

## ORIGINAL ARTICLE

# A specific CD4 epitope bound by tregalizumab mediates activation of regulatory T cells by a unique signaling pathway

Bianca Helling<sup>1</sup>, Martin König<sup>1</sup>, Benjamin Dälken<sup>1</sup>, Andre Engling<sup>1</sup>, Wolfgang Krömer<sup>1</sup>, Katharina Heim<sup>1</sup>, Holger Wallmeier<sup>2</sup>, Jürgen Haas<sup>3</sup>, Brigitte Wildemann<sup>3</sup>, Brigitte Fritz<sup>3</sup>, Helmut Jonuleit<sup>4</sup>, Jan Kubach<sup>4</sup>, Theodor Dingermann<sup>5</sup>, Heinfried H Radeke<sup>6</sup>, Frank Osterroth<sup>1</sup>, Christoph Uherek<sup>1</sup>, Niklas Czeloth<sup>1,7</sup> and Jörg Schüttrumpf<sup>1,7</sup>

CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) represent a specialized subpopulation of T cells, which are essential for maintaining peripheral tolerance and preventing autoimmunity. The immunomodulatory effects of Tregs depend on their activation status. Here we show that, in contrast to conventional anti-CD4 monoclonal antibodies (mAbs), the humanized CD4-specific monoclonal antibody tregalizumab (BT-061) is able to selectively activate the suppressive properties of Tregs *in vitro*. BT-061 activates Tregs by binding to CD4 and activation of signaling downstream pathways. The specific functionality of BT-061 may be explained by the recognition of a unique, conformational epitope on domain 2 of the CD4 molecule that is not recognized by other anti-CD4 mAbs. We found that, due to this special epitope binding, BT-061 induces a unique phosphorylation of T-cell receptor complex-associated signaling molecules. This is sufficient to activate the function of Tregs without activating effector T cells. Furthermore, BT-061 does not induce the release of pro-inflammatory cytokines. These results demonstrate that BT-061 stimulation via the CD4 receptor is able to induce T-cell receptor-independent activation of Tregs. Selective activation of Tregs via CD4 is a promising approach for the treatment of autoimmune diseases where insufficient Treg activity has been described. Clinical investigation of this new approach is currently ongoing.

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## INTRODUCTION

Regulatory T cells (Tregs) represent a subpopulation of CD4<sup>+</sup> T cells, which downregulate the immune response to self and non-self antigens.<sup>1</sup> Tregs are essential for the maintenance of peripheral tolerance and for the prevention of autoimmunity by suppressing the activation of other immune cells and by modulating the activity of activated effector T cells (Teffs).<sup>2,3</sup> The absence or dysfunction of Tregs can lead to autoimmunity and allergies.<sup>4</sup> The experimental restoration of defective or absent Treg cell numbers and/or function therefore represents an approach for the treatment of autoimmune diseases.<sup>5,6</sup> Moreover, manipulation of Treg activity may have a role in the prevention of organ transplant rejection.<sup>7</sup> The IL-2 receptor alpha chain (CD25) and the intracellular transcription factor FOXP3 serve as phenotypic markers for Treg identification, with FOXP3 being known as the master regulator in the development and function of Tregs.<sup>5,6</sup> Although Tregs are anergic *in vitro*, when activated under physiologic conditions they are able to suppress the proliferation and cytokine production of bystander Teffs<sup>7,8</sup> through both cell-to-cell contact, and

the secretion of immunosuppressive cytokines.<sup>9</sup> Tregs require activation via the T-cell receptor (TCR) complex to become suppressive.<sup>6</sup> However, Tregs remain unable to proliferate in response to TCR-mediated stimulation.<sup>10</sup>

TCR cross-linking by anti-CD3 or anti-CD28 monoclonal antibodies (mAbs) can induce Treg activation.<sup>6,11</sup> However, these mAbs also induce the activation of Teffs and the secretion of pro-inflammatory cytokines, most notably TNF- $\alpha$  and IFN- $\gamma$ . This can cause a variety of symptoms including capillary leakage, leukocyte sequestration and flu-like symptoms.<sup>12,13</sup>

As recently shown, the human immunodeficiency virus-1 (HIV-1) envelope protein gp120 can also mediate activation of Tregs by binding and signaling through the CD4 molecule, demonstrating that CD4-mediated manipulation of Tregs can induce tolerance.<sup>14</sup>

Anti-CD4 mAbs are used for the treatment of autoimmune diseases. In model systems, they are able to induce tolerance,<sup>15,16</sup> and they mediate immunomodulatory effects through different mechanisms.<sup>17</sup> It is known that CD4 binding by anti-CD4 mAbs can lead to the

<sup>1</sup>Biotest AG, Dreieich, Germany; <sup>2</sup>Condor Scientific Computing and Consulting, Sulzbach, Germany; <sup>3</sup>Division of Molecular Neuroimmunology, Department of Neurology, University Hospital Heidelberg, Heidelberg, Germany; <sup>4</sup>Department of Dermatology, Johannes Gutenberg University, Mainz, Germany; <sup>5</sup>Institute of Pharmaceutical Biology, Goethe University Frankfurt, Frankfurt, Germany and <sup>6</sup>Institute of Pharmacology and Toxicology, Clinic of the Goethe University, Frankfurt, Germany

<sup>7</sup>These authors contributed equally to this work.

Correspondence: Dr B Dälken, Project Management, Biotest AG, Landsteinerstraße 5, Dreieich 63303, Germany.

E-mail: benjamin\_daelken@biotest.de

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induction of anergy by modulating antigenic stimulation through the TCR.<sup>18–20</sup> Anti-CD4 mAbs can also induce CD4 downmodulation caused by internalization or stripping of the CD4 receptor from the cell surface. This receptor modulation reduces overall CD4/MHC class II interactions, resulting in reduced T-cell activation.<sup>21–23</sup> By antigenic modulation, anti-CD4 mAbs induce differentiation of naive T cells into FOXP3<sup>+</sup> Tregs, which control autoimmunity through TGF- $\beta$  and IL-10 release.<sup>24–26</sup> Furthermore, anti-CD4 mAbs can induce depletion of CD4<sup>+</sup> T cells by apoptosis, antibody-dependent cytotoxicity or complement-mediated lysis of mAb-coated CD4<sup>+</sup> T cells.<sup>27,28</sup> However, depleting mAbs increase the susceptibility to infections, as they mediate immunosuppression rather than immunomodulation. Moreover, CD4<sup>+</sup> T-cell depletion may not be essential for efficacy, and clinical effects of non-depleting mAbs have been reported.<sup>29,30</sup> To date, the use of neither non-depleting nor depleting anti-CD4 mAbs has been less successful in clinical trials, as they inactivate and/or remove inflammatory CD4<sup>+</sup> T cells, rather than reprogram the overactive immune system by Treg activation.<sup>17,23,31</sup>

Tregalizumab (BT-061) is the first humanized anti-CD4 mAb that selectively induces Treg activation. In contrast to anti-CD3 or anti-CD28 mAbs, BT-061 selectively activates the suppressive properties of the Tregs without activating Teffs. In addition, BT-061 does not induce antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity, and is therefore non-depleting in humans.<sup>32</sup>

## RESULTS

### The humanized anti-CD4 mAb BT-061 exhibits the unique functionality of Treg activation that is not shown by other anti-CD4 mAbs

As previously demonstrated, the agonistic signal mediated by the binding of BT-061 to CD4 is necessary and sufficient to activate the suppressive features of Tregs.<sup>32–34</sup> In order to assess whether this property of BT-061 is unique among anti-CD4 mAbs, we analyzed the suppressive activity of freshly isolated Tregs (CD4<sup>+</sup>CD25<sup>+</sup>) treated with BT-061, seven other conventional anti-CD4 mAbs and the anti-CD3 antibody OKT-3. As it is known that the activation of the suppressive properties of Tregs requires stimulation via the TCR complex,<sup>6</sup> the incubation of Tregs with OKT-3 is considered to be an optimal stimulus to induce the suppressive activity of Tregs. However, in contrast to BT-061, OKT-3 activates both Tregs and Teffs.<sup>6</sup> OKT-3-treated Tregs reduced the proliferation of a mixed lymphocyte reaction to  $55.3 \pm 13.1\%$  of control, which was significantly greater ( $P=0.0033$ ) than that induced by untreated Tregs ( $84.5 \pm 13.2\%$ ). BT-061-treated Tregs also strongly reduced the proliferation of a mixed lymphocyte reaction ( $64 \pm 11.1\%$ ), although this was slightly lower than OKT-3 (Figure 1a). Furthermore, BT-061-treated Tregs reduced the proliferation of a mixed lymphocyte reaction in a dose-dependent manner (Figure 1b). In contrast to BT-061, none of the other analyzed anti-CD4 mAbs showed significant activation of Tregs. Hence, BT-061 was the only anti-CD4 mAb that acted agonistically via CD4 to induce functional activation of Tregs. Notably, Tregs exhibited no significant proliferation themselves when treated with BT-061 alone and thus, remained anergic (Figure 1c). Typical markers of Treg activation (CD25, CD39, CD44, CD45RO, CD69, CD73, CD103, CD127, CD197, CTLA-4, GARP, LAG-3 and HLA-DR) remained unmodulated after BT-061 treatment. However, BT-061 induced the upregulation of latency-associated peptide (LAP) on Tregs (Supplementary Figure 1a). Moreover, BT-061 did not activate Teffs by modulation of activation markers (CD25, CD44, CD45RO, CD69, CD127 and HLA-DR) (Supplementary Figure 1b). These results were further supported by experiments, demonstrating that BT-061, like

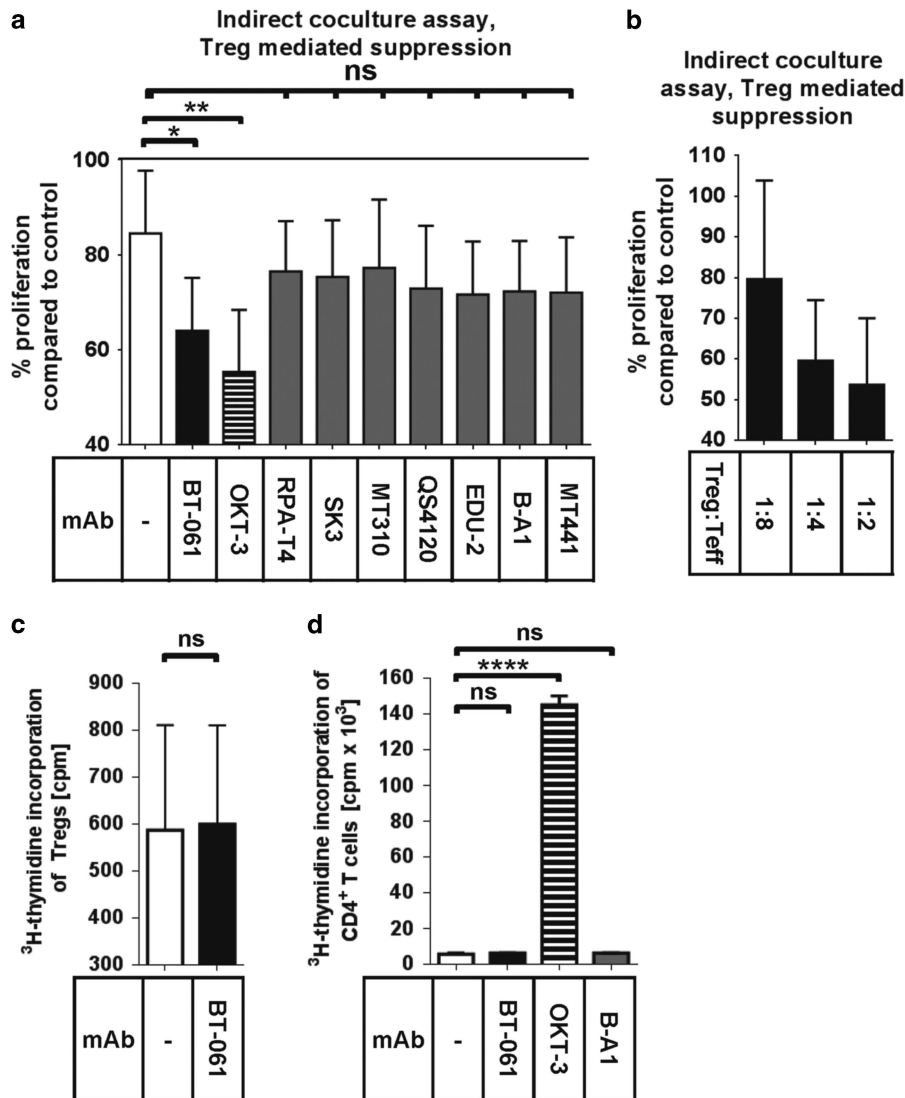
B-A1 (control anti-CD4 mAb), did not induce proliferation of Teffs in the presence of autologous peripheral blood mononuclear cells (PBMCs), in contrast to OKT-3 (Figure 1d). In summary, BT-061 activated the suppressive properties of Tregs without stimulating Teffs.

### The recognition of a unique, conformational epitope on domain 2 of CD4 discriminates BT-061 binding from other anti-CD4 mAbs

BT-061 is the only tested anti-CD4 mAb that activated the suppressive properties of Tregs, therefore the domain specificity of BT-061 and other anti-CD4 mAbs was examined. The CD4 molecule has four extracellular immunoglobulin domains (D<sub>1</sub>–D<sub>4</sub>), a single transmembrane region and a short C-terminal cytoplasmic tail.<sup>35</sup> OKT-3 does not compete for the binding of CD4, it binds the  $\epsilon$  component of the CD3 signal-transduction complex,<sup>36</sup> hence it was used as a negative control. The anti-CD4 mAb OKT-4 binds to D<sub>3</sub> of CD4.<sup>37</sup> BT-061 and the other anti-CD4 mAbs did not compete with OKT-4 for binding to CD4 (Figure 2a). The anti-CD4 mAb SK3 binds to D<sub>1</sub>.<sup>37</sup> BT-061, MT441 and OKT-4 did not compete with SK3 for binding to D<sub>1</sub> (Figure 2b). However, MT310, QS4120, B-A1 and EDU-2 all competed with SK3, indicating that they also bind to D<sub>1</sub>.<sup>38,39</sup> RPA-T4 demonstrated partial competition with SK3 ( $69 \pm 7.1\%$  binding), a finding supported by published literature indicating binding to D<sub>1</sub>.<sup>40</sup> In conclusion, all analyzed anti-CD4 mAbs bind to D<sub>1</sub>, except OKT-4, MT441 and BT-061. Finally, competition assays with BT-061 revealed that MT441, but none of the other anti-CD4 mAbs, competed with BT-061 for binding to CD4 (Figure 2c). As previously published, MT441 binds to D<sub>2</sub> of CD4.<sup>41</sup> We thus assumed that, although no Treg stimulating activity was shown for MT441 (Figure 1a), BT-061 may also bind to D<sub>2</sub>.

As a result of these findings, we investigated whether the Treg activating potential of BT-061 may be mediated by binding of a special epitope on D<sub>2</sub>. An analysis of the 2.9 Å-resolution crystal structure of BT-061 in complex with CD4 revealed that BT-061 binds to a non-linear epitope on D<sub>2</sub>. BT-061 binds to the amino acids 148–154, 164–168, 185, 187, 189–190 and 192 (Figure 2d). The CD4 amino acids 26, 156, 159, 161 and 192 remain available for potential additional interactions with charge complementary amino acids in the light and heavy chain of BT-061 (Supplementary Figure 2b). The crystallographic analysis revealed that BT-061 binds to a different epitope on D<sub>2</sub> than MT441 (amino acids 148–150, 164–166) (Figure 2d) and other anti-CD4 mAbs tested (Table 1). In addition, it could be deduced that BT-061 binds to the opposite side of the extracellular part of CD4 than the MHCII molecule, which binds to D<sub>1</sub> of CD4.<sup>42</sup> Hence, BT-061 does not sterically interfere with its binding (Figure 2f).

BT-061 closely interacts with CD4 by engaging both the light and heavy chain. Only two of the three complementarity-determining regions of the light and heavy chain of BT-061 are involved in CD4 binding (Supplementary Figure 2a and c). The interactions between CD4 and BT-061 are mainly hydrogen bonds, complemented by van der Waals contacts and polar interactions (Supplementary Figure 2d, 2e). There are three binding pockets on the CD4 surface that contribute significantly to the binding of BT-061. First, Tyr105 on BT-061 heavy chain is essential for the interaction with CD4, as its side chain fits perfectly into a pocket on the surface of CD4. On the CD4 molecule, the amino acids S152, V153, Q154, Q164, G165, T185, V186, L187 and K192 build polar interactions and hydrogen bonds to Tyr105 and form the corresponding binding pocket. Second, Tyr34 on BT-061 light chain is a further important residue for the interaction with CD4. The CD4 amino acids S150, P151, S152 and K167 form a binding pocket for Tyr34 via hydrogen bonds. Finally, BT-061 shows a number of intra-molecular salt bridges. One of them, formed between



**Figure 1** BT-061 is the only anti-CD4 antibody that activates Tregs. (a) Tregs were isolated and pre-incubated for 16–24 h at 37 °C with plate-bound BT-061, OKT-3 other anti-CD4 mAbs ( $1 \mu\text{g ml}^{-1}$ ), or medium and transferred to a mixed lymphocyte reaction using allogeneic, CD3-depleted, irradiated PBMCs ( $2 \times 10^5$  cells per well) to activate the proliferation of Teffs ( $10^5$  cells per well, Treg:Teff: 1:4). The proliferation of Teffs with pre-treated Tregs is shown compared with the proliferation without Tregs ( $n=6$ ). (b) Tregs were isolated and pre-incubated for 48 h at 37 °C with plate-bound BT-061 ( $1 \mu\text{g ml}^{-1}$ ) and transferred to a mixed lymphocyte reaction using allogeneic, CD3-depleted, irradiated PBMCs ( $2 \times 10^5$  cells per well) to activate the proliferation of Teffs ( $10^5$  cells per well). The proliferation of Teffs with pre-treated Tregs is shown compared with the proliferation without Tregs ( $n=3$ ). (c) Tregs were isolated and pre-incubated for 48 h at 37 °C with plate-bound BT-061 ( $1 \mu\text{g ml}^{-1}$ ) or medium and transferred to allogeneic, CD3-depleted, irradiated PBMCs ( $2 \times 10^5$  cells per well). The  $^3\text{H}$ -thymidine incorporation of Tregs is shown ( $n=6$ ). (d)  $\text{CD4}^+$  T cells ( $5 \times 10^4$  cells per well) and allogeneic, CD3-depleted PBMCs ( $1.5 \times 10^5$  cells per well) were incubated for 3 days at 37 °C with BT-061 ( $1 \mu\text{g ml}^{-1}$ ), B-A1 ( $1 \mu\text{g ml}^{-1}$ ), OKT-3 ( $0.5 \mu\text{g ml}^{-1}$ ) or medium. The  $^3\text{H}$ -thymidine incorporation of  $\text{CD4}^+$  T cells is shown ( $n=3$ ). Data are represented as mean+s.d.

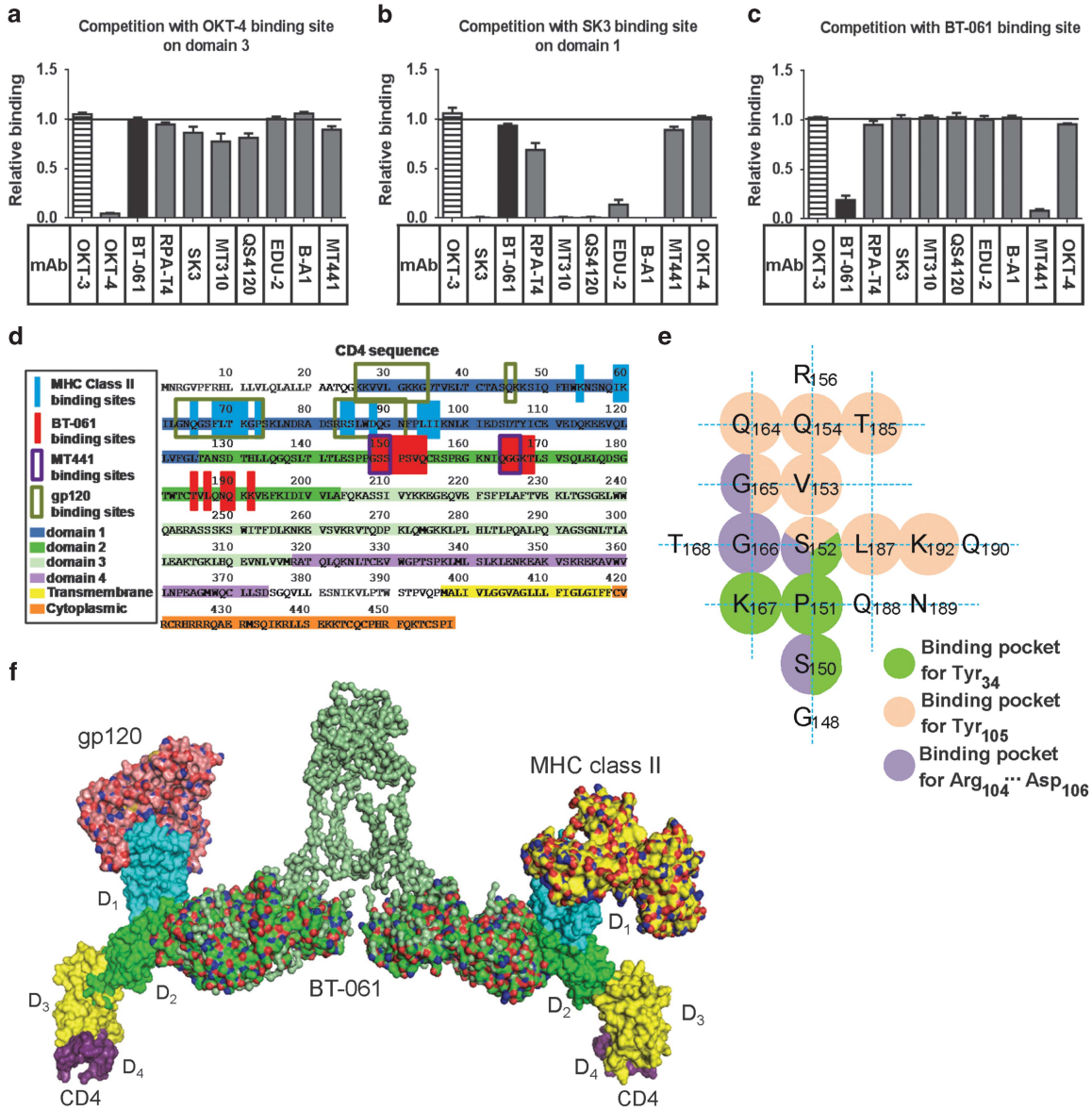
Arg104 and Asp106 of the BT-061 heavy chain, confers polar interactions with a pocket on CD4 formed by the amino acids S150, S152, G165 and G166 (Figure 2e).

The contributions of the individual amino acids to the binding of BT-061 to CD4 was confirmed by *in vitro* experiments, demonstrating that single-point mutations strongly decreased CD4 binding, biological activity (potency to downmodulate CD4), and Treg-activating properties (Supplementary Figure 3). Single mutants A63G, R33K and L98I as well as double mutants R33K+A63G, L98I/R33K and A55G/R33K retained activity, although the mutants G33A/R33K, Y105W and S101T strongly reduced binding of CD4, CD4 downmodulation and Treg activation. Binding of the mutants Y105W and S101T was almost completely prohibited. Thus, no affinity could be determined. In

conclusion, the residues surrounding Arg104, Tyr105 and Asp106 in the heavy chain and Tyr34 in the light chain of BT-061 are crucial for binding. These results indicate that BT-061 recognizes a unique conformational epitope on  $\text{D}_2$  of the CD4 molecule that is not recognized by the other anti-CD4 mAbs analyzed. We suggest that binding of this unique epitope is critical for the induction of Treg-activating capacities of BT-061.

#### An incomplete engagement of the TCR pathway differentiates BT-061 from other anti-CD4 mAbs

As BT-061 binds to a different epitope than other anti-CD4 mAbs, and it is known that the effects of anti-CD4 mAbs on T-cell signal-transduction pathways vary depending on the CD4 epitope



**Figure 2** BT-061 recognizes a unique, conformational epitope on domain 2 of CD4. Isolated CD4<sup>+</sup> T cells (10<sup>5</sup> cells per well) were incubated for 2 h at 4 °C in the presence of the indicated mAbs (1 μg ml<sup>-1</sup>). The binding of (a) OKT-4-Alexa Fluor 647, (b) SK3-APC or (c) BT-061-FITC was measured using flow cytometry. The relative binding of the indicated mAbs is shown compared with the untreated control. Data are represented as mean±s.d. (n=2, n=6 for BT-061). (d) The peptide sequence and domain structure of human CD4 as well as the binding regions of MHCII, BT-061, MT441 and gp120 are shown. (e) Amino acids forming the binding pocket for Tyr<sub>34</sub>, Arg<sub>104</sub>, Tyr<sub>105</sub> and Asp<sub>106</sub>. (f) Representation of the CD4/BT-061 crystal structure superimposed with a crystal structure of a CD4/MHCII and a CD4/gp120 complex. BT-061: pale green with elements: C: green, N: blue, O: red; D<sub>1</sub> of CD4: light blue, D<sub>2</sub> of CD4: green, D<sub>3</sub> of CD4: yellow, D<sub>4</sub> of CD4: purple; MHCII elements: C: yellow, N: blue, O: red; gp120 elements: C: rose, N: blue, O: red.

recognized,<sup>43</sup> we analyzed whether BT-061 also induces unique signaling that differs from conventional anti-CD4 mAbs. BT-061 induces downstream signals, which diverge in Teffs and Tregs, resulting in Treg-specific Ca<sup>2+</sup> flux, TGF-β secretion and increases in cAMP (Czeloth N *et al.*, 2014, manuscript submitted).<sup>34</sup> Nonetheless, after treatment with BT-061, we found no significant differences in the phosphorylation of 16 analyzed intracellular signaling molecules in Tregs and Teffs (Figure 3a). Therefore, we focused on total CD4<sup>+</sup> T cells to further analyze signaling effects. As the signaling induced by CD3-specific antibodies evokes proliferation, cytokine secretion and the activation of Teffs,<sup>12</sup> we analyzed the signaling

induced by the anti-CD4 mAbs in relation to the signaling induced by OKT-3. During our studies we identified two groups of anti-CD4 mAbs according to the signaling observed. The first group of anti-CD4 mAbs-including RPA-T4, SK3, MT310 and QS4120 (represented by RPA-T4 in Figures 3b, 3c), and the second group of anti-CD4 mAbs-including B-A1, EDU-2, MT441 and OKT-4 (represented by B-A1 in Figures 3b and c), induced a similar phosphorylation-intensity within their groups. BT-061-induced signaling was unique when compared with OKT-3 and the other anti-CD4 mAbs tested (Figure 3b and Supplementary Figure 5). BT-061-induced phosphorylation of Lck, PLC-γ and SLP-76 was similar to OKT-3, EDU-2, B-A1, MT441 and

**Table 1 Domain usage of anti-CD4 mAbs**

Isotype Domain on CD4	SK3 (Leu3a)									
	Tregalizumab (BT-061)		RPA-T4	MT310	QS4120	EDU-2	B-A1	MT441	OKT-4	Ibalizumab
Epitope	hIgG <sub>1</sub> D <sub>2</sub>	mIgG <sub>1</sub> D <sub>1</sub>	mIgG <sub>1</sub> D <sub>1</sub>	mIgG <sub>1</sub> D <sub>1</sub>	mIgG <sub>2a</sub> D <sub>1</sub>	mIgG <sub>2a</sub> D <sub>1</sub>	mIgG <sub>2b</sub> D <sub>2</sub>	mIgG <sub>2b</sub> D <sub>3</sub>	hIgG <sub>4</sub> D <sub>1/D<sub>2</sub></sub>	D <sub>1</sub>
Amino acids	148–154, 164–168, 185–192	148–150, 164–166	148–150, 164–166	148–150, 164–166	148–150, 164–166	148–150, 164–166	148–150, 164–166	148–150, 164–166	102, 104, 121, 146, 147, 188	26–34, 45, 63–73, 83–91
Source	G148, S149, S150, P151, S152, V153, Q154, Q164, G165, G166, K167, T168, T185, L187, N189, Q190, K192	G148, S149, S150, Q164, G165, G166	G148, S149, S150, Q164, G165, G166	G148, S149, S150, Q164, G165, G166	G148, S149, S150, Q164, G165, G166	G148, S149, S150, Q164, G165, G166	G148, S149, S150, Q164, G165, G166	G148, S149, S150, Q164, G165, G166	E102, S104, L121, P146, P147, Q188	K26, K27, V28, V29, L30, G31, K32, K33, G34, Q45, G63, N64, Q65, G66, S67, F68, C69, T70, K71, G72, P73, R83, R84, S85, L86, W87, D88, Q89, G90, N91
	Crystal structure	Crystal structure	Crystal structure	Crystal structure	Crystal structure	Crystal structure	Crystal structure	Crystal structure	Crystal structure	Crystal structure
	40	37	39	38	Figure 2	Figure 2	41	37	41	50

Abbreviations: mIgG, mouse immunoglobulin G; hIgG, human immunoglobulin G. Table showing the binding epitope of the different anti-CD4 mAbs and gp120 on the CD4 molecule.

OKT-4, but was reduced when compared with RPA-T4, SK3, MT310 and QS4120. In addition, BT-061-induced phosphorylation of ZAP70, Pyk2, MEK, LAT, SHP-2 and MAPK was reduced when compared with all other anti-CD4 mAbs and OKT-3. Finally, unlike OKT-3 and the other anti-CD4 mAbs, BT-061 did not induce phosphorylation of PKC, ERK, Itk, IKK, JNK, Akt and NF- $\kappa$ B. Moreover, we observed that the phosphorylation induced by BT-061 was reduced to baseline values after 60 min, whereas that induced by OKT-3 and the other analyzed anti-CD4 mAbs had a longer duration and remained above baseline values at 60 min (Figure 3c and Supplementary Figure 4). Considering the observed unique phosphorylation-intensity and -duration, BT-061-induced signaling is entirely different compared with that of the other anti-CD4 mAbs and OKT-3 (Figure 4). This might lead to downstream effects in Tregs, resulting in Ca<sup>2+</sup> flux, TGF- $\beta$  secretion and cAMP increase, which result in BT-061-mediated selective activation of Tregs.

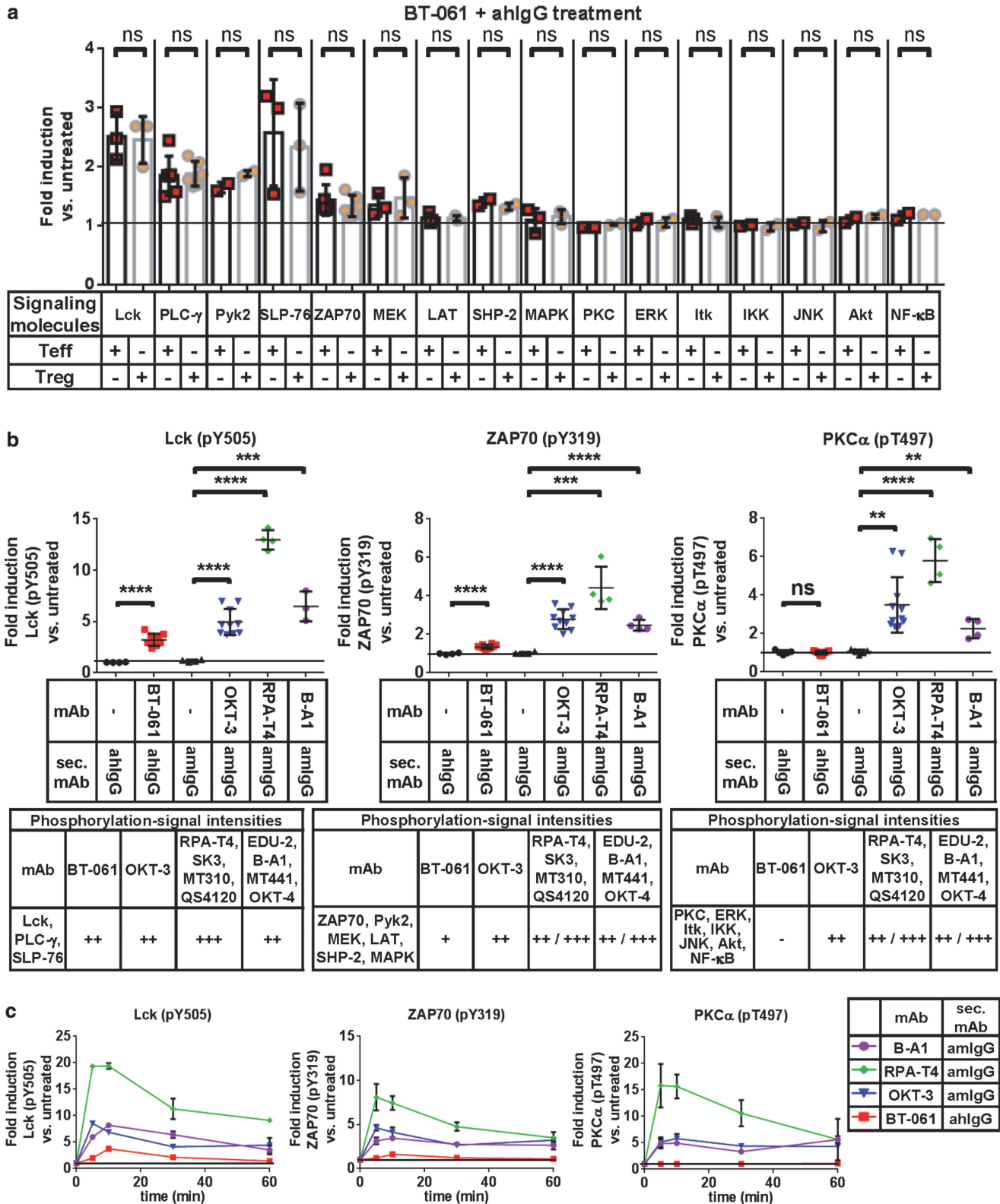
### BT-061, RPA-T4, QS4120, B-A1, MT441 and OKT-4 do not induce pro-inflammatory cytokine release

As OKT-3 and anti-CD28 mAbs induce secretion of pro-inflammatory cytokines,<sup>12,13,44</sup> we analyzed the cytokine release induced by BT-061. Compared with other anti-CD4 mAbs, BT-061 did not induce pro-inflammatory cytokines (Figure 5). As expected, OKT-3 induced the release of GM-CSF, IFN- $\gamma$ , TNF- $\alpha$ , IL-2 (only after 20 h), IL-5 and IL-10. SK3, MT310 and EDU-2 also caused the induction of GM-CSF, TNF- $\alpha$  (only after 20 h) and IL-10. By contrast, RPA-T4, QS4120, B-A1, MT441 and OKT-4 did not induce pro-inflammatory cytokine release. This indicates that the unique phosphorylation of signaling molecules induced by BT-061 does not stimulate Teffs.

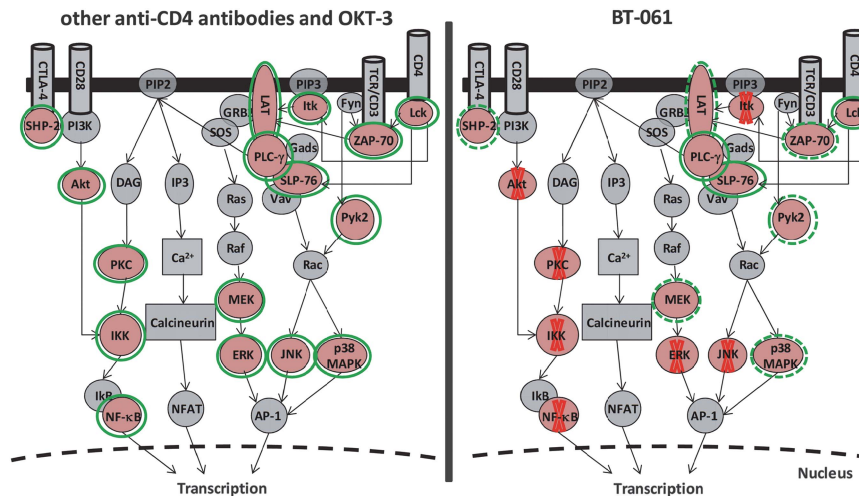
### DISCUSSION

Here we report the specific and unique mechanism by which the humanized CD4-specific mAb tregalizumab (BT-061) activates the suppressive properties of Tregs without stimulating Teffs. In this study, we demonstrated that BT-061 activates Tregs via CD4 by a mechanism not shared by other anti-CD4 mAbs. Although Tregs cultured in medium were able to suppress a mixed lymphocyte reaction suboptimally, as also shown by Haas *et al.*,<sup>45</sup> BT-061 significantly enhanced the suppressive properties of the Tregs, an effect not observed with the other anti-CD4 mAbs tested. As previously reported, treatment with the anti-CD4 mAb OKT-4 does not result in suppression of CD8<sup>+</sup> T-cell proliferation via Tregs.<sup>34</sup> BT-061 did not induce proliferation of Teffs or Tregs. In addition, BT-061 did not stimulate Teffs to modulate activation markers or to secrete pro-inflammatory cytokines. Although typical Treg activation markers remained unmodulated after BT-061 treatment, upregulation of LAP on Tregs was observed. LAP is known as the N-terminal region of pro-TGF- $\beta$  and contributes to the suppressor activity of Tregs.<sup>46,47</sup> Pro-TGF- $\beta$  is intracellularly processed by a furin-like pro-protein convertase to produce the latent TGF- $\beta$  complex. In this complex, the N-terminal region of pro-TGF- $\beta$  (LAP) associates non-covalently with the C-terminal region of pro-TGF- $\beta$ , which constitutes mature TGF- $\beta$ .<sup>48</sup> TGF- $\beta$  must be released from this complex to elicit its biological activity.<sup>49</sup> The BT-061 induced-upregulation of LAP on Tregs is in agreement with the finding that BT-061-induced Treg-specific TGF- $\beta$  secretion (Czeloth N. *et al.*, 2014, manuscript submitted), which may contribute to the mechanism of Treg-mediated inhibition.

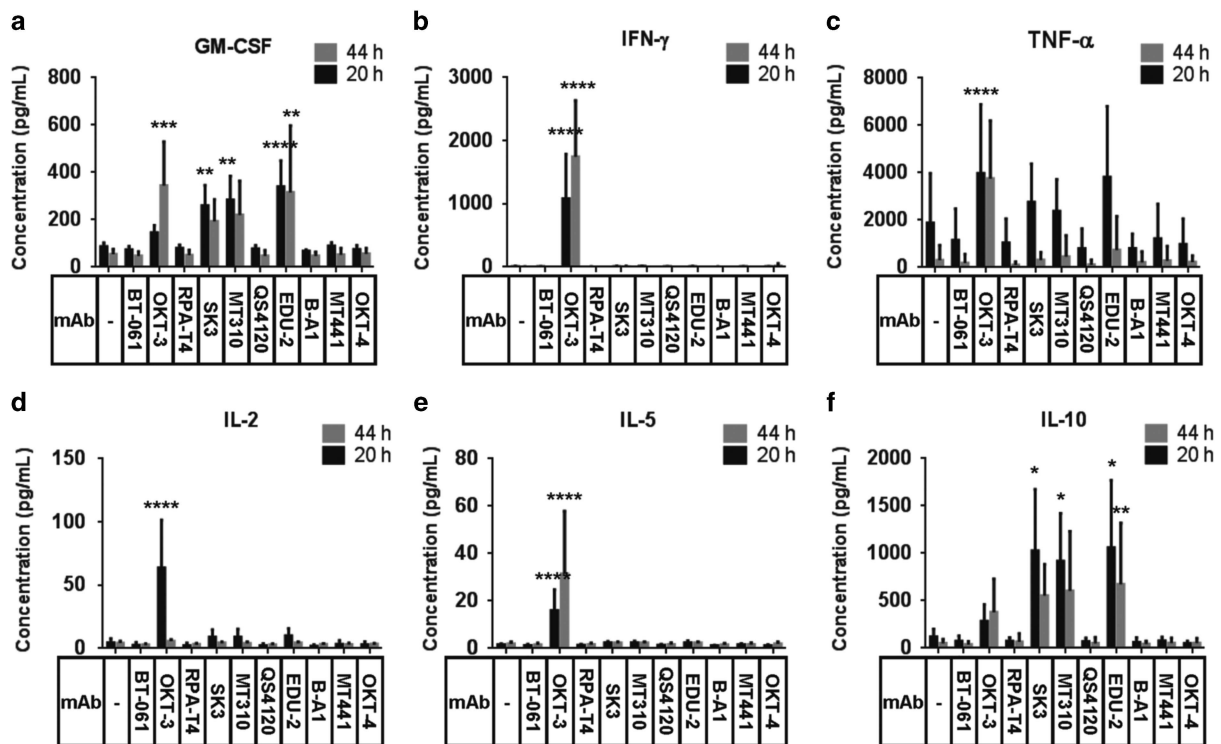
We assume that the ability of BT-061 to selectively activate Tregs is mediated by binding of a special epitope on CD4. Using crystal



**Figure 3** BT-061 induces unique phosphorylation of signaling molecules compared with other anti-CD4 mAbs. (a) Teffs or Tregs ( $10^5$  cells per well) were pre-incubated for 30 min at room temperature with BT-061 ( $1 \mu\text{g ml}^{-1}$ ). After cross-linking by anti-human IgG (ahlgG) ( $20 \mu\text{g ml}^{-1}$ ) for 10 min at  $37^\circ\text{C}$  phosphorylation of different signaling molecules was measured by intracellular staining and flow cytometry ( $n=2-6$ ). (b)  $\text{CD4}^+$  T cells ( $10^5$  cells per well) were pre-incubated with BT-061, OKT-3 or other anti-CD4 mAbs and cross-linked by either ahlgG ( $20 \mu\text{g ml}^{-1}$ ) or anti-mouse IgG (amIgG) ( $10 \mu\text{g ml}^{-1}$ ) for 10 min ( $n=3-10$ ). (c) The  $\text{CD4}^+$  T cells were stimulated for 5, 10, 30 or 60 min with the secondary antibody prior to the intracellular staining. The induction of the phosphorylation of the indicated molecules is shown compared with the untreated control ( $n=2$ ). Data are represented as mean  $\pm$  s.d.



**Figure 4** An incomplete engagement of the TCR pathway differentiates BT-061 from other anti-CD4 mAbs. The major signal-transduction pathways downstream of the TCR and the molecules induced by other anti-CD4 mAbs and OKT-3 or BT-061 are shown. A green circle indicates signal induction, a dashed green circle displays a reduced signal induction and a red cross demonstrates no signal induction. The molecules marked gray were not analyzed.



**Figure 5** BT-061 does not induce pro-inflammatory cytokine secretion. (a–f) PBMCs ( $4 \times 10^5$  cells per well) were incubated for 20 h and 44 h at  $37^\circ\text{C}$  with BT-061, OKT-3, other anti-CD4 mAbs ( $2 \mu\text{g ml}^{-1}$ ) or medium. After the indicated stimulation, cell-free supernatant was collected and analyzed for pro-inflammatory cytokine induction. Data are represented as mean+s.d. (20 h:  $n=3$ , 44 h:  $n=6$ ). The  $P$ -value refers to the untreated control:  $*P<0.05$ ,  $**P<0.01$ ,  $***P<0.005$ ,  $****P<0.0001$ .

structure analysis, we demonstrated that BT-061 recognizes a unique conformational epitope on  $D_2$  of the CD4 molecule (amino acids 148–154, 164–168, 185, 187, 189–190 and 192) that is not recognized by the other anti-CD4 mAbs analyzed. The HIV-1 envelope protein gp120 can also mediate activation of Tregs, supporting CD4-mediated manipulation of Tregs for tolerance induction.<sup>14</sup> However, gp120

binds to the  $D_1$  of CD4 (amino acids 26–34, 45, 63–73 and 83–91) (Figures 2d and f)<sup>50</sup> and thus, to a different epitope on the CD4 molecule than BT-061. Hence, we suggest that the Treg activation could be related not only to one particular CD4 epitope. In addition, BT-061 and gp120 both induce a Treg-specific cAMP increase,<sup>14,34</sup> a key event of Treg activation and Treg-mediated suppression,<sup>51,52</sup>

confirming the assumption that upregulation of cAMP is a crucial Treg activating event.

The anti-CD4 mAbs RPA-T4, SK3, MT310 and QS4120 also bind to D<sub>1</sub> of CD4, thereby inhibiting gp120 binding to CD4.<sup>37–40</sup> MAb reactive with the D<sub>1</sub> of CD4 compete with gp120 binding and, thereby, block virus infectivity.<sup>37</sup> As EDU-2 and B-A1 competed with SK3 binding to D<sub>1</sub>, we assume that they also may inhibit gp120 binding. In contrast to the other anti-CD4 mAbs, OKT-4 does not inhibit gp120 binding as it binds to D<sub>3</sub>.<sup>37</sup> Although the humanized anti-CD4 mAb ibalizumab (amino acids 102, 104, 121, 146–147 and 188) and MT441 bind to D<sub>2</sub>, they also block HIV-1 infection by inhibiting a post-binding step required for viral entry.<sup>41</sup> MT441 is less potent in blocking HIV-1 infection compared with ibalizumab, which may be the result of MT441, recognizing an epitope that is further down on D<sub>2</sub> compared with ibalizumab. This may result in less interference with some critical steps in viral entry.<sup>41</sup> As BT-061 binds to an epitope even further down on D<sub>2</sub> compared with MT441, the effect of BT-061 on gp120 binding should be studied in the future.

MAbs which bind to D<sub>1</sub> (RPA-T4, SK3, MT310, QS4120, EDU-2 and B-A1) interfere with the binding of MHCII and so block T-cell activation, possibly leading to the induction of anergy. RPA-T4 has been shown previously to inhibit Teff proliferation independent of Tregs through CD4 blockade.<sup>53</sup> Gp120 also inhibits T-cell activation by MHCII competition.<sup>54</sup> In contrast, the crystallization of BT-061 and CD4 showed that the binding sites of BT-061 and the MHCII molecule lie on opposite sides of the extracellular part of CD4, excluding steric interference. Hence, BT-061 does not inhibit T-cell activation by antagonism of CD4/MHCII interactions.

Tregs show several properties that suggest their intracellular signaling may differ from that of Teffs. For example, Tregs fail to proliferate on their own or to produce IL-2 as well as pro-inflammatory cytokines in response to TCR stimulation.<sup>55</sup> As BT-061 selectively activates Tregs, we hypothesized that BT-061 induces a unique signaling in Tregs compared with Teffs. However, after treatment with BT-061, we found no significant differences concerning the phosphorylation of 16 signaling molecules in Tregs and Teffs. In comparison with all other anti-CD4 mAbs and OKT-3, the signaling cascade used by BT-061 resulted in a reduced and transient phosphorylation of ZAP70, Pyk2, MEK, LAT, SHP-2 and MAPK. In addition, the signaling cascade used by BT-061 did not involve PKC, ERK, Itk, IKK, JNK, Akt or NF- $\kappa$ B in either Tregs or Teffs. OKT-3, B-A1, EDU-2, MT441 and OKT-4 induce comparable signaling events, but only OKT-3 can activate Tregs. Thus, the integration of signaling events into functional responses is complex. These findings support the notion that the interaction with different epitopes on the CD4 molecule can have different outcomes on T-cell signal-transduction pathways.<sup>43</sup> BT-061 binds to a different epitope and induces unique signaling compared with other anti-CD4 mAbs. We suggest that the unique phosphorylation-intensity and -duration of signaling molecules result in the selective activation of the suppressive function of Tregs without stimulating Teffs mediated by BT-061. Tregs have a lower activation threshold than Teffs,<sup>56</sup> which may explain why Tregs can be sufficiently activated by suboptimal TCR signaling in contrast to Teffs.

Although Treg activation can also be induced by stimulation with the anti-CD3 mAb OKT-3 or anti-CD28 mAbs,<sup>6,11</sup> these mAbs induce the activation of Teffs and the secretion of pro-inflammatory cytokines.<sup>12,13</sup> After treatment with BT-061, no such increase in pro-inflammatory cytokines was observed. Hence, the unique phosphorylation of signaling molecules induced by BT-061 does not correlate with pro-inflammatory cytokine induction. Although the

anti-CD4 mAbs RPA-T4, QS4120, B-A1, MT441 and OKT-4 induced phosphorylation of signaling molecules observed for OKT-3, they also did not induce the release of pro-inflammatory cytokines. Furthermore, although phosphorylation of signaling molecules induced by EDU-2 is reduced compared with SK3 and MT310, the cytokine release induced by the three anti-CD4 mAbs is similar. Thus, cytokine release does not correlate with the phosphorylation-intensity. The reasons underlying these differences will be an interesting issue of further studies.

In conclusion, the data presented herein add detail to the molecular mechanisms underlying BT-061-mediated Treg activation. We demonstrated that engagement of the epitope on the domain 2 of CD4 recognized by BT-061 induces unique phosphorylation of signaling molecules in CD4<sup>+</sup> T cells compared with other anti-CD4 mAbs. This unique signaling is sufficient to activate Tregs functionally without also stimulating Teffs. Therefore, the mode of action of BT-061 represents a potentially novel way to treat diseases where insufficient Treg activity is postulated as a pathophysiologic mechanism, including autoimmune diseases. BT-061 is currently in clinical development in rheumatoid arthritis and psoriasis supporting the concept of Treg activation for the treatment of autoimmune diseases.<sup>33,57</sup>

## METHODS

### Isolation of T cells

To enrich CD4<sup>+</sup> T cells from fresh human PBMCs, they were purified by negative selection using the Dynabeads Untouched Human CD4 T cells (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Tregs and Teffs were separated by CD25-positive selection using the Dynabeads Regulatory CD4<sup>+</sup> CD25<sup>+</sup> T cell Kit (Life Technologies).

### Anti-CD4 mAbs

The following anti-human CD4 mAbs were used: BT-061 (Biotest AG, Dreieich, Germany), OKT-3 (BioLegend, San Diego, CA, USA), RPA-T4 (BD Pharmingen, San Jose, CA, USA), SK3 (BioLegend), MT310 (Santa Cruz Biotechnology, Dallas, TX, USA), QS4120 (Abnova, Neihu District, Taipei City, Taiwan), B-A1 (Acris Antibodies, San Diego, CA, USA), EDU-2 (Santa Cruz Biotechnology), MT441 (Ansell Corporation, Bayport, MN, USA), OKT-4 (BioLegend).

### Co-culture assay

Tregs were stimulated for 16–24 h at 37 °C with plate-bound BT-061, RPA-T4, SK3, QS4120, B-A1, MT441, MT310 or EDU-2 (1  $\mu$ g ml<sup>-1</sup>). Medium-treated Tregs served as a negative control and anti-CD3-treated (OKT-3, 1  $\mu$ g ml<sup>-1</sup>) Tregs as a positive control. 10<sup>5</sup> syngeneic Teffs were added to 96-well plates containing allogeneic, irradiated PBMCs (2  $\times$  10<sup>5</sup> cells per well). Pre-stimulated Tregs were then transferred to Teffs at a ratio of 1:4. 1  $\mu$ Ci of <sup>3</sup>H-thymidine was added on day 6. Sixteen hours later, cells were harvested and incorporated radioactivity was measured. All tests were performed in triplicates/quadruplicates and mean values were calculated.

### Competition

CD4<sup>+</sup> T cells were pre-incubated for 2 h at 4 °C with BT-061, OKT-3, RPA-T4, SK3, QS4120, B-A1, MT441, MT310, EDU-2 or OKT-4 (1  $\mu$ g ml<sup>-1</sup>). The CD4<sup>+</sup> T cells were washed and the binding of OKT-4-Alexa Fluor 647 (BioLegend), SK3-APC (Becton Dickinson, Franklin Lakes, NJ, USA) or BT-061-FITC (Biotest AG) was measured using flow cytometry.

### Analysis of protein phosphorylation

CD4<sup>+</sup> T cells, Teffs or Tregs (10<sup>5</sup> cells per well) were incubated for 30 min at room temperature with BT-061, OKT-3, RPA-T4, SK3, QS4120, B-A1, MT441, MT310, EDU-2 or OKT-4 (1  $\mu$ g ml<sup>-1</sup>). The mAbs were cross-linked for 5, 10, 30 or 60 min at 37 °C either by anti-human IgG (20  $\mu$ g ml<sup>-1</sup>)



(Life Technologies) or anti-mouse IgG (amIgG) ( $10 \mu\text{g ml}^{-1}$ ) (Thermo Scientific, Waltham, MA, USA). For intracellular staining, cells were treated with fixation buffer, permeabilized with perm buffer III and stained with anti-Zap-70 (pY319)/Syk (pY352), anti-PLC- $\gamma$ 2 (pY759), anti-ERK1/2 (pT202/pY204), anti-NF- $\kappa$ B p65 (pS529), anti-PKC $\alpha$  (pT497), anti-p38 MAPK (pT180/pY182), anti-Btk (pY551)/Itk (pY511), anti-MEK1 (pS298), anti-Pyk2 (pY402), anti-SLP-76 (pY128), anti-Lck (pY505), anti-Akt (pS473), anti-LAT (pY226), anti-JNK (pT183/pY185), anti-SHP-2 (pY542) or anti-IKK $\gamma$  (pS376) according to the manufacturer's instructions (all BD Phosflow, San Jose, CA, USA) before being analyzed on a FACS Canto II. Fold induction was calculated by dividing the mean fluorescence intensity of the measured value by the mean fluorescence intensity of the untreated control.

### Analysis of cytokine secretion

Fresh PBMCs ( $4 \times 10^5$  cells per well) were stimulated for 20 h and 44 h at 37 °C with BT-061, OKT-3, RPA-T4, SK3, QS4120, B-A1, MT441, MT310, EDU-2 or OKT-4 ( $2 \mu\text{g ml}^{-1}$ ). Medium-treated PBMCs served as a negative control. After stimulation, cell-free supernatant was collected and analyzed by Human Cytokine 10-Plex Panel according to the manufacturer's instructions (Life Technologies). All tests were performed in duplicates and mean values were calculated.

### Flow cytometry

Flow cytometry measurement was performed on a FACS Canto II (Becton Dickinson). The control staining of purified Tregs and Teffs was performed using antibodies against CD3, CD4, CD25 and CD127 (Becton Dickinson). The lymphocyte population was gated according to cell size and granularity (FSC/SSC). CD3<sup>+</sup>CD4<sup>+</sup> T cells were gated and distinguished by either the expression of CD25<sup>+</sup>CD127<sup>low</sup> for Tregs or CD25<sup>+</sup>CD127<sup>high</sup> for Teffs. The Teffs and Tregs showed a purity of ~90–98%. Data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

### Crystal structure analysis

Recombinant human CD4 was produced by using different constructs of CD4 that were cloned by standard procedures into vectors for heterologous expression in insect cells followed by purification via NiNTA. The Fab fragment of BT-061 was cleaved from the intact antibody using the protease papain and purified by protein A. The Fab fragment was further purified by size exclusion chromatography. The CD4/Fab complex was formed by mixing the purified proteins with a molar surplus of CD4 and further purification by size exclusion chromatography. The crystals of the CD4/BT-061 complex were prepared by co-crystallization. Crystals were flash-frozen and measured at a temperature of 100 K. The X-ray diffraction data were collected from co-crystals of CD4 with the Fab fragment of BT-061 at the SWISS LIGHT SOURCE (SLS, Villigen, Switzerland) using cryogenic conditions. The structure was solved and refined to a final resolution of 2.9 Å. Data were processed using the programs XDS and XSCALE. Subsequent model building and refinement was performed according to standard protocols with the software packages CCP4 and COOT.

### Statistical analysis

Statistical analyses to calculate *P*-values were performed with Prism 6.01 (GraphPad, La Jolla, CA, USA) using a one-way analysis of variance or two-tailed unpaired *t*-test. Mean values  $\pm$  s.d. are shown, \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.005, \*\*\*\**P* < 0.0001.

### CONFLICT OF INTEREST

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