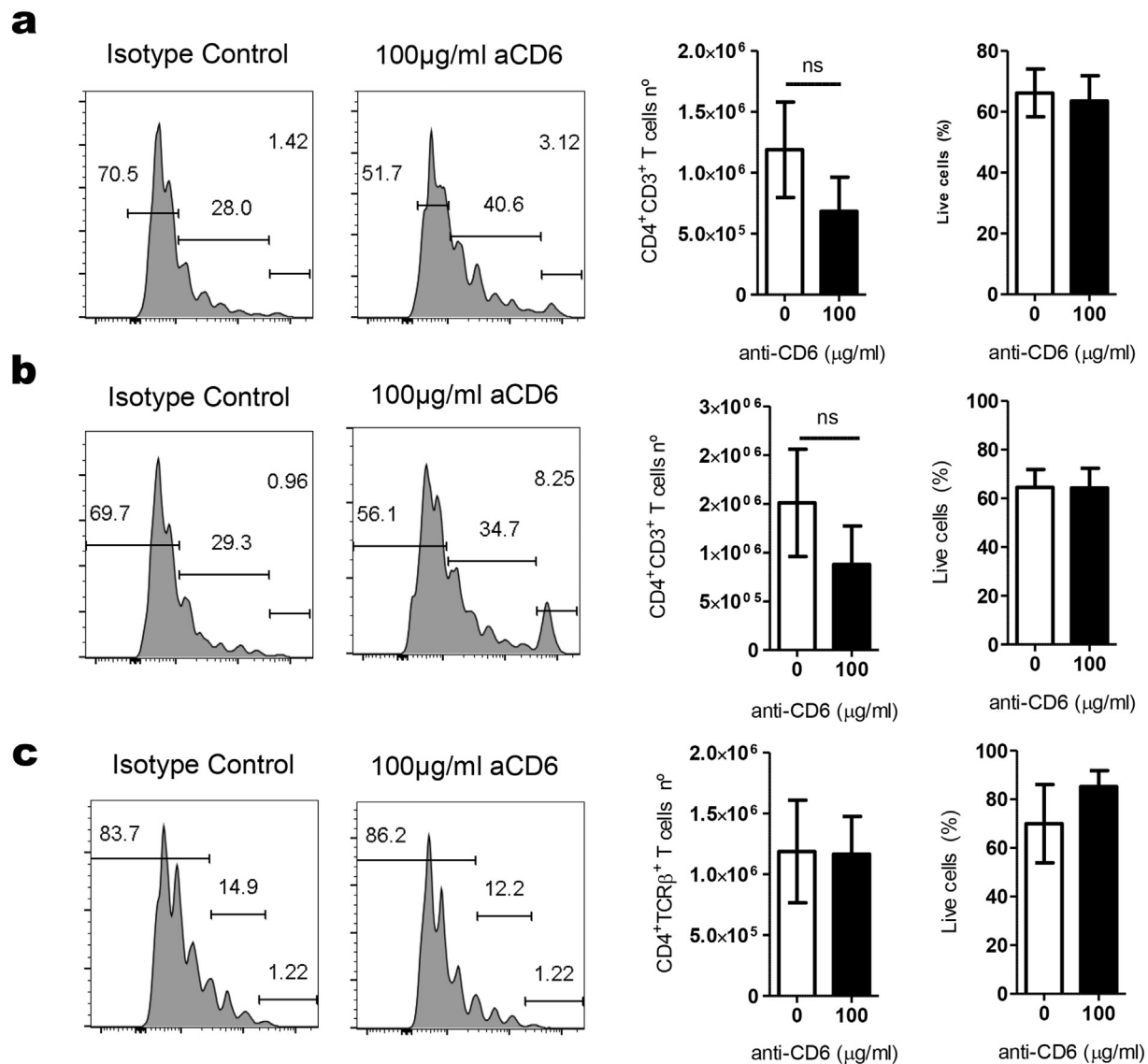
We also found that polarization towards a Th1 phenotype responded to CD6 d1-targeting in an opposite way, with higher doses of anti-CD6 d1 leading to greater frequency of Th1 cells (Fig. 2f,g), again without significant impact on T cell survival or proliferation (Fig. 2h–j).

The polarization of uncommitted CD4 T cells towards Th2 and Th17 phenotypes remained unaffected by anti-CD6 d1, even at the highest doses (Fig. 2k).

### 3.3. Alteration of T cell functional specialization by anti-CD6 d1 is a consequence of abrogation of CD6-CD166 interactions

We then investigated whether the observed impact of anti-CD6 d1 on T cell polarization was a consequence of steric hindrance of CD166 binding to CD6.

First, we stimulated T cells, under cytokine conditions favouring Treg polarization, by providing anti-CD3 and anti-CD28 in the absence of APCs (and, consequently, without CD166 provision at the T cell – APC

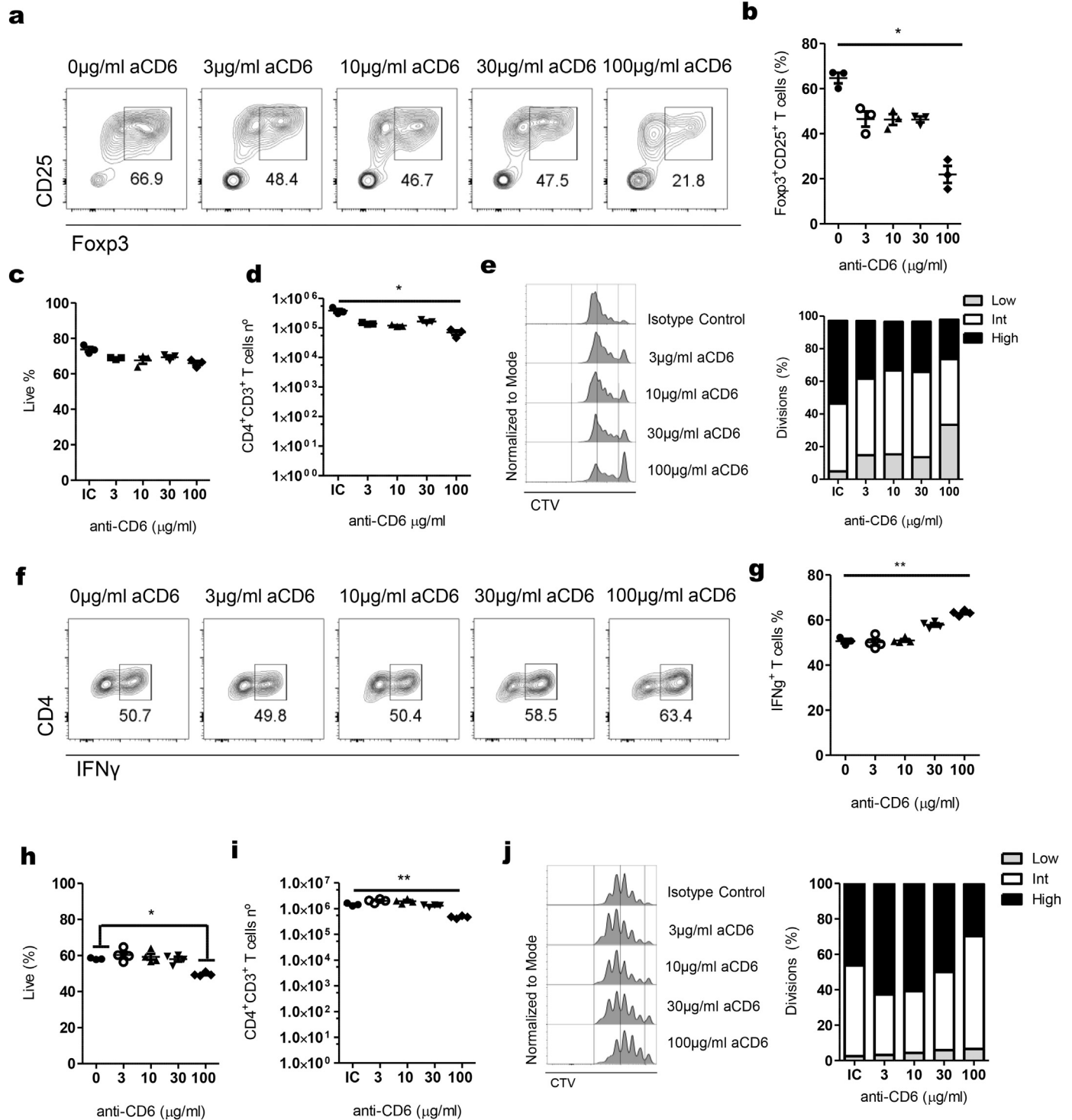


**Fig. 4.** Impact of itolizumab on human CD4 T cell proliferation and survival. Peripheral blood mononuclear cells (PBMCs) from healthy donors were used as source of naïve T cells (responders). Those naïve T cells were labelled with CTV and stimulated with syngeneic irradiated PBMC plus anti-CD3 (a); with allogeneic irradiated PBMCs (b); or with anti-CD3 and anti-CD28 in the absence of stimulating cells (c). Responder naïve CD4 T cells were cultured for 5 days in a ratio of 1:2 with the irradiated PBMCs (except in C) in non-polarizing conditions with IL-2. (a–c) Representative histograms of cultures with and without itolizumab and bar graphs showing the number of recovered CD4 T cells (left) and their viability (right). The graphs represent the pooled data from seven independent experiments, each with triplicates. Statistical test: Mann-Whitney.

interface). We found that addition of anti-CD6 d1 to those conditions did not change the polarization of uncommitted CD4 T cells (Fig. 3a).

However, the different stimulation regime (APCs vs. anti-CD3/anti-CD28) leads in itself to a different polarization efficiency. As a consequence, we addressed this issue with a more comparable stimulatory

regime. We stimulated uncommitted CD4 T cells under the same conditions as described in Fig. 2, but now using soluble CD6 to prevent CD6 interactions with CD166 on APCs. We found that the addition of soluble CD6 led to a dose-dependent impact on Treg polarization similar to what we observed with anti-CD6 (Fig. 3b,c). Therefore, anti-CD6



**Fig. 5.** Itolizumab inhibits Treg polarization while enhancing acquisition of Th1 phenotype. (a–e) PBMCs from healthy donors were used as source of naïve CD4 T cells (responders), labelled with CTV and stimulated 5 days with syngeneic irradiated PBMC (at a 1:2 ratio) with added anti-CD3, TGF-β, and IL-2 for Treg polarization; or (f–j) with IL-12, IL-2 and anti-IL4 for Th1 polarization. (a) Representative contour plots of CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells and (b) graph with pooled data. (c) Viability of CD4 T cells at the end of culture in Treg polarizing conditions and (d) number of recovered cells. (e) Representative histograms showing T cell proliferation (CTV dilution) under Treg polarizing conditions and pooled data with the frequency of T cells within the represented low, intermediate and high cell division gate. (f) Representative contour plots of IFNγ producing CD4 T cells and (g) graph with pooled data. (h–j) Viability, number of recovered CD4 T cells, and T cell proliferation at the end of cultures under Th1-polarizing conditions. Data are representative of two independent experiments, each of them run with triplicates. Statistical test: Kruskal-Wallis. \**p* < .05 \*\**p* < .01.

modulation of T cell functional specialization upon activation appears to be a consequence of displacement of CD6-CD166 interactions.

### 3.4. CD6-targeting in human T cells with itolizumab reduces proliferation and Treg cell induction

We then investigated whether itolizumab, a humanized monoclonal antibody targeting human CD6 d1, can also influence the acquisition of effector functions by activated human CD4 T cells. With human experiments, due to the inability to use populations of T cells with a defined TCR, we sort-purified naïve CD4 T cell that were then stimulated with soluble anti-CD3 in the presence of antigen presenting cells (APCs), or by direct stimulation with allogeneic APCs. In addition, we also used plate-bound anti-CD3 as a strategy to activate T cells in the absence of APCs. We also confirmed that virtually all human CD4 cells constitutively display CD6 on their surface (Supplementary Fig. 1b).

First, we investigated the impact of CD6-targeting with itolizumab following T cell stimulation in presence of APCs. We found that when T cell stimulation was provided with soluble anti-CD3 added to syngeneic irradiated peripheral blood mononuclear cells (PBMCs, Fig. 4a), or when allogeneic irradiated PBMCs were used as stimulators (Fig. 4b), itolizumab tended to reduce T cell proliferation, as assessed by CTV dilution, leading to a reduced number of T cells at the end of the culture. The figure shows data from seven independent experiments, each with triplicates. A statistically significant impact on proliferation was observed in approximately half the experiments, but when all data were pooled, the reduction in proliferation did not reach statistical significance. No significant impact on T cell survival, assessed as the percentage of live cells, was observed (Fig. 4a,b).

We also performed *in vitro* assays providing T cell stimulation in conditions where APCs were absent (Fig. 4c). Under those conditions T cell proliferation was not affected by itolizumab, as assessed by CTV dilution, with similar numbers of T cells recovered at the end of the cultures and with no significant impact on T cell viability.

We also found that anti-CD6 had a small impact on the activation of CD4 T cells, as upregulation of CD69 (used as a surrogate marker for T cell activation) was affected in presence of anti-CD6 (Supplementary Fig. 3).

Finally, we investigated whether CD6 targeting with Itolizumab would have a similar impact on T cell functional specialization as we have observed with murine antibodies targeting the same CD6 d1. We cultured sorted human naïve CD4 T cells in conditions favouring Treg or Th1 polarization. We found a dose-dependent reduction on the frequency of induced Treg cells when itolizumab was added to the cultures (Fig. 5a,b). There was no significant impact on T cell viability (Fig. 5c), and only a small, albeit significant, impairment in T cell proliferation at the highest doses of itolizumab (Fig. 5d,e). Conversely, addition of itolizumab to Th1-polarizing cultures led to a dose-dependent increase of Th1 cells emerging at the end of the culture (Fig. 5f,g). A similar impact on proliferation and survival was observed (Fig. 5h–j). Overall, we found a similar impact of CD6 d1-targeting in human and murine cells.

## 4. Discussion

Taken together, our data show that a monoclonal antibody targeting CD6 d1 can have a strong impact on the functional specialization of T cells, affecting different lineages in a distinct way: while increasing concentration of anti-CD6 d1 impair Treg differentiation, it favours Th1. Importantly, both mice and human T cells, presented similar results.

Ongoing clinical trials of itolizumab for the treatment of RA reported, in initial dose finding studies, that patients treated with the highest dose of itolizumab responded worse than patients treated with lower doses [35]. Such observations are consistent with the *in vivo* outcome of animals treated with anti-CD6 d1 at the time of EAE induction, where high doses of the therapeutic antibody appear to be less effective. These *in vivo* results contrast with the outcome of anti-CD4 administration. Indeed, neutralizing anti-CD4 antibodies (devoid of lytic function)

can prevent EAE at all tested doses – what has been interpreted as partial disruption of the immune synapse. Such protective effect was shown to be dependent on Treg induction [36], although the same approach of CD4-blockade can rely on Foxp3-independent tolerance for soluble proteins [37,38].

In order to address whether disruption of CD6-CD166 interactions could explain the effect of anti-CD6 d1, and if such disruption had an impact on Treg induction, we used soluble CD6 to directly disrupt CD6-CD166 binding. We found that soluble CD6 could recapitulate the dose-dependent outcome of anti-CD6 d1. However, contact-independent strong stimulation with anti-CD3/CD28 was not affected with addition of anti-CD6. Our observations are in line with a recent report that showed the absence of difference for *in vitro* Treg induction, between CD6<sup>-/-</sup> and CD6<sup>+/+</sup> T cells, when under contact-independent and supra-physiological conventional conditions [39]. It was previously reported that Treg cells from CD6-deficient mice have reduced suppressive function [39]. We did not assess the function of Treg cells polarized under the presence of anti-CD6. However, irrespective of a possible Treg functional impact of CD6-targeting we found a major quantitative effect on the number of polarized Treg cells.

It has been previously reported that the physical binding of antibodies to CD6 expressed in Jurkat cells, induces inhibitory signals decreasing cell proliferation [40]. We found that anti-CD6 d1 antibodies appear to directly modulate T cell activation independently of CD166 ligation, inferred from CD69 upregulation. These data suggest that the overall impact of anti-CD6 may combine blockade of CD6-CD166 interactions with a small direct effect on signalling.

It should be noted, however, that a putative interference on T cell activation signalling by anti-CD6 d1 cannot be major, but rather a subtle impact. Major interferences in T cell activation would be expected to result in changes in cell proliferation and possibly viability, if that was the case. Although in human studies we observed some response diversity regarding impact on T cell proliferation following T cell activation in presence of anti-CD6 d1, in both human and murine cells that trend is present (especially a delay in proliferation) but without reaching statistical significance. By contrast we observed a major impact on T cell polarization, restricted towards specific functional subsets. While Tregs and Th1 cells were very significantly affected by anti-CD6 d1, Th2 and Th17 were not. Such specificity towards distinct functional subsets are probably related to different levels of activation favouring alternative polarization phenotypes [41].

Other studies have also shown a protective effect of CD6 manipulation in EAE, as well as a suppression of Th1 and Th17 responses by anti-CD6 or CD6 gene deletion [19,42]. A direct comparison between the published reports is difficult given the diversity of experimental protocols, namely distinct genetic backgrounds, CD6-gene ablation *versus* antibody-targeting of CD6, different antibodies targeting distinct domains, and different dosages and treatment schedules. It should be stressed that distinct outcomes may be a consequence of different affinities, avidities and binding specificities of distinct anti-CD6 antibodies [43]. However, our observation highlights the importance of antibody dose in modulating T cell functional specialization. This observation is novel and may be important for other therapeutic targets, in particular molecules important for T cell activation.

Overall, our results show that therapeutic antibodies, such as anti-CD6 d1, may have paradoxical effects at different doses due to distinct impact on CD4 T cell functional specialization: while a low dose anti-CD6 d1 favours regulation, a higher dose may lead to opposite outcome by preventing Treg induction while favouring a Th1 fate. As such, dose selection is important, and the same compound may be therapeutically useful for different indications depending on its dose.

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### Author contributions

RFF designed the research, performed experiments, analysed data, and wrote the manuscript. AB, SCPA, RFS, CMG, TC, and JCO performed experiments and reviewed the manuscript. AMC, VGO, and KL designed the research and reviewed the manuscript. LG designed the research and wrote the manuscript.

### Declaration of Competing Interest

Dr. Graca reports grants from Fundação para a Ciência e Tecnologia, during the conduct of the study. All other authors have nothing to disclose.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2019.08.008>.

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