PREVENTIVE ANTI-CD2 TREATMENT DOES NOT IMPAIR PARASITE CONTROL IN A MURINE TOXOPLASMOSIS MODEL

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Targeting human CD2 with the monoclonal antibody (mAb) CB.219 reduces intestinal inflammation in a colitis model where T cells carry human CD2. Here, we asked whether this mAb has adverse effects on infection control.

Mice expressing human CD2 on T cells (huCD2tg) were orally infected with Toxoplasma (T.) gondii and treated with the human CD2-specific mAb CB.219 in a preventive setting. The intestinal T. gondii loads in CB.219 treated mice did not differ from the control group. Histologically, huCD2tg mice showed moderate ileal inflammation that did not change with CB.219 treatment. In the ileum, CB.219 treatment reduced the protein levels of interferon-γ, transforming growth factor β and interleukin-6, whereas interleukin-18 mRNA was slightly increased. The infiltration of neutrophils, macrophages, and T cells into the ileum was unaffected by CB.219 treatment. However, CB.219 treatment decreased the numbers of forkhead box P3+ regulatory T cells (Treg) in ileum and liver of huCD2tg mice. This was confirmed in vitro using human peripheral blood mononuclear cells.

Taken together, targeting CD2+ T cells by the human CD2 mAb CB.219 does not prevent beneficial immune reactions necessary for pathogen control. Further experiments will address gut specificity, underlying mechanisms, and general applicability of CB.219 treatment.

Keywords: CD2, inflammatory bowel disease, infection model, experimental ileitis

Introduction

The glycoprotein CD2 is expressed on virtually all T cells and natural killer cells, and its principle ligand, the lymphocyte function-associated antigen-3 (CD58), on hematopoietic and nonhematopoietic cells [1]. CD2 acts as an adhesion molecule during the formation of the immunological synapse between T cells and antigen-presenting cells, and it is accessory in lowering the threshold of antigen concentration for specific T-cell responses [1]. Being important in antigen-dependent and -independent T-cell activation, CD2 is a potential target in therapy of various diseases. The humanized anti-CD2 monoclonal antibody (mAb) sipilizumab is permitted for treatment of psoriasis in adults [2], in preventing renal allograft rejection [3] and in treating acute graft-versus-host disease [4]. CD2-directed therapy has also been suggested for leukemia, lymphoma, and mature T-cell malignancies with abnormal levels of CD2 expression [5]. The better understanding of the role of CD2 in intestinal homeostasis is critical since two scenarios are possible. First, since targeting CD2 by mAb in transfer colitis models ameliorated colonic inflammation by induction of anergy in CD2+ cells, this strategy might reveal a novel therapeutic target for inflammatory bowel disease (IBD) [6, 7]. Second, a reduced interferon (IFN)γ secretion was observed by intestinal lymphocytes following treatment with a CB.219 mAb specific for human CD2 [6]. However, IFNγ is also critical for immunity against bacterial, viral, and protozoal infections [8]. Hence, targeting CD2 might be associated with an increased risk for intestinal infections.

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We specifically addressed this point in an infection model with the obligate intracellular parasite *Toxoplasma (T.) gondii*, that can invade and replicate in almost any nucleated cell in the host [9]. In this toxoplasmosis model, orally infected mice develop small intestinal inflammation [10]. Cytokines like IFNγ, tumour necrosis factor (TNF) α, interleukin (IL)-12, IL-18, IL-22, and IL-23 but not IL-17 contribute to the intestinal immunopathology [11–13]. Mice expressing human CD2 exclusively on thymocytes and circulating T cells (huCD2tg) while all other immune cell compartments of these mice remain unaffected [14] are an excellent tool to study the effect of CD2-specific mAb in vivo. Using experimental toxoplasmosis in huCD2tg mice, we here specifically addressed the effects of the anti-human CD2 CB.219 on the immunity against infection.

**Materials and methods**

**Animals**

Human CD2 transgenic (huCD2tg) mice [14] obtained from D. Kioussis (London, UK) were bred under specific pathogen-free conditions at the Forschungseinrichtungen für Experimentelle Medizin (Berlin, Germany). Wildtype C57BL/6 mice (WT) were purchased from the Jackson Laboratory (Bar Harbor, USA). During infection C57BL/6 mice (WT) were purchased from the Jackson Laboratory (Bar Harbor, USA). During the experiments, animals were killed by carbon dioxide anaesthesia. All experiments were performed in accordance with the German legislation on the protection of animals (G0207/05).

**Human blood cells**

Anonymized samples of peripheral blood mononuclear cells (PBMC) of healthy donors were obtained from leukocyte filters after leukapheresis as approved by the ethics committee of the Charité – Universitätsmedizin Berlin (EA1-157-13).

**Infection with T. gondii and antibody treatment**

Mice were infected orally with 100 cysts of the *T. gondii* strain ME49 as described previously [15]. The clinical course was assessed daily by weight as well as overall behavior and appearance. Mice were electively killed if they lost more than 20% of their initial weight and/or behaved lethargic and/or had ruffled coat. Human CD2-specific mAb CB.219 (200 μg) [6] or polyclonal mouse immunoglobulin (IgG) (200 μg; Dianova, Hamburg, Germany) was applied into the peritoneum (i.p.) simultaneous to oral *T. gondii* infection (day 0). Antibody treatment was repeated on days 3 and 5. Mice were sacrificed on day 7, and small intestines, mesenteric lymph nodes, spleens, and livers were removed.

**Ex vivo organ culture**

Livers were perfused in situ with 2 ml of a prewarmed digestion medium (RPMI 1640 supplemented with 5% foetal calf serum, 2 mg/ml collagenase IV, and 0.2 mg/ml DNase I) injected into the portal vein. Samples of 1 cm³ of liver tissue and 1-cm segments of the terminal ileum were rinsed in sterile phosphate-buffered saline (PBS; PAA Laboratories, Cölbe, Germany) and placed in 48-well tissue-culture plates containing 500 μl basal medium (RPMI1640, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM l-glutamin; all from PAA Laboratories; 50 μM β-mercaptoethanol; Sigma-Aldrich, Taufkirchen, Germany). After 24 h, culture supernatants were collected, snap frozen over liquid nitrogen, and stored at −80 °C.

**Histopathology**

The remaining small intestines and the right liver lobules were fixed in 4% formaldehyde and embedded in paraffin. Paraffin sections (1–2 μm) were stained with haematoxylin and cosin (H&E), and histomorphology was scored in a blinded manner. An approved scoring scheme was used to address ileal inflammation [15, 16]. Inflammation of the liver was assessed using a modified score [17] as follows: lobular inflammation was scored as 1) low inflammatory infiltrate; 2) increased inflammatory cells but less pyknotic necrosis; 3) marked increase in inflammatory cells and lots of pyknotic necroses; 4) marked inflammatory infiltration and necrotic areas; and 5) severe inflammation with bridging necrosis. Portal inflammation was scored as 1) mild inflammation; 2) moderate inflammation; 3) severe inflammation; and 4) severe inflammation which disperse into the parenchyma. The sum of the scores on lobular and portal inflammation made up for the hepatitis score.

**Cytometric bead array**

Supernatants of ex vivo organ cultures were examined by cytometric bead array (CBA) for the following cytokines: IL-2, IL-10, IFNγ, TGFβ, IL-17A, IL-1β, and TNFα as well as regarding chemokines C-C chemokine ligand 3 (CCL3), CC-chemokine ligand 2 (CCL2), and chemokine (C-X-C motif) ligand 1 (CXCL1) using Flex sets following the manufacturer’s instructions (BD Bioscience, Freiburg, Germany).

**Real-time PCR**

RNA was isolated from ileum tissue samples, reversely transcribed, and analyzed for cytokine specific mRNA as

*European Journal of Microbiology and Immunology*
described previously [13]. Mouse IFNγ and IL-18 mRNA expressions were detected and analyzed using Light Cycler Data Analysis Software (Roche, Mannheim, Germany). Expression levels were calculated in relation to the hypoxanthine phosphoribosyl transferase (HPRT) expression.

**Immunohistochemistry**

For immunohistochemistry, paraffin sections were subjected to a heat- or, in the case of F 4/80, to a proteolytic-induced epitope retrieval step. Sections were incubated with polyclonal rabbit anti-human myeloperoxidase (MPO; #A0398; Dako, Glostrup, Denmark) or rat anti-mouse F4/80 (clone BM8, eBioscience, San Diego, California). This was followed by incubation with secondary antibodies (biotinylated donkey anti-rabbit and biotinylated donkey anti-rat, respectively; both from Dianova, Hamburg, Germany). Biotin was detected by the alkaline phosphatase (AP)-labeled streptavidin, and AP was visualized using Fast Red as chromogen (Dako). For detection of regulatory T cells, sections were incubated with rat anti-mouse FoxP3 (clone FJK-16s, eBioscience) followed by incubation with rabbit anti-rat (Dianova). The EnVision+ System-HRP Labelled Polymer Anti-Rabbit (Dako) was used for detection of rabbit IgG. HRP was visualized with diaminobenzidine (Dako) as chromogen. Proteins were then inactivated by pressure cooking, and sections were incubated with polyclonal rabbit anti-CD3 (#IR50361-2; Dako) followed by detection employing the Dako REAL™ Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse (Dako). Nuclei were stained by hematoxylin, and slides were covered with glycerol gelatin (both from Merck, Darmstadt, Germany). Negative controls were performed by omitting the primary antibodies. Images were acquired using an AxioImager Z1 microscope equipped with a change-coupled device camera and processed with the AxioVision software (all from Carl Zeiss MicroImaging, Jena, Germany). For quantification, positively stained cells in 10 high power fields (hpf; 0.237 mm²) were counted.

**In vitro cultures**

Leukocytes from mouse mesenteric lymph nodes and spleens were prepared as described elsewhere [18]. Nonparenchymal liver cells were isolated by grinding liver tissue through a metal sieve followed by rinsing with prewarmed digestion medium (RPMI 1640, 5% foetal calf serum, 2 mg/ml collagenase IV, 200 μg/ml DNase I; all from Sigma-Aldrich) and incubation for 15 min at room temperature. This liver suspension was passed through a 70 μm-mesh cell strainer (BD Biosciences, Heidelberg, Germany), centrifuged, and washed with phosphate-buffered saline (Biochrom, Berlin, Germany) prior to lysis of erythrocytes. After Percoll density-gradient centrifugation (Sigma-Aldrich), nonparenchymal liver cells were collected from the interphase, washed, and resuspended in RPMI1640 supplemented with 10% foetal bovine serum. Human PBMC were prepared by Ficoll density gradient centrifugation (ρ = 1.078 g/ml) according to the manufacturer’s instructions (GE Healthcare, Frankfurt, Germany). Cells (10⁶) were activated by plate-bound anti-CD3 (10 μg/ml; clone OKT3; Sigma-Aldrich), soluble anti-CD28 (1 μg/ml; clone CD28.2; BD Biosciences), and IL-2 (50 U/ml; Merck-Millipore, Darmstadt, Germany) in the absence or presence of CB.219. For detection of intracellular factors, cultures received 5 μg/ml brefeldin A (Sigma-Aldrich) during the last 6 h of the culture. After 24 h, cells were counted by Trypan-blue exclusion (Biochrom, Berlin, Germany).

**Flow cytometry**

Directly conjugated mAb for the following human antigens were purchased from BD Bioscience if not stated otherwise: CD3 (clone 145-2C11), CD4 (clone GK1.5), CD8 (clone 53-6.7), CD11a, CD11b (clone M1/70), CD11c (clone HL3), CD40L (clone MRI; Miltenyi Biotec, Bergisch Gladbach, Germany), CD69 (clone H1.2F3), and Foxp3 (clone MF23). Before intranuclear staining of forkhead box protein (Foxp3), cells were fixed and permeabilized using the Mouse Foxp3 Buffer Set (BD Bioscience) according to the manufacturer’s instructions.

Directly conjugated mAb for the following human antigens was purchased from BioLegend (San Diego, CA): C-C chemokine receptor (CCR)7 (clone G043H7), CD4 (clone RPA-T4), CD45RA (clone HI100), CD8α (clone RPA-T8), Foxp3 (clone 259D), IFNγ (clone 4S.B3), IL-10 (clone JES3-9D7), latency-associated protein addressing TGFβ (clone TW4-2F8), and TNFα (clone Mab11). Before intracellular cytokine or Foxp3 staining, cells were fixed and permeabilized using the FIX & PERM™ Cell Fixation & Cell Permeabilization Kit (BioLegend) according to the manufacturer’s instructions. Stained cells were assessed by multicolor flow cytometry using a FACS Canto II device with the FACS Diva software (both from BD Biosciences). During data analysis by the FlowJo software (FlowJo, LLC, Ashland, Oregon), viable lymphocytes were defined by their forward/sideward scatter properties.

**Statistical analysis**

Nonparametric tests (Mann–Whitney test) were carried out using GraphPad Prism 6 (GraphPad, La Jolla, CA), and differences with p ≤ 0.05 were considered significant.

**Results**

**HuCD2tg mice are susceptible to infection with T. gondii**

We first tested the susceptibility of the huCD2tg mice for infection with *T. gondii*. Infected huCD2tg mice continu-
Anti-CD2 treatment does not impair parasite control

European Journal of Microbiology and Immunology

Recently lost weight and were indistinguishable by clinical signs from infected wildtype mice. All infected mice had a ruffled coat and appeared haggard and tremulous at the end of the experiment (data not shown). The analysis of the immune cell compositions in mesenteric lymph nodes (mLN), spleen, and liver with regard to the ratio of CD4+/CD8+ T cells and the relative contents of FoxP3+CD4+ regulatory T cells (Treg) did not reveal differences between huCD2tg and WT mice (Table 1). In addition, the mLN and the spleens of WT and huCD2tg mice did not differ in the relative content of CD3+ T cells and in the activation of CD4+ T cells as assessed by CD40L and CD69 (Table 1).

CB.219 does not affect clearance of the intestinal pathogen in huCD2tg mice

In order to evaluate the effect of the CD2-directed mAb CB.219 on infection control in huCD2tg mice after oral infection with T. gondii, the huCD2tg mice displayed comparable intestinal damage of the small intestine as WT mice (Fig. 1A).

Table 1. Effect of the huCD2 transgene on the composition of the T-cell compartment upon T. gondii infection. WT and huCD2tg mice were infected with 100 cysts of T. gondii by gavage. After 7 days, cells from mLN, spleen, and liver were stained for CD3, CD4, CD8, and FoxP3 and analyzed by flow cytometry. Preliminary experiment with n = 3–5

| Organ | Group | CD3+ (% lymphocytes) | Ratio CD4+/CD8+ | FoxP3+ (%CD4+) | CD40L (%CD4+) | CD69 (%CD4+) |
|-------|-------|----------------------|----------------|---------------|---------------|--------------|
| mLN   | WT    | 60.2                 | 1.9            | 4.6           | 27.6          | 5.9          |
|       | huCD2tg | 56.6                | 2.6            | 4.3           | 35.2          | 6.2          |
| Spleen| WT    | 25.9                 | 2.2            | 2.0           | 13.3          | 2.9          |
|       | huCD2tg | 24.5                | 2.6            | 1.6           | 10.9          | 1.7          |
| Liver | WT    | 12.8                 | 2.5            | 0.6           | 8.3           | 4.5          |
|       | huCD2tg | 41.0                | 2.1            | 0.6           | 5.9           | 10.4         |

Table 2. Effect of the huCD2 transgene on the composition of the myeloid cell compartment upon T. gondii infection. WT and huCD2tg mice were infected as described for Table 1. After 7 days, cells from mLN, spleen, and liver were stained for CD11a-c and analyzed by flow cytometry. Preliminary experiment with n = 3–5

| Organ | Group | Myeloid cells (% FSClow SSClow) |
|-------|-------|-------------------------------|
|       |       | CD11a+ | CD11b+ | CD11a/b+ | CD11c+ |
| mLN   | WT    | 5.1    | 2.5    | 0.7      | 2.9    |
|       | huCD2tg | 5.2    | 4.6    | 0.9      | 3.2    |
| Spleen| WT    | 11.0   | 6.7    | 3.7      | 6.5    |
|       | huCD2tg | 12.3   | 6.6    | 4.0      | 7.1    |
| Liver | WT    | 9.8    | 3.3    | 1.8      | 10.7   |
|       | huCD2tg | 34.2   | 8.7    | 6.4      | 16.8   |

Table 3. Spontaneous cytokine and chemokine secretion of ileum cultures from mice treated with CB.219 during T. gondii infection. Mean ± SD concentrations of cytokines and chemokines in supernatants of ileum cultures from mice on day 7 postinfection. Concentrations normalized to the total protein content of the supernatants are given in pg/μg protein

| Factor | Control IgG (n = 10) | CB.219 (n = 8) |
|--------|---------------------|---------------|
| IL-4   | 711 ± 490           | 326 ± 236     |
| IL-10  | 35 ± 17             | 32 ± 23       |
| IL-1β  | 74 ± 39             | 72 ± 41       |
| TNFα   | 33 ± 17             | 24 ± 15       |
| CXCL1  | 292 ± 299           | 156 ± 133     |
| CCL3   | 93 ± 49             | 57 ± 31       |
| IL-12  | 146 ± 154           | 114 ± 13      |

European Journal of Microbiology and Immunology
administration of *T. gondii*, a preventive regimen that simultaneously administered the antibody was applied. Overall survival did not differ between the groups treated with either the control IgG or the CB.219 mAb (data not shown). Interestingly, CB.219 treatment had no effect on small intestinal pathogen load as determined by comparable *T. gondii* DNA amounts in both groups (Fig. 1B). Furthermore, CB.219 treatment did not change the severity of small bowel inflammation compared to control IgG-treated mice (Fig. 1C). Moderate diffuse inflammatory cell infiltrates within the mucosa, accompanied by comparably moderate changes in the epithelial cell layers, and the overall mucosal architecture were the hallmarks of the small intestinal pathology in both groups. Thus, targeting human CD2 by CB.219 did not reduce the small intestinal damage and did not interfere with the control of the parasite.

**Preventive CB.219 treatment mainly reduces local IFNγ**

Spontaneous cytokine secretion of ileum organ cultures was comparable between CB.219-treated and control animals with regard to the pro-inflammatory cytokines IL-1β, IL-4, IL-12, and TNFα; the chemokines CXCL1 and CCL3; and the anti-inflammatory cytokine IL-10 (Table 3). Of notice, IFNγ, TGFβ, and IL-6 were significantly reduced in CB.219-treated animals (Fig. 2A). Diminished IFNγ protein concentrations from ileum organ cultures were confirmed by reduced IFNγ mRNA extracted from ileal tissue (Fig. 2C). IL-18 was increased at the mRNA level after CB.219 treatment (Fig. 2C). As in the small intestine, IFNγ in the liver was lower following CB.219 treatment while TGFβ and IL-6 remained comparable to the control group (Fig. 2B). While unaffected in the ileum, the chemoattractants CCL2 and CCL3 were found to be reduced in the liver after CB.219 treatment (Fig. 2B).

Although the histopathology was not markedly improved upon CB.219 treatment, the mAb decreased the pro-inflammatory cytokine and chemokine production in ileum and liver.

**CB.219 reduces Treg in the small intestine and liver**

The differences in the chemokine expression upon CB.219 treatment led us to analyze the immune cell composition in the small intestine and liver. F4/80+ macrophages, MPO+

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**Fig. 1.** Small intestinal damage and pathogen load. (A) Small intestinal tissue sections were stained with H&E in wildtype (WT) and human CD2 transgenic (huCD2tg) mice following oral *T. gondii* infection. Original magnification ×100; scale bar represents 100 μm. (B, C) In a preventive setting, huCD2tg mice received 200 μg CB.219 or control IgG (ctrl) i.p. simultaneously to the oral *T. gondii* infection. (B) DNA was isolated from ileum tissue, and *T. gondii* DNA was assessed by qPCR. (C) Histological scores for control (ctrl) and CB.219-treated huCD2tg mice (CB.219) following oral *T. gondii* infection. Mean value ± 95% confidence interval of *n* = 10 in each group.
Anti-CD2 treatment does not impair parasite control

Fig. 2. Cytokine production within ileum and liver. HuCD2tg mice were infected with T. gondii and treated with CB.219 or control IgG (ctrl) as described for Fig. 1B, C. At day 7, (A) ileum or (B) liver tissue was cultured for 24 h before cytokines and chemokines from the supernatants were assessed by CBA. (C) RNA was prepared from ileum tissue samples and subjected to IFNγ- or IL-18-specific qPCR. Values were normalized to HPRT as housekeeping gene. Mean value ± 95% confidence interval of \( n = 5 \) in each group; \(^* p < 0.05\), \(^{**} p < 0.01\), \(^{***} p < 0.001\)
neutrophils, and CD3⁺ T cells were comparably distributed in both organs and in both treatment groups (Fig. 3A, B). In the small intestine, macrophages were sporadic and scattered throughout the mucosa. Most of the cells in dense

![Image](image_url)

**Fig. 3.** Immune-cell infiltrations in small intestine and liver. Representative images for (A) small intestine and (B) liver tissue of control (ctrl, \( n = 10 \)) and CB.219-treated (CB.219, \( n = 10 \)) mice following oral *T. gondii* infection showing F4/80⁺ macrophages, MPO⁺ neutrophils, or CD3⁺ T cells in combination with FoxP3 for Treg. Original magnification ×400; arrows indicate positive cells, and arrowheads indicate CD3⁺FoxP3⁺ Treg. FoxP3⁺CD3⁺ Treg were counted in 10 high power fields (hpf) in (C) small intestine and (D) liver tissue sections. Mean value ± 95% confidence interval of \( n = 5 \) in each group; *\( p < 0.05 \), **\( p < 0.01 \)

*European Journal of Microbiology and Immunology*
infiltrates were neutrophils and T cells (Fig. 3A). Macrophages and neutrophils were found evenly distributed within the liver tissue. T cells clearly accumulated in the proximity of vessels (Fig. 3B). The only cell population distinguishing the group of mice treated with CB.219 from the control IgG group was the FoxP3+ Treg that were significantly reduced in number in the small intestine (Fig. 3C) and liver of mice treated with CB.219 (Fig. 3D).

The effect of CB.219 on Treg infiltration in the small intestine and liver raised the question whether this was associated with a change in circulating Treg in humans. To address this, T cells isolated from peripheral blood of healthy donors were stimulated in vitro with CD3 and CD28 in the presence or absence of CB.219. Here, FoxP3+ Treg were reduced in the cultures containing CB.219 (control: 9.83 ± 1.44; 1 μg/ml CB.219: 7.24 ± 1.12*; mean values ± SEM * p < 0.05 compared to the control) (Table 4). The percentages of IFNγ- and of TNFα-producing CD4+ and CD8+ T cells were increased after incubation with the CD2-specific mAb, while the IL-10 and TGFβ producers remained in the same range as the control cultures (Table 4).

Taken together, our data from T. gondii-infected huCD2tg mice treated with CB.219 indicate that this mAb specific for human CD2 does not alter the capacity of the effector T cells to deal with the parasite, and hence, treatment is not associated with an increased risk during the course of parasite infection when compared to controls.

### Discussion

Therapeutic treatment of transfer colitis with the human CD2 mAb CB.219 ameliorates colonic inflammation in huCD2tg mice suggesting a therapeutic potential for this mAb [6]. We here focused on the question whether CB.219 has adverse effects on the clearance of parasite infections that might challenge this avenue. The obligate intracellular parasite T. gondii can invade virtually all cells and tissues of the body including the small intestine [10]. Thus, the model of T. gondii infection in huCD2tg mice provides the opportunity to address effects of CB.219 on the local immune-cell compartments in intestinal inflammation in general and for pathogen control. Histologically, CB.219 treatment did not alter the inflammation in the small intestine and in the liver caused by T. gondii. Lower levels of IFNγ in small intestine and liver and increased systemic IFNγ compared to the control group sufficiently supported the clearance of the pathogen. Besides reduced numbers of Treg in the small intestine and liver, CB.219 also showed no interference with the composition of the immune-cell compartments. In line with this, human peripheral blood mononuclear cells stimulated in the presence of CB.219 showed a reduction in Treg number. We therefore assume that treatment with CB.219 is not associated with an increased risk with regard to parasite infections.

Successful CD2-directed therapy strongly depends on the disease entity, the type of mainly targeted cells, and also on the subtype as well as on the specific epitopes targeted by a given antibody. Since CD2-mediated stimuli are mandatory for murine Treg survival [19], an effect on this compartment requires antibodies neutralizing CD2. Combination of anti-CD2 and conventional therapy to treat experimental visceral leishmaniasis induced IFNγ production in CD4+ lymphoblasts ensuring parasite killing [20]. Depleting T cells by non-activating anti-CD2 inhibits graft infiltration in experimental transplantation [21]. Anti-CD2 treatment also affects natural killer (NK) cells [22–24]. In huCD2tg mice, only circulating T cells, thymocytes and megakaryocytes express human CD2 while the murine counterpart is found on the natural killer cells [14]. Human peripheral NK cells did not respond to CB.219 stimulation in vitro (data not shown), so we assumed that the function of murine NK cells was also not affected by the mAb in vivo. This inherent characteristic of the huCD2tg mice allowed for focusing the study on the role of CD2 on T cells during infection of the small intestine in experimental toxoplasmosis. Treatment with

### Table 4. Effect of CB.219 on the cytokine production of human T cells in vitro. PBMC were activated via CD3 and CD28 in the absence or presence of the anti-CD2 mAb CB.219 and received brefeldin A (5 μg/ml) during the last 6 h of culture. After 24 h, cells were fixed and stained for CD4, CD56, and cytokine expression. Staining was assessed by flow cytometry. Mean values ± SEM of n = 5 with triplicate determinations. Relative cell numbers are given as (% of CD4+ or CD4-CD56- lymphocytes)

| Cell type | Cytokine | CB.219 (μg/ml) |
|-----------|----------|----------------|
|           | 0        | 1              | 10             |
| CD4+ T cells | IFNγ    | 3.05 ± 0.53    | 3.76 ± 0.61*   | 3.86 ± 0.65* |
|           | TGFβ     | 0.45 ± 0.17    | 0.05 ± 0.12    | 0.28 ± 0.17  |
|           | TNFα     | 3.38 ± 0.59    | 6.01 ± 1.05*   | 6.49 ± 1.16* |
|           | IL-10    | 0.86 ± 0.24    | 0.86 ± 0.39    | 0.90 ± 0.43  |
| CD8+ T cells | IFNγ    | 3.11 ± 0.73    | 4.67 ± 1.04*   | 3.96 ± 0.98* |
|           | TGFβ     | 0.39 ± 0.24    | 0.46 ± 0.12    | 0.46 ± 0.12  |
|           | TNFα     | 1.89 ± 0.34    | 2.48 ± 0.35*   | 2.88 ± 0.35* |

* p < 0.05 compared to the control without CB.219
CB.219 also diminishes IFNγ secretion in intestinal lymphocytes in a transfer colitis model with huCD2tg mice [6]. This reduction was also seen here in huCD2tg mice with toxoplasmosis. Not questioning the central role of IFNγ in infection control [8], little local and systemic IFNγ is a hallmark for protection from inflammation of the small intestine [25]. In IL-22-deficient mice, weak inflammation of the small intestine associates with low levels of the Th1 cytokine IL-18 [26]. Increased intestinal production of the IFNγ-regulating factor IL-18 combined with reduced IFNγ in ileum and liver upon treatment of T. gondii-infected huCD2tg mice with CB.219 might reflect different kinetics eventually leading to the equally moderate small intestinal inflammation. Reduction or absence of Treg in concert with low TGFβ levels can worsen intestinal inflammation [27]. In summary, the counterbalancing effect of reduced local IFNγ concentrations and Treg counts in the small intestine might be responsible for the overall not improved histopathology upon CB.219 treatment.

Although T. gondii-induced inflammation is mainly driven by T helper (Th)1 immune responses [9] and strongly depend upon IL-12 and IFNγ [28], IL-12 was very low and not affected by targeting huCD2 on T cells. Specific CD4+ and CD8+ effector T cells develop during acute and long-term infections with T. gondii, and all cell compartments are mandatory for proper pathogen control [25, 29]. CB.219 did not alter the ratios of human CD4+ and CD8+ T cells as well as the effector status of these cells in vitro. We therefore think that it is safe to assume that, despite a reduced ileal IL-6 production, the mAb did not impede the main T cell-driven pathogen specific effector functions. These were shown by comparable and low levels of T. gondii DNA in the control group of huCD2tg mice and in the group treated with CB.219.

Earlier studies in a transfer colitis model showed that therapeutic targeting of CD2 improved overall survival with WT and huCD2tg mice [6, 7]. The lack of effect of anti-CD2 and CD2 deficiency treatment on infection control in WT mice [7, 30] is shown here for the humanized counterpart. CCL3 and CCL2 crucial for chemotaxis of myeloid cells can be considered markers for innate immune responses against T. gondii [29, 31]. While both chemokines were not altered in the small intestine, their decreased concentration found in the liver upon CB.219 treatment did not translate into a reduction of macrophage recruitment into this tissue.

Taken together, targeting CD2 by the human CD2-specific mAb CB.219 abrogates and reverts colonic inflammation [6] but does not interfere with small intestinal pathology upon T. gondii infection and does not prevent the control of the pathogen.

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European Journal of Microbiology and Immunology

References

1. Koyasu S, Lawton T, Novick D, Recny MA, Siliciano RF, Wallner BP, Reinherz EL: Role of interaction of CD2 molecules with lymphocyte function-associated antigen 3 in T-cell recognition of nominal antigen. Proc Natl Acad Sci U S A 87, 2603–2607 (1990)

2. Langley RG, Papp K, Bissonnette R, Toth D, Matheson R, Hultquist M, White B: Safety profile of intravenous and subcutaneous sipiluzumab, an anti-CD2 monoclonal antibody, for the treatment of plaque psoriasis: results of two randomized, double-blind, placebo-controlled studies. Int J Dermatol 49, 818–828 (2010)

3. Pruett TL, McGory RW, Wright FH, Pescevovicz MD, Yang H, McClain JB: Safety profile, pharmacokinetics, and pharmacodynamics of sipiluzumab, a humanized anti-CD2 monoclonal antibody, in renal allograft recipients. Transplant Proc 41, 3655–3661 (2009)

4. Adkins D, Ratnatharathorn V, Yang H, White B: Safety profile and clinical outcomes in a phase I, placebo-controlled study of sipiluzumab in acute graft-versus-host disease. Transplantation 88, 198–202 (2009)

5. Arun I, Wulu JA, Janik JE, Jasper GA, Yuan CM, Venzon D, Stetler-Stevenson M: Visual inspection versus quantitative flow cytometry to detect aberrant CD2 expression in malignant T cells. Cytometry B Clin Cytom 78, 169–175 (2010)

6. Erben U, Pawlowski NN, Doerfel K, Loddenkemper C, Hoffmann JC, Siegmund B, Kuhl AA: Targeting human CD2 by the monoclonal antibody CB.219 reduces intestinal inflammation in a humanized transfer colitis model. Clin Immunol 157, 16–25 (2015)

7. Pawlowski NN, Kakirman H, Kuhl AA, Liesenfeld O, Grollich K, Loddenkemper C, Zeitz M, Hoffmann JC: Alpha CD 2 mAb treatment safely attenuates adoptive transfer colitis. Lab Invest 85, 1013–1023 (2005)

8. Schroder K, Hertzog PJ, Ravasi T, Hume DA: Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 75, 163–189 (2004)

9. Munoz M, Liesenfeld O, Heimesaat MM: Immunology of Toxoplasma gondii. Immunol Rev 240, 269–285 (2011)

10. Liesenfeld O: Oral infection of C57BL/6 mice with Toxoplasma gondii: a new model of inflammatory bowel disease? J Infect Dis 185 Suppl 1, S96–101 (2002)

11. Liesenfeld O, Kang H, Park D, Nguyen TA, Parkhe CV, Watanabe H, Abo T, Sher A, Remington JS, Suzuki Y: TNF-alpha, nitric oxide and IFN-gamma are all critical for development of necrosis in the small intestine and early mortality in genetically susceptible mice infected perorally with Toxoplasma gondii. Parasite Immunol 21, 365–376 (1999)

12. Vossenkamper A, Struck D, Alvarado-Enquivel C, Went T, Takeda K, Akira S, Pfeffer K, Alber G, Lochner M, Forster I, Liesenfeld O: Both IL-12 and IL-18 contribute to small intestinal Th1-type immunopathology following oral infection with Toxoplasma gondii, but IL-12 is dominant over IL-18 in parasite control. Eur J Immunol 34, 3197–3207 (2004)

13. Munoz M, Heimesaat MM, Danker K, Struck D, Lohmann U, Plickert R, Bereswill S, Fischer A, Dunay IR, Wolk K, Loddenkemper C, Krell HW, Libert C, Lund LR, Frey O, Holscher C, Iwakura Y, Ghilardi N, Ouyang W, Kamradt T, Sabat R, Liesenfeld O: Interleukin (IL)-23 mediates Toxo-
Anti-CD2 treatment does not impair parasite control

plasma gondii-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 but independent of IL-17. J Exp Med 206, 3047–3059 (2009)

14. Lang G, Wotton D, Owen MJ, Sewell WA, Brown MH, Mason DY, Crompton MJ, Kioussis D: The structure of the human CD2 gene and its expression in transgenic mice. EMBO J 7, 1675–1682 (1988)

15. Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, Jahn HK, Dunay IR, Moter A, Gescher DM, Schumann RR, Gobel UB, Liesenfeld O: Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with Toxoplasma gondii. J Immunol 177, 8785–8795 (2006)

16. Erben U, Loddenkemper C, Doerfel K, Spieckermann S, Haller D, Heimesaat MM, Zeitz M, Siegmund B, Kuhl AA: A guide to histomorphological evaluation of intestinal inflammation in mouse models. Int J Clin Exp Pathol 7, 4557–4576 (2014)

17. Batts KP, Ludwig J: Chronic hepatitis. An update on terminology and reporting. Am J Surg Pathol 19, 1409–1417 (1995)

18. Hoffmann JC, Pawlowski NN, Grollich K, Loddenkemper C, Zeitz M, Kuhl AA: Gammadelta T lymphocytes: a new type of regulatory T cells suppressing murine 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis. Int J Colorectal Dis 23, 909–920 (2008)

19. Kashiwakura Y, Sakurai D, Kanno Y, Hashiguchi M, Kobayashi A, Kuros A, Tokudome S, Kobata T, Kojima H: CD2-mediated regulation of peripheral CD4(+)CD25(+) regulatory T-cell apoptosis accompanied by down-regulation of Bim. Immunology 139, 48–60 (2013)

20. Bimal S, Sinha S, Singh SK, Narayan S, Kumar V, Verma N, Ranjan A, Sinha PK, Das VN, Pandey K, Kar SK, Das P: Leishmania donovani: CD2 biased immune response skews the SAG mediated therapy for a predominant Th1 response in experimental infection. Exp Parasitol 131, 274–282 (2012)

21. Brady JL, Sutherland RM, Hancock M, Kitsoulis S, Ladhoud MH, Phillips PM, Hawthorne WJ, d’Apice AJ, Cowan PJ, Harrison LC, O’Connell PJ, Lew AM: Anti-CD2 producing pig xenografts effect localized depletion of human T cells in a huSCID model. Xenotransplantation 20, 100–109 (2013)

22. Kim EO, Kim TJ, Kim N, Kim ST, Kumar V, Lee KM: Homotypic cell to cell cross-talk among human natural killer cells reveals differential and overlapping roles of 2B4 and CD2. J Biol Chem 285, 41755–41764 (2010)

23. Kim TJ, Kim N, Kim EO, Choi JR, Bluestone JA, Lee KM: Suppression of human anti-porcine natural killer cell xenogeneic responses by combinations of monoclonal antibodies specific to CD2 and NKG2D and extracellular signal-regulated kinase inhibitor. Immunology 130, 545–555 (2010)

24. Zheng X, Wang Y, Wei H, Sun R, Tian Z: CD2 and CD2 synergize for the Erk1/2 activation in the Natural Killer (NK) cell immunological synapspe. J Biol Chem 284, 21280–21287 (2009)

25. Benevides L, Cardoso CR, Milanezi CM, Castro-Filice LS, Barenco PV, Sousa RO, Rodrigues RM, Mineo JR, Silva JS, Silva NM: Toxoplasma gondii soluble tachyzoite antigen triggers protective mechanisms against fatal intestinal pathology in oral infection of C57BL/6 mice. PLoS One 8, e75138 (2013)

26. Munoz M, Eideneschken C, Ota N, Wong K, Lohmann U, Kuhl AA, Wang X, Manzanillo P, Li Y, Rutz S, Zheng Y, Diehl L, Kayagaki N, van Looikeren-Campagne M, Liesenfeld O, Heimesaat M, Ouyang W: Interleukin-22 induces interleukin-18 expression from epithelial cells during intestinal infection. Immunity 42, 321–331 (2015)

27. Boehm F, Martin M, Kesselring R, Schiechl G, Geissler EK, Schlitt HJ, Fichtner-Feigl S: Deletion of Fopx3+ regulatory T cells in genetically targeted mice supports development of intestinal inflammation. BMC Gastroenterol 12, 97 (2012)

28. Gazzinelli RT, Wysocka M, Hayashi S, Denkers EY, Hieny S, Caspar P, Trinchieri G, Sher A: Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with Toxoplasma gondii. J Immunol 153, 2533–2543 (1994)

29. Luangsay S, Kasper LH, Rachinel N, Minns LA, Mennechet FJ, Vandewalle A, Buzoni-Galet D: CCR5 mediates specific migration of Toxoplasma gondii-primed CD8 lymphocytes to inflammatory intestinal epithelial cells. Gastroenterology 125, 491–500 (2003)

30. Pawlowski NN, Struck D, Grollich K, Kuhl AA, Zeitz M, Liesenfeld O, Hoffmann JC: CD2 deficiency partially prevents small bowel inflammation and improves parasite control in murine Toxoplasma gondii infection. World J Gastroenterol 13, 4207–4213 (2007)

31. Khan WI, Motomura Y, Wang H, El-Sharkawy RT, Verdu EF, Verma-Gandhu M, Rollins BI, Collins SM: Critical role of MCP-1 in the pathogenesis of experimental colitis in the context of immune and enterochromaffin cells. Am J Physiol Gastrointest Liver Physiol 291, G803–811 (2006)