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A CD28 superagonistic antibody elicits 2 functionally distinct waves of T cell activation in rats

Nora Müller,1 Jens van den Brandt,2 Francesca Odoardi,3,4 Denise Tischner,2 Judith Herath,2 Alexander Flügel,3,4 and Holger M. Reichardt1,2

1Institute for Virology and Immunobiology, University of Wuerzburg, Wuerzburg, Germany. 2Department of Cellular and Molecular Immunology, University of Goettingen Medical School, Goettingen, Germany. 3Department of Neuroimmunology, Max Planck Institute of Neurobiology, Martinsried, Germany. 4Institute for Immunology, Ludwig Maximilians University Munich, Munich, Germany.

Introduction
Efficient activation of naive T cells by antigen-presenting cells requires the concomitant engagement of the TCR and the costimulatory molecule CD28 (1). These 2 signals result in an upregulation of cell-surface receptors such as CD25 (IL-2Rα), CD69, CD134 (αβL integrin), diminished levels of the sphingosine 1-phosphate (S1P) receptor endothelial differentiation sphingolipid G protein–coupled receptor 1 and L selectin levels were downregulated, and the cells lost their responsiveness to sphingosine 1–phosphate–directed migration. These proadhesive alterations were accompanied by signs of strong activation, including upregulation of CD25, CD69, CD134, and proinflammatory mediators. However, this did not lead to a cytokine storm similar to the clinical trial. While most of the early changes disappeared within 48 hours, we observed that CD4+CD25+FoxP3+ regulatory T cells experienced a second phase of activation, which resulted in massive cell enlargement, extensive polarization, and increased motility. These data suggest that CD28 superagonists elicit 2 qualitatively distinct waves of activation.

Administration of the CD28 superagonistic antibody JJ316 is an efficient means to treat autoimmune diseases in rats, but the humanized antibody TGN1412 caused devastating side effects in healthy volunteers during a clinical trial. Here we show that JJ316 treatment of rats induced a dramatic redistribution of T lymphocytes from the periphery to the secondary lymphoid organs, resulting in severe T lymphopenia. Live imaging of secondary lymphoid organs revealed that JJ316 administration almost instantaneously (<2 minutes) arrested T cells in situ. This reduction in T cell motility was accompanied by profound cytoskeletal rearrangements and increased cell size. In addition, surface expression of lymphocyte function–associated antigen-1 was enhanced, endothelial differentiation sphingolipid G protein–coupled receptor 1 and L selectin levels were downregulated, and the activated T cell population (2–6). In contrast to classical costimulation, CD28 superagonistic antibodies, such as JJ316 and CD62L (L selectin), synthesis of effector cytokines and chemokines, a dynamic rearrangement of the F-actin cytoskeleton, and finally, clonal expansion of the activated T cell population (2–6). In contrast to classical costimulation, CD28 superagonistic antibodies, such as JJ316 and TGN1412 in humans, allow for the activation and expansion of T cells without the need of TCR engagement (7). Previous studies indicated that these antibodies preferentially address CD4+CD25+FoxP3+ Tregs (8), a lymphocyte subpopulation that contains bona fide Tregs by CD28 superagonistic antibodies has been proposed to underlie the beneficial effects of these drugs in the treatment of autoimmune diseases such as EAE in rats (10, 11). While the efficiency in animal models is undoubtedly, administration of the humanized CD28 superagonistic antibody TGN1412 to healthy volunteers caused devastating side effects (12). Within a few hours, a single i.v. dose induced severe lymphopenia and a systemic inflammatory response leading to multiorgan failure. Recent data suggest that the massive cytokine release encountered after TGN1412 administration might be a specific feature of human immune cells (13). However, whether such species differences also apply to the other adverse effects of TGN1412 remains elusive. Stimulated by the apparent discrepancy between the beneficial effects observed in animals and the outcome of the recent clinical trial in humans, we reinvestigated the properties of the CD28 superagonistic antibody JJ316 in rats. Our results demonstrate that the effects of JJ316 follow a biphasic course. Almost instantaneously after i.v. infusion, T cells are arrested within the secondary lymphoid organs and form clusters. This leads to a dramatic redistribution from the periphery to the spleen and lymph nodes, resulting in severe T lymphopenia similar to the clinical trial in humans. The trapped T cells become strongly activated, as indicated by cytoskeletal rearrangements, an altered density of cell surface receptors, and increased mRNA expression of proinflammatory cytokines and chemokines. Contrasting to the scenario in humans, however, cytokine serum levels remain moderate. These immediate changes are later followed by a second qualitatively different phase of activation that mainly contains bona fide Tregs. Thus, CD28 superagonistic antibodies elicit 2 distinct waves of T cell activation.

Results
JJ316 causes profound T lymphopenia by inducing redistribution of T cells to the secondary lymphoid organs. To analyze the effects of JJ316 on
JJ316 almost instantaneously abrogates T cell motility in the secondary lymphoid organs of living animals. To study rapid effects of JJ316 on T cell motility, we performed intravital video recording of T cells in blood, liver, lung, and spleen within the primary lymphoid organs of living animals.

To confirm trapping of T cells in the secondary lymphoid organs, we adoptively transferred CFSE-labeled lymphocytes to wild-type rats and studied their redistribution after JJ316 injection. Within 10 hours, the frequency of the CFSE+ T cells was reduced in blood, liver, and lung while their cellularity in spleen remained unaltered (Figure 1D). Thus, CD28 superagonistic antibodies lead to the redistribution of T lymphocytes from the periphery to secondary lymphoid organs.

Figure 1 Administration of JJ316 induces T lymphopenia and leads to the redistribution of CD4+ T cells. (A) Blood samples from rats i.v. injected with 1.0 mg, 0.2 mg, or 0.04 mg JJ316 were taken at the indicated time points and analyzed for the percentages of circulating T cells (left panel) and granulocytes (right panel) by flow cytometry. n = 6; mean ± standard error of the mean. Statistical analysis refers to the individual comparison of each time course to untreated control rats by 1-way ANOVA. (B) Leukocytes were isolated from lymph nodes, spleen, lung, and liver 10 and 72 hours after injection of 1.0 mg JJ316 or PBS as a control. Subsequently, the total number CD4+ T cells was determined by microscopic counting followed by flow cytometric analysis of CD4 and TCRβ expression. n = 3; mean ± standard error of the mean. (C) The relative frequencies of CD4+ naive (CD45RC−RT6.1+), activated (CD45RC−RT6.1+), and memory T cells (CD45RC−RT6.1− CD45RC−RT6.1+) was determined 10 and 72 hours after application of JJ316 or PBS. Thy-1+ RTEs (recent thymic emigrants) were excluded from the analysis. n = 3; mean ± standard error of the mean. *P < 0.05; **P < 0.01; ***P < 0.001.

JJ316 not only affects T cell motility in the secondary lymphoid organs, but also profoundly reduces the frequency and number of CD4+ T cells in blood and the periphery, as shown by flow cytometry and microscopic counting followed by flow cytometric analysis of CD4 and TCRβ expression.
myelin basic protein (MBP), which were retrovirally engineered to express green fluorescent protein (TMBP-GFP cells) (16). As reported earlier (5), these effector T cells distributed throughout the red pulp and T cell areas in the white pulp of the spleen within 60 hours after transfer (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI32698DS1). The majority of the T cells permanently moved within the tissue in a seemingly random fashion as indicated by the orientation and the low vector sum of their trajectories (Figure 2A and Supplemental Video 1). A few scattered “stationary” T cells displayed a confined motility and seemed to be attached to anchoring points.

Infusion of JJ316 dramatically changed the behavior of the effector T cells. Within 2 minutes most of the TMBP-GFP cells (>80%) slowed down and reduced their average speed from 6–7 μm/min to 1–2 μm/min (Figure 2B and Supplemental Video 1). Consecutively, their cell trajectories were shortened and the number of stationary T cells rose to 70%–80% (Figure 2A and D). Some of the T cells became aggregated and began to form clusters (Supplemental Video 1). This process was completed after 3–4 minutes and remained stable for around 90 minutes. Subsequently, T cell motility slowly recovered and reached control levels within 3–24 hours after JJ316 infusion (Figure 2C and D, and Supplemental Video 1).
This effect of JJ316 was not dependent on the antigen specificity or the phenotype of the T cells. CD4+ effector T cells directed against ovalbumin (T_{OVA-GFP} cells) or freshly purified T cells from the spleen (consisting of ~80% naïve αβ TCR+ cells) behaved in all respects like T_{MBP-GFP} cells after infusion of the CD28 superagonistic antibody (Figure 2). Furthermore, treatment with JJ316 did not affect the spatial distribution of the transferred effector T cells within the spleen (Supplemental Figure 1). These data indicate an extremely rapid but transient impact of JJ316 on T cell motility.

**Figure 3** Analysis of JJ316-induced cytoskeletal rearrangements ex vivo by confocal microscopy and SEM. (A) CD4+ T cells, CD8+ cells, and B cells were isolated at the indicated time points after treatment with 1.0 mg JJ316 and stained with phalloidin–Alexa Fluor 594 (red) to visualize F-actin polymerization. Double staining with an anti-FoxP3 antibody in combination with a secondary anti-rat Alexa Fluor 488 antibody (green) allowed distinguishing between CD4+ Th and Tregs. Scale bar: 5 μm. (B) The diameters of 150 individual cells per subtype and the time point were quantified by a computer-aided method; mean ± standard error of the mean. Statistical analysis by 2-way ANOVA. The asterisks refers to the Tregs (shown on top of the graph), the pound signs to the 3 other cell types (shown below the graph). ***P < 0.001; ###P < 0.001. (C) CD4+ T cells were purified before and 12 hours after JJ316 administration and analyzed by SEM. Scale bar: 5 μm. (D) Western blot analysis of cofilin-phosphorylation in CD4+CD25− Th and CD4+CD25+ Tregs magnetically purified 72 hours after PBS or JJ316 injection. Staining with an anti-cofilin antibody served as a loading control. One representative experiment out of 3 is shown. (E) CD4+CD25− Th and CD4+CD25+ Tregs were purified 72 hours after JJ316 administration and analyzed by SEM (left panel). Scale bar: 5 μm. To visualize microtubules and to study the localization of the microtubule-organizing center, Th and Tregs were stained with an anti-β-tubulin antibody (right panel). Scale bar: 5 μm.
often accompanied by cytoskeletal rearrangements (2). This led us to investigate the impact of JJ316 on morphological characteristics by confocal microscopy. Ex vivo analysis revealed profound activation of all lymphocyte subpopulations, including CD4+ T cells, CD8+ cells, and B cells within 12 hours after JJ316 infusion (Figure 3A). The F-actin cytoskeleton became polymerized, the originally round cells adopted an irregular shape, and the cell size significantly increased. These changes were most pronounced in CD4+ T cells expressing FoxP3 (Figure 3, A and B). Interestingly, the observed cytoskeletal alterations were not accompanied by cellular polarization. Scanning electron microscopy (SEM) analysis of CD4+ T cells 12 hours after JJ316 injection revealed a round morphology and no evidence of uropod formation (Figure 3C).

While activation occurred very rapidly, it was also transient. After 24 hours, the size of most lymphocyte subtypes was significantly reduced again and remained unaltered for the entire observation period of 72 hours (Figure 3, A and B). Nevertheless, at this stage bona fide CD4+FoxP3+ Tregs remained significantly enlarged and started to adopt a polarized phenotype (Figure 3, A and B). These changes became more pronounced at 72 hours after JJ316 infusion, which was confirmed by SEM analyses (Figure 3E).

Purified CD4+CD25+ FoxP3+ Th cells exhibited only moderate morphological alterations, characterized by radially located large lamellipodia and microvilli structures. In contrast, CD4+CD25–FoxP3+ Tregs isolated from JJ316-treated rats showed dramatic modifications such as the complete loss of microvilli, a smooth surface, and long membrane–protrusions (Figure 3E). Further immunohistochemical stainings revealed relocalization of the microtubule-organizing center (MTOC) to the trailing edge in CD4+CD25–FoxP3+ Tregs but not CD4+CD25+ FoxP3+ Th cells (Figure 3E). Flow cytometric analysis indicated that FoxP3 was indeed redefined to CD4+CD25– T lymphocytes, which efficiently inhibited T cell proliferation in an in vitro suppression assay (Supplemental Figure 2). These data confirm that CD4+CD25–FoxP3+ T cells isolated from JJ316-treated rats functionally represent Tregs. Notably, these data are in line with previous studies, which demonstrated selective activation of Tregs by JJ316 (10).

The polarized shape of the Tregs after JJ316 infusion further led us to analyze cofilin dephosphorylation, which is thought to underlie F-actin remodeling and cellular polarization (17). Cofilin was strongly dephosphorylated in CD4+CD25–FoxP3+ Tregs of JJ316-treated rats while the level of phospho-cofilin in CD4+CD25+ FoxP3+ Th cells was much less affected (Figure 3D). This was confirmed by densitometry, revealing 37±3% lower amounts of phospho-cofilin in Th cells after antibody treatment while its level was reduced by 79%±8% in Tregs (n = 3; mean ± standard error of the mean).

In vitro stimulation with JJ316 induces 2 distinct phases of activation. Administration of JJ316 in vivo leads to early morphological changes in all analyzed immune cells irrespective of their expression of the CD28 molecule. To distinguish between direct and indirect effects of JJ316, we investigated changes in lymphocyte morphology and size in vitro. Lymphocyte subpopulations were magnetically purified and stimulated with JJ316 on microscopic slides. Within 30 minutes, all CD4+ cells as well as the CD8+ T lymphocytes adopted a different shape and were significantly enlarged (Figure 4, A and B). These changes, which qualitatively resemble the ones observed after application of JJ316 in vivo, were transient since lymphocyte size and morphology reverted to the original state within 2 hours (Figure 4, A and B). In contrast to T lymphocytes, B cells did not respond to JJ316 stimulation at all, supporting the argument that indirect effects must account for the activation of these cells in vivo (Figure 4, A and B). Interestingly, prolonged stimulation with the CD28 superagonistic antibody for 7 hours resulted again in the polarization and a significant increase in the size of CD4+FoxP3+ T cells while no such effect was seen in the other 3 subtypes (Figure 4, A and B). Notably, purified CD4+CD25+ T cells responded in a similar way to JJ316 stimulation as the FoxP3+ population present in mixed CD4+ T cell cultures (Figure 4, A and B). In line with this finding, FoxP3 expression was largely refined to the CD4+CD25+ T lymphocytes and did not change in the presence of JJ316 (Figure 4C). Thus, in vitro stimulation with JJ316 clearly reflects the biphasic activation of CD4+CD25+FoxP3+ Tregs also seen in animals.

To gain insight into the signaling pathways underlying JJ316 action, we stimulated CD4+ T cells with JJ316 in the presence of kinase inhibitors and subsequently analyzed the cells for polarization as a measure of activation. Bona fide Tregs were again identified by immunohistochemical staining for FoxP3. While almost all T cells polarized within 30 minutes, the PI3K inhibitor LY294002 prevented this effect in FoxP3+ as well as in FoxP3– cells. In contrast, the MEK inhibitor U0126 did not impact on either cell type (Figure 4D). In agreement with previous findings (18), no phosphorylation of Akt could be demonstrated after JJ316 treatment (data not shown), indicating that PI3K signaling must be mediated through an Akt-independent pathway. Our findings suggest that JJ316 induces similar signaling pathways in all CD4+ T cells.

**JJ316 administration induces T cell adhesion to fibronectin while the motility of Tregs is selectively enhanced at later time points.** In view of the very transient T cell arrest observed by video microscopy (<3 hours) and the prolonged T lymphopenia (>24 hours), we studied T cell adhesion to extracellular matrix components ex vivo by live imaging and confocal microscopy. We purified CD4+ T cells 12 hours after JJ316 treatment and studied their behavior on slides coated with fibronectin, a ligand for β1 integrins (19). T cells from control animals displayed slow floating over the surface without adopting a migratory phenotype such as the formation of lamellipodia (Figure 5A). In contrast, cells isolated from JJ316-treated rats firmly attached to fibronectin, and consequently, free floating was almost completely abrogated (Figure 5A). Furthermore, we analyzed CD4+CD25–Th and CD4+CD25+ Tregs isolated 72 hours after administration of JJ316. Th cells displayed only a slow movement with little sign of polarization similar to control cells (Figure 5, A and B). In contrast, Tregs were crawling along the matrix and most cells displayed a migratory phenotype that was characterized by repeated lamellipodia formation and retraction (Figure 5B). These data indicate that JJ316 transiently increases the adhesiveness of all CD4+ T cells to fibronectin while it selectively enhances the motility of Tregs at later time points.

**JJ316 impacts on cell adhesion molecules and interferes with T cell egress from secondary lymphoid organs.** Stimulated by our finding that JJ316 increases T cell adhesion to fibronectin, we next studied surface expression of the proadhesive molecule, LFA-1, a β2-integrin that is required for the firm interaction of T lymphocytes with APCs and endothelial cells (4). Flow cytometric analysis revealed a significant upregulation of LFA-1–surface expression on CD4+ T cells 12 hours after JJ316 infusion (Figure 6A). Additionally, we found that L selectin (CD62L), mediating the rolling on endothelial interfaces before stable arrest and cellular extravasation (20), became almost undetectable on the cell surface of CD4+ T lymphocytes after 12 hours (Figure 6A). Thus, simulation with JJ316 induces
similar changes as seen after classical costimulation through engagement of the TCR and CD28.

Next we analyzed the expression of the S1P receptor 1 (EDG-1), which mediates the egress of T lymphocytes from secondary lymphoid organs (21). We found that within 2 hours after JJ316 injection Edg1 mRNA levels went steeply down and remained low for around 12 hours. Subsequently, the expression slowly returned to original levels (Figure 6B). At the peak of Edg1 downregulation, i.e., 12 hours after JJ316 infusion, the migratory capacity of T cells along an S1P gradient was virtually lost. While S1P at a concentration of 0.001 μg/ml induced an almost 4-fold higher transmigration index of T cells from control rats, the migratory capacity of T cells after JJ316 injection was unchanged (Figure 6C). In addition, CD69-surface expression was strongly increased after infusion of JJ316 (Figure 6D), which is in line with the recent observation that CD69 diminishes EDG-1 expression and thereby impairs S1P chemotactic func-
tion (22). Notably, treatment of animals with FTY720, a synthetic compound that renders T cells nonresponsive toward S1P (23), induced T lymphopenia and granulocytosis in peripheral blood with almost identical magnitude and kinetics as JJ316 (Figure 6E). This supports a potential role of S1P receptor downregulation for JJ316-induced lymphopenia and T cell redistribution.

JJ316 rapidly upregulates T cell activation markers and proinflammatory mediators but induces only moderate cytokine release. Infusion of the CD28 superagonistic antibody TGN1412 led to strong T cell activation and a cytokine storm in human volunteers (12). Therefore we studied induction of membrane activation markers and expression of proinflammatory mediators by JJ316. Fluorescently labeled naive and effector T cells were adoptively transferred into wild-type rats, and 4 hours after JJ316 or PBS treatment they were reisolated from the spleen by preparative flow cytometry and analyzed by quantitative PCR. In both cell types, JJ316 induced strongly elevated mRNA expression of the proinflammatory cytokines Ifng and Il17 and the chemokines Rantes and Mcp1 as well as Il2ra (CD25) (Figure 7A). Similar effects were seen in T cells reisolated from the lymph nodes (data not shown). Furthermore, Edg1 mRNA levels were dramatically downregulated on naive and effector T cells (Figure 7A), confirming our previous results (Figure 5B). Thus, administration of the CD28 superagonistic antibody induces broad and fulminant T cell activation in rats.

To confirm induction of activation markers on the protein level, we performed a flow cytometric analysis. CD25 (IL-2Rα) and
CD134 (Ox40) were strongly upregulated on all CD4+ T cells within 12 hours, thus following the transcriptional changes with some delay (Figure 7B). The effect on CD25 expression was only transient, and after 24 hours its expression level had already returned by half (Figure 7B). In contrast, expression of the chemokine receptor CXCR3 was unaffected by JJ316 at all time points as revealed by confocal microscopy (data not shown). Most surprisingly, however, only very moderately elevated serum levels of the proinflammatory cytokines TNFα and IFNγ were measured within the first 24 hours after JJ316 treatment while MCP-1 was not detectable at all (Figure 7C). Notably, the serum concentrations reached by CD28 superagonist treatment in rats were 2 to 3 orders of magnitude lower as compared with those measured after injection of a nonlethal dose of LPS (Figure 7C). A lower dose of 0.2 mg JJ316 did not result in any detectable TNFα and IFNγ release into the serum at all (data not shown), although it caused marked T lymphopenia (Figure 1A). In addition, we never observed any signs of discomfort in rats treated with JJ316, such as scruffy fur, diminished locomotion, or reduced food uptake. Morphological analysis of major organs failed to reveal any pathological effect of JJ316 treatment. Thus, despite strong T cell activation, systemic cytokine release is very limited after JJ316 administration and therefore, constitutes a major difference between rats and humans.

**Discussion**

CD28 superagonistic antibodies have the unique ability to address T cells in the absence of concurrent TCR ligation (7). Previous analyses had suggested that this leads to the preferential expansion of Tregs as compared with other T cells (8). Whereas this characteristic is thought to underlie the remarkable efficiency of such antibodies in the treatment of autoimmune diseases in rodents (10, 11), a recent clinical trial led to devastating side effects in 6 human volunteers (12). This raised the question of whether the rapid effects were unique to the human system or whether the
rodent CD28 superagonistic antibody induces similar changes. Our results indicate that JJ316 led to a redistribution of T cells to secondary lymphoid organs and thereby induced T lymphopenia, which is reminiscent of the effects of TGN1412 in healthy volunteers. In contrast to the clinical trial in humans, we were now able to study the mechanisms underlying lymphocyte redistribution after CD28 superagonist injection in detail. Within 10 hours, CD4+ T cell counts in liver and lung dropped dramatically while their numbers remained unaffected in spleen and lymph nodes. This was confirmed by our finding that the CD28 superagonistic antibody did not induce a memory phenotype as well as by tracking experiments showing that CD4+ T cells were depleted from the extralymphoid organs after JJ316 treatment. In contrast, B cell homing was only marginally affected. Our finding that the cellularity of the secondary lymphoid organs was not increased despite the redistribution of T cells from peripheral tissues can be explained by the low absolute number of blood-residing lymphocytes (24) and a modest induction of T cell apoptosis by JJ316 (data not shown). In summary, CD28 superagonistic antibodies have the ability to trap T cells in the secondary lymphoid organs. T lymphopenia occurs remarkably rapidly and is maintained over a 24–48 hours period. Indeed, intravital video microscopy revealed that T cells were almost instantaneous arrested in spleen and lymph nodes of living animals following JJ316 injection. Within 2 minutes, their velocity dropped dramatically and the number of stationary cells increased to around 80%. These data suggest that T cells become trapped in secondary lymphoid organs immediately after administration of the CD28 superagonistic antibody as a consequence of their reduced motility. However, since locomotion in situ was restored a few hours after antibody administration,
additional changes must occur in T cells explaining the prolonged lymphopenia for more than 24 hours. We have identified a number of mechanisms that presumably contribute to this effect. Firstly, we observed cytoskeletal rearrangements that include F-actin polymerization and an extensive increase in cell size but no cellular polarization occurred similar to that seen after antigen challenge (2). Secondly, the β2 integrin LFA-1 was upregulated while L-selectin expression was reduced by JJ316. These are typical changes that were also induced upon costimulation of antigen-specific T cells and strengthened the interaction with APCs while interfering with lymphocyte recirculation. Using a functional assay, we could further show that T cell interaction with fibronectin was strongly enhanced by JJ316. Since fibronectin is a ligand for β1 integrins, the observed firm adhesion could also contribute to the impaired T cell recirculation. Thus, several mechanisms might be involved in promoting the trapping of T cells in the secondary lymphoid organs. In addition, our findings revealed that CD28 superagonistic antibodies not only retained T cells in spleen and lymph nodes but also interfere with T cell egress. This is mainly mediated through EDG-1, the S1P receptor that is predominantly expressed on T lymphocytes and guides their egress from thymus and secondary lymphoid organs in response to S1P present at elevated levels in the circulation (21). We could show that JJ316, similar to costimulation of antigen-specific cells, strongly downregulates EDG-1 levels on T lymphocytes, which impairs their ability to migrate along a S1P gradient. This is presumably the consequence of increased CD69-surface expression following infusion of the CD28 superagonistic antibody and closely resembles the effects of FTY720, a S1P receptor antagonist that is presently tested for its application in the treatment of inflammatory conditions in humans (25). Our experiments confirmed that induction of T lymphopenia by JJ316 and FTY720 occurs with similar kinetics and magnitude, indicating that EDG-1 might indeed account for the prolonged trapping of T cells in spleen and lymph nodes. Taken together, we suggest that impaired egress from secondary lymphoid organs, in combination with increased cell-cell and cell-matrix interactions, underlies at least in part the prolonged T lymphopenia induced by CD28 superagonistic antibodies.

In addition to the proadhesive alterations, JJ316 also strongly induced expression of membrane activation markers and proinflammatory mediators. Within a few hours after antibody infusion, mRNA expression of the cytokines Ifng and Il17, the chemokines Mep1 and Rantes as well as of Il2ra was dramatically elevated in both, naive and effector T cells. This indicates broad and fulminant T cell activation and is in agreement with our previous observation that IFNγ is upregulated after in vitro stimulation with JJ316 (10). With some delay, cell-surface expression of CD25 (IL-2Rα), CD69, and CD134 (Ox40) were also strongly enhanced by JJ316. Surprisingly, however, the concentrations of TNFα and IFNγ in the serum of rats treated with JJ316 were only very modestly increased while MCP-1 was not detectable at all. Importantly, peak cytokine concentrations were 2 to 3 orders of magnitude below the levels achieved by injection of a nonlethal dose of LPS and were not accompanied by notable abnormalities in the appearance, behavior, or organ morphology of the rats (12, 26). At a 5-fold lower dose of JJ316, cytokines could not be measured in the serum at all. This suggests that secretion rather than the synthesis of proinflammatory mediators constitutes the main difference between rats and humans and confirms that the massive cytokine storm encountered in the clinical trial could not have been foreseen on the basis of experiments in rodents. However, it is noteworthy that such studies are generally performed in young animals that grow up under virtually pathogen-free conditions.

For that reason we additionally tested diabetes-prone BB rats, 6- to 9-month-old Lewis rats, and rats that had suffered from EAE but were unable to identify any pathological effects of JJ316 (data not shown). Nevertheless, we are fully aware that these conditions still do not compare by any means to human adults having experienced 20 years of recurrent infections.

While systemic cytokine levels are definitively low, it is still conceivable that their local concentrations are elevated. Possibly, such an effect could contribute to the activation of bystander cells such as B lymphocytes that are unable to directly respond to CD28 engagement. In line with this notion, we have found extensive cytoskeletal rearrangements and a strong increase in the size of B cells isolated from JJ316-treated rats. However, stimulating B cells in culture with JJ316 did not induce any alterations.

Interestingly, the first wave of T cell activation induced by CD28 superagonistic antibodies is transient in nature. Within 24–48 hours, the changes in cell size, F-actin polymerization, CD25 expression, and the increased adhesion to fibronectin disappeared in most lymphocytes. In contrast, CD4+CD25+FoxP3+ bona fide Tregs experienced a second activatory phase at this stage. Their morphology changed from an unpolared to a polarized phenotype and after 3 days, the difference in size and motility between Treg and Th cells was quite remarkable. As shown previously, Tregs also proliferate more vigorously at this time point, leading to their strong enrichment (11). Thus, a first wave of phenotypic alterations affecting all lymphocytes is followed by a second wave of effects that preferentially pertains to Tregs. While these 2 phases are not clearly intercepted in vivo, our in vitro analyses are clearly in support of such a notion. All T cells were rapidly activated by JJ316, but their size returned to basal levels after 2 hours. Subsequently, preexisting CD4+CD25+FoxP3+ bona fide Tregs experienced a second phase of activation characterized by a marked increase in size while the other T cell subsets did not respond any longer. FoxP3 expression remained unaffected by the CD28 superagonistic antibody during the course of the experiment. Nevertheless, it does not appear that different signal transduction pathways are engaged by JJ316 in Th as compared with Tregs (27). Our results rather suggest that the first activation phase is similar in all T cell subsets, resulting in more sustained signaling and the observed second activation phase. Finally, cytokines that may locally be released after JJ316 stimulation could preferentially act on Tregs, thereby, inducing their restimulation. This, however, could only be tested in knockout models that are not available in rats. In summary, CD28 superagonistic antibodies cause a biphasic activation of T cells despite apparently inducing similar signaling pathways.

We believe that our observations contribute to a better understanding of what happened during the clinical trial of TGN1412 in
March 2006 (12). We assume that the rapidly occurring effects of the humanized CD28 superagonistic antibody were a consequence of the first wave of T cell activation, with the exception that rats do not experience a massive cytokine storm. During the second wave of T cell activation, the effects of CD28 superagonistic antibodies on Tregs dominate and are likely to account for the beneficial effects observed in rodent models of autoimmune diseases. Therefore, these antibodies are a 2-sided sword and require extensive investigation before they may be successfully applied to humans.

Methods

Animal experimentation. Lewis rats were bred in our own animal facility and kept in individually ventilated cages. Male rats were used for experimentation at an age of 8 weeks (+250 g). J316 (dissolved in 0.3 ml PBS) and FTY-720 (dissolved in 0.1 ml 50% EtOH) were injected into the tail vein, and LPS (serotype 011:B4) was injected i.p. at a concentration of 4 mg/kg. To track lymphocytes in vivo (see Figure 1D), they were purified from donor rats and labeled with 5 μM CFSE for 8 minutes at room temperature. Cells (4 × 10⁶ per rat) were injected into the tail vein under deep anesthesia. To study resident leukocytes in lung and liver, the rats were perfused with PBS prior to dissecting these organs. The procedures for performing animal experiments as well as animal care were in accordance with the principles of the Bavarian and Lower Saxony state regulations and approved by the Regierung von Unterfranken (Würzburg, Germany), the Regierung von Oberbayern (Munich, Germany), and the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Oldenburg, Germany).

Antibodies and reagents. The following antibodies were used for immunofluorescence and western blotting: anti–phospho-cofilin (Ser3) from Cell Signaling, anti-cofilin (N19) and anti–rabbit-HRP from Santa Cruz, anti–β-tubulin (TUB2.1) from Sigma-Aldrich, anti-FoxP3 (FJK-16a) from eBiosciences, anti-rabbit/rat/mouse Alexa Fluor 488/594 and phallolidin–Alexa Fluor 488/594 from Molecular Probes, and anti–goat-HRP from Dianova. Antibodies against cell-surface molecules used for flow cytometry were obtained from BD Pharmingen: CD4 (Ox38), CD8α (Ox8), CD25 (Ox39), CD45RC (Ox22), CD134 (Ox40), CD62L (HL1), β1–TCCR (R73), granulocyte antigen (His48), LFA-1/CD11a (WT.1), Thy-1 (Ox7), RT6.1 (P4/16), CD69 (Yuggu-F6); kindly provided by Jung-Hyun Park, NIH, Bethesda, USA), Poly-γ-Lysine, S1P, LPS, CFSE, LY294002, and U0126 were purchased from Cell Signaling, Sigma-Aldrich, fibronectin from TebuBio, FTY720 from Biozol, and CMTMR [5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethyl-rhodamine] from Invitrogen. J316 is derived from a hybridoma cell line and was provided by Thomas Hünig (University of Wuerzburg).

Magnetic cell sorting. Single-cell suspensions were prepared from lymph nodes or spleen followed by magnetic cell sorting using an AutoMacs machine (Miltenyi) as previously described (10). CD4+ T cells were obtained by depleting B cells (Ox33), γδT cells (V65), NK cells (10/78), and CD8α cells (Ox8). Th and Tregs were further separated by positive selection for CD25 expression (Ox39); the purity of both subpopulations was greater than 90% based on flow cytometric analysis of FoxP3. The same strategy was employed to isolate CD8α+ and B cells, with the exception that Ox35 (specific for CD4+ cells) was substituted for Ox8 or Ox33, respectively.

FACS analysis. Lymphocytes were isolated by passing the freshly isolated organs through a 40 μm nylon mesh, washed in FACS buffer (PBS with 0.5% BSA and 0.05% NaN3), counted, and stained using various antibody combinations. In case of liver and lung, the lymphocytes were isolated by density centrifugation following perfusion with NaCl. The dissected tissue was passed through a metal mash, and after centrifugation the homogenate was resuspended in 28 ml of 40% Percoll, overlaid on 12 ml of 80% Percoll, and spun for 20 minutes (950 g, 4°C). Finally, the lymphocytes were harvested at the interface between the layers. To remove erythrocytes, blood samples were treated with OptiLyse (Beckman Coulter) after staining. Analysis by 4- and 6-color flow cytometry was performed using a BD FACS Calibur or a BD FACS Canto II machine, respectively, in combination with DiVa and CELLQUEST software.

Intravital imaging of T cell locomotion. CD4+ effector TSBP-GFP and TOWA-GFP lines were established and tested for their phenotype and antigen specificity as reported previously (16). Splenic T cells, containing greater than 80% naive lymphocytes, were purified by depleting monocytes/macrophages (Ox42) and B cells (Ox33). Then, they were labeled with 3 μM of the red fluorescent dye, CMTMR, by incubating the cells for 20 minutes at 37°C. For intravital imaging, 3 × 10⁶ splenic CMTMR+ T cells were conjugated with 5 × 10⁶ GFP effector T cells. Intravital imaging was performed 60 hours later.

Animals were anesthetized and preparation of the spleen was performed as described previously (5). Video time-lapse recordings were performed using an inverted microscope (Axiovert 200M; Zeiss) equipped with a 20×0.4 NA objective (Zeiss). A Coolscan-HQ camera (Photometrics) was used to acquire images in 30-second intervals. MetaMorph (Visitrion Systems) software was used for processing the data. Cell trajectories and velocities were evaluated as described previously (5) using ImageJ software (http://rsb.info.nih.gov/ij/).

Immunofluorescence analysis by laser scanning confocal microscopy. Freshly isolated T cells were attached on poly-L-lysine–coated 8-well chamber slides (Nunc) for 20 minutes at 37°C at a density of 3 × 10⁶ cells in 100 μl medium per chamber. After adding 4% PFA for 10 minutes, the cells were permeabilized with 0.01% Triton-X100 for 5 minutes, blocked with 5% BSA, and incubated with an anti-FoxP3 antibody diluted in 1% BSA overnight at 4°C. After washing, the slides were stained with anti–rat Alexa Fluor 488 secondary antibody, together with phallolidin–Alexa Fluor 594 for 45 minutes at room temperature. The slides were mounted with Fluoromount-G medium (Southern Biotech) and analyzed by confocal microscopy. For visualizing tubulin structures, T cells were fixed with PHEMO-fix (3.7% Parformaldehyde, 0.05% Glutaraldehyde, 0.5% Triton-X100 in PHEMO buffer) for 10 minutes at 37°C, washed twice with PHEMO buffer (68 mM PIPES, 25 mM HEPES, pH 6.9, 15 mM EGTA, 3 mM MgCl2, 10% DMSO) at 37°C, followed by 50 mM NH4Cl and PBS. Subsequently, the cells were stained with an anti–β-tubulin antibody. Imaging was performed using a Zeiss LSM 410 Meta confocal microscope equipped with 63× oil objective (numerical aperture 1.4) and laser lines 488 and 543. Image acquisition was performed with Zeiss LSM software 3.2 SP2. For each quantification, 100–200 individual cells were randomly chosen and analyzed.

In vitro stimulation of lymphocyte subsets. Eight-well chamber slides were coated with 5 μg/ml sheep anti-mouse IgG for 1 hour at 37°C. Slides without anti-mouse IgG were used as control. We incubated 3 × 10⁶ cells/ml with 10 μg/ml J316 for 1 hour on ice, and 100 μl of each cell suspension was incubated at 37°C on prewarmed slides. Stimulation was stopped by adding 4% PFA for 10 minutes, followed by immunofluorescence staining. To analyze the impact of kinase inhibitors, T cells were preincubated with 30 μM LY294002 or 10 μM U0126 for 20 minutes at 37°C and cooled on ice, followed by J316 incubation as described above.

Scanning electron microscopy. Purified T cells were settled on Poly-L-lysine coated coverslips (12 mm) in a 24-well plate at a density of 2 × 10⁶ cells/ml, fixed with 6.25% Glutaraldehyde in 50 mM phosphate buffer, pH 7.2, for 10 minutes at room temperature, and subsequently, incubated overnight at 4°C. After washing with phosphate buffer, the samples were dehydrated stepwise in acetone, critical-point dried, and sputtered with platinum/palladium. Cells were investigated by SEM analysis, using a Zeiss DSM 962 scanning electron microscope.

Fibronectin induced locomotion. For ex vivo live cell imaging, 6-channel μ-slides VI (ibidi) were coated with 20 μg/ml fibronectin in PBS, containing MgCl2/CaCl2, overnight at 4°C. Slides were washed with PBS and blocked.

with 2.5% BSA at 37°C, washed again with PBS, kept in 0.5% BSA at 4°C, and prewarmed before analysis. Purified T cells were resuspended in 0.5% BSA. Imaging was immediately started after adding 1 x 10^6 T cells in a volume of 30 μl into a channel at a 1 minute time frame for 10 minutes. The temperature of the samples was maintained at 37°C using an objective table heater. Data analysis of time stacks was performed using ImageJ software and plugins for manual tracking and chemotaxis (http://rsb.info.nih.gov/iij/).

**SI P chemotaxis assay.** The response of T cells to S1P was studied using 6.5 mm transwell inserts with a 3 μm pore size (Costar). T cells were resuspended in RPMI/0.5% fatty acid free BSA. To assess chemotaxis, 1 x 10^6 T cells in 100 μl volume were placed into each insert. The lower wells of a 24-well plate were filled with 600 μl RPMI/0.5% fatty acid free BSA containing the indicated concentrations of S1P. After 3 hours at 37°C, T cells that had transmigrated into the lower well were harvested and counted using CaliBRITE beads (BD Biosciences) by flow cytometry.

**Cytokine bead array.** Cytokine serum levels were determined by Cytokine bead array (Becton Dickinson Biosciences). The procedure was previously described, amplified using specific primers, and normalized to the amount of the housekeeping gene Actb (29). Data were obtained by independent duplicate measurements.

**Western blot analysis.** T cells were centrifuged and lysed in denaturing sample buffer. After mechanical shearing of DNA, lysates were heated at 95°C for 5 minutes and separated on a 10% SDS-PAGE gel. After transfer, the PVDF membrane was stained with the indicated primary antibodies, visualized with an HRP-conjugated secondary antibody, and developed using ECL as chemiluminescence substrate (Pierce).

**Statistics.** Comparison of multiple experimental groups was performed by 2-way ANOVA followed by a post-hoc Bonferroni multiple comparison test (Figure 3B and Figure 4B). Individual time courses were compared using the repeated measure ANOVA followed by a post-hoc Bonferroni multiple comparison test (Figure 1A and Figure 6D). To compare 2 experimental conditions, the Student’s t test was employed (Figure 1, B and D, Figure 2, B–D, Figure 6, B and C, and Figure 7, A and C). Differences in lymphocyte subtype composition were analyzed using the χ^2 test (Figure 1C). P < 0.05 was considered significant.

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Address correspondence to: Holger M. Reichardt, University of Göttingen Medical School, Department of Cellular and Molecular Immunology, Humboldtallee 34, 37073 Goettingen, Germany. Phone: 49-551-393365; Fax: 49-551-395843; E-mail: hreichardt@med.uni-goettingen.de.