Amplification of Regulatory T Cells Using a CD28 Superagonist Reduces Brain Damage After Ischemic Stroke in Mice

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Background and Purpose—Neuroinflammation plays an important role in ischemic brain injury. Regulatory T cells (Treg) are important endogenous immune modulators. We tested the hypothesis that Treg amplification with a CD28 superagonistic monoclonal antibody (CD28SA) reduces brain damage in murine cerebral ischemia.

Methods—Cerebral ischemia was induced by coagulation of the distal middle cerebral artery or by 60 minutes filament occlusion of the proximal middle cerebral artery in C57BL6 mice. 150 μg CD28SA was injected intraperitoneally 3 or 6 hours after ischemia onset. Outcome was determined by infarct volumetry and behavioral testing. Brain-infiltrating leukocyte subpopulations were analyzed by flow cytometry and immunohistochemistry 3 and 7 days after middle cerebral artery occlusion.

Results—CD28SA reduced infarct size in both models and attenuated functional deficit 7 days after stroke induction. Mice treated with CD28SA increased numbers of Treg in spleen and brain. Tregs were functionally active and migrated into the brain where they accumulated and proliferated in the peri-infarct area. More than 60% of brain infiltrating Treg produced interleukin-10 in CD28SA compared with 30% in control.

Conclusions—In vivo expansion and amplification of Treg by CD28SA attenuates the inflammatory response and improves outcome after experimental stroke. (Stroke. 2015;46:212-220. DOI: 10.1161/STROKEAHA.114.007756.)

Key Words: brain ischemia ■ regulatory T cells
In this study, we tested the hypothesis that in vivo application of murine CD28SA in experimental ischemic stroke causes preferential expansion and activation of Foxp3-expressing Treg and that these augmented and activated Treg cells improve outcome.

### Materials and Methods

#### Animals

All experiments were performed according to national guidelines and to the ARRIVE guidelines and were approved by the governmental authorities (Regierungspräsidium Karlsruhe, Germany). Age-matched 8- to 12-weeks-old male mice (C57BL/6J; Charles River Laboratories) were used for the experiments. IL-10 knockout mice were purchased from The Jackson Laboratory. Mice were kept in the pathogen-free animal facility of the University of Heidelberg. Two mice died after filament middle cerebral artery occlusion (fMCAO) and 4 mice died after permanent middle cerebral artery occlusion (pMCAO) from CD28SA- and IgG control antibody–treated group.

#### In Vivo Application of Antibodies

Unless stated otherwise, mice were treated with 150 μg of rat antimouse CD28 monoclonal (D665) or isotype control antibodies intraperitoneally (IP) 3 hours after middle cerebral artery occlusion (MCAO). CD28SA antibody in PBS was obtained from Exbio (Prague, Czech Republic). The dose was chosen according to previous work showing the expansion of Treg cells in vivo in response to CD28SA. IgG1 isotype control antibody was purchased from eBioscience. Animals were randomly allocated to treatment groups.

#### Experimental Stroke Models

Details for the pMCAO by transtemporal electrocoagulation and the fMCAO for 60 minutes are presented in the online-only Data Supplement.

#### Analysis of Cytokine Expression

Cytokine concentration in serum samples were measured by Cytometric Bead Array (BD Biosciences) following the manufacturer’s instructions using an LSRII flow cytometer (BD). Results were analyzed using FCAP Array software (Soft Flow, Inc.).

#### Measurement of Infarct Volume

Infarct volume was determined on cresyl violet stained, 20 μm thick coronal cryosections (online-only Data Supplement).

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Figure 1. CD28 superagonistic monoclonal antibody (CD28SA) treatment increases the number of regulatory T cell (Treg) cells in the spleen after permanent middle cerebral artery occlusion (pMCAO). **A**, Flow cytometric analysis of the homing receptor CD103+ in Foxp3+ Tregs per CD4 splenocytes. Three hours after pMCAO, mice were treated with either isotype antibody (control) or CD28SA antibody. Splenocytes were analyzed by flow cytometry 7 days later. **B**, The cell number of indicated cell populations in spleens. *P*<0.05 (t test, n=8 per group, 2 individual experiments). **C**, Kinetic analysis of Treg percentage among CD4 cells after CD28SA. *P*<0.05 as compared with IgG control treated mice (n=5–8 per group). **D**, Cytokine concentrations in sera from isotype control, 50, 150, or 300 μg CD28 treated mice at 1, 3, and 7 days after pMCAO. *P*<0.05 (ANOVA with post hoc Tukey’s test, n=5 per group). IFN indicates interferon; IL, interleukin; and TNF, tumor necrosis factor.
**Functional Outcome Test**
To evaluate sensory-motor deficit, the corner\(^{21}\) and cylinder tests\(^{22}\) were performed 1 day before as well as 1, 3, and 7 days after pMCAO (online-only Data Supplement).

**Leukocyte Isolation From Brain and Flow Cytometry Analysis**
Mice were anesthetized and perfused transcardially with saline. Brain hemispheres were mechanically homogenized. The cell suspension was incubated in dissociation buffer (180 U collagenase IV and 250 U DNase in PBS) and overlaid on percoll gradients of 1.03 and 1.088 g/mL density. A single layer of leukocytes was collected from the interphase and analyzed by flow cytometry (online-only Data Supplement).

**Immunohistochemistry**
Immunohistochemical staining was performed on 12 \(\mu\)m-thick coronal cryosections taken at the level of the bregma. Slides were fixed in 4% paraformaldehyde. Sections were incubated with anti-Foxp3 (FJK-16s) followed by antirat IgG-biotinylated secondary antibody (Vector). Immunoreactivity was visualized by the avidin–biotin complex method (Vector), and sections were developed in diaminobenzidine (Vector) and mounted on coverslips. We captured images on a Zeiss Axiosvert 200 mW microscope.

**Statistical Analysis**
Analysis was performed using GraphPad Prism 6.0 (GraphPad Software). Bar graphs are represented as mean± SD. Numbers of animals (n=9) to detect infarct size were determined via a priori calculation (effect size \(\delta\) [by ANOVA] of 0.6 with \(\alpha\leq0.05\) and a power of 0.80) according to previous results.\(^7\)

**Results**

**CD28SA Treatment Causes Treg Expansion After Cerebral Ischemia**
First, we examined whether CD28SA expands Treg after cerebral ischemia. CD28SA was injected 3 hours after pMCAO, and splenocytes were analyzed by flow cytometry 7 days later. CD28SA antibody increased Treg in spleen by 4-fold compared with control. Because polyclonal Treg expansion after CD28SA induces homing markers and migration to inflamed sites,\(^23\) we also analyzed the expression of the tissue homing marker CD103\(^{24,25}\) in splenic Treg. We observed a 3-fold increase of the percentage of CD103 expressing Treg compared with control mice (Figure 1A and 1B). Kinetic analysis revealed that expansion of Treg was present already 3 days after CD28SA administration (Figure 1C).

Sera from isotype control or different doses (50, 150, and 300 \(\mu\)g) of CD28SA-treated mice were analyzed for the presence of circulating IL-2, IL-4, IL-6, IFN-\(\gamma\), TNF-\(\alpha\), and IL-10 at 1, 3, and 7 days after pMCAO (Figure 1D). One day after treatment, we could hardly detect any cytokine expression after CD28SA treatment. At 3 days after pMCAO, we...
detected a significant upregulation of IL-6, IFN-γ, and TNF-α in the 300 μg CD28SA group. However, the mean amount of IL6, IFN-γ, and TNF-α (15, 7, and 18 pg/mL, respectively) found in the serum was rather low. In our present study, circulating IL-10 serum levels were significantly increased in the 150 and 300 μg CD28SA groups, which presumably resulted from expansion of Treg cells. At day 7 after CD28SA treatment, the expression of cytokines had returned to baseline compared with control.

**CD28SA Reduces Infarct Size**

Administration of CD28SA 3 hours after pMCAO significantly reduced infarct volume at day 7 compared with isotype control–treated mice (Figure 2A). CD28SA treatment also improved behavioral outcome compared with the control group 7 days after pMCAO as determined by the corner test and the cylinder test (Figure 2B). CD28SA did not affect mortality in this model (2 mice in CD28SA and 2 mice in IgG).

To describe the kinetics of the effect of CD28SA in more detail, infarct volumes were measured at 1, 3, and 7 days after pMCAO in separate experiments with different doses (50, 150, 300 μg) of CD28SA. Infarct volumes did not differ at 1 day after pMCAO (data not shown), whereas CD28SA-treated mice had a significantly smaller infarct size in 150 g- and 300 μg-treated mice 3 days (Figure 2C, left) and 7 days after pMCAO (Figure 2C, right).

To examine a potentially longer therapeutic time window, CD28SA was injected 6 hours after pMCAO treatment. CD28SA still reduced infarct size 7 days after pMCAO (Figure 2D).

To validate the beneficial effect of CD28SA in another experimental model of cerebral ischemia, transient MCAO was induced for 60 minutes using an endovascular filament. The mortality in this model was 5% in both groups. In accordance with previous reports, we observed a significantly decreased absolute cell number of total CD4+CD3+ T cells in the spleen in both CD28SA- and isotype-treated control mice 3 days after MCAO in this stroke model, which causes substantially larger infarcts (Figure 3A and 3B). However, after CD28SA treatment, absolute Foxp3+ Treg splenic cell counts as well as their percentage within the CD4+ T cell population were significantly increased compared with the control group (Figure 3A and 3B). Infarct volume was significantly reduced in CD28SA-treated mice compared with control 3 days after fMCAO (Figure 3C).

**Tregs in the Peri-Infarct Area Are Increased by CD28SA**

To determine the effect of CD28SA on brain-infiltration of Foxp3+ Treg after stroke, mice were treated with CD28SA or isotype antibody, and 7 days after pMCAO, leukocytes were isolated from the brain and analyzed by flow cytometry. Cells were gated as CD45high to distinguish infiltrating leukocytes from microglia. T-lymphocytes were subdivided into CD3+CD4+ subsets, and Treg cells were identified by expression of Foxp3 (Figure 4A and 4B). Although the absolute cell number of CD3+ T cells was not affected in both groups, the number of Treg was significantly higher in brains of CD28SA-treated mice compared with controls (Figure 4C). The proliferation of Treg cells was specified by expression of Ki-67 (Figure 4A). In isotype-treated mice, Foxp3+ Treg cells represented 23±7.4% of CD4+ T cells, and 72%±8.3% of Treg cells were proliferating. In CD28SA-treated mice, 60%±2.3% of CD4+ T cells in the brain were Foxp3+. Moreover, 92%±2.4% of Foxp3+ Treg were Ki-67+, indicating that the vast majority of Treg in the ischemic brain was proliferating and thereby supporting Treg activation on CD28SA stimulation (Figure 4B). To investigate the topographical distribution of Foxp3+ Treg in the ischemic brain, cryosections of brain from CD28SA and control mice were stained for Foxp3. Treg were located mainly in the peri-infarct area of ischemic brains in both groups. The number of Foxp3+ Treg was substantially higher in CD28SA-treated mice (69±8/section; n=4; P<0.05, t test) compared with control mice (24±4.7/section; n=4; Figure 4D).

**CD28SA Reduces Activation of Macrophages**

To examine the effect of amplified Treg on other immune cells after ischemia, we analyzed the activation state of brain infiltrating macrophages by flow cytometry.
7 days after pMCAO. Similar to previous publications, we used the expression of CD45 to distinguish between CD11b+ microglia, expressing only a low level of CD45, and CD11b+ macrophages/dendritic cells, expressing high levels of CD45 after ischemic stroke. To exclude dendritic cells, CD11b+CD11c− cells were gated and the activation of macrophages was determined by the expression of MHCII (Figure 5A). The expression of MHCII on macrophages was reduced in CD28SA-treated mice compared with control mice (Figure 5B, left). In contrast, MHCII expression on CD45 low microglial cells was similar in both groups (Figure 5B, right). Additionally, we also analyzed the infiltration and activation of granulocytes using Ly6G as marker and observed that the percentage of Ly6G and also mean fluorescence intensity of the activation marker CD11b was not altered by CD28SA treatment (Figure 5C).

**Brain Invading Treg Produce More Interleukin-10 After CD28SA Treatment**

To investigate the functional activity of brain infiltrating Treg, isolated leukocytes from pMCAO mice were stained for IL-10 expression and analyzed by flow cytometry. Consistent with previous reports showing that CD28SA induces IL-10 production in Treg in the periphery, we observed a significant increase of the proportion of IL-10 producing Treg in the brain after pMCAO (Figure 6A). The main source of IL-10-producing cells within the CD4+ T cells in the brain was Foxp3+ Treg (Figure 6A). After CD28 treatment, 61%±2.7% of Foxp3+ Treg produced IL-10 compared with 33%±4% in control, suggesting that Treg in the brain were functional and highly activated (Figure 6A). We also analyzed IFN-γ and TNF-α expression in brain infiltrating cells. In CD28SA-treated mice, the expression of IFN-γ was reduced significantly in CD4 cells compared with control mice. TNF-α expression was lower in CD4 T cells and infiltrating macrophages (Figure 6B and 6C).

To further investigate the effect of IL-10 in CD28SA treatment, IL-10 knockout mice were treated with isotype antibody or CD28SA following pMCAO. Although Treg cells expanded in IL-10 knockout mice after CD28SA (Figure 6D, left), infarct size did not differ (Figure 6D, right). Clearly, this supports an essential role of IL-10 for the effect of CD28SA in this model.
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Discussion

The present study yields 5 new findings: (1) injection of CD28SA induces a strong expansion of Treg in the periphery after cerebral ischemia; (2) treatment with CD28SA within 6 hours after ischemia onset improves outcome and (3) leads to increased brain immigration of Treg after cerebral ischemia; (4) CD28SA treatment increases IL-10 production by Treg; and (5) in mice treated with CD28SA, macrophages in the brain seem to be less activated.

Previous work from our group suggested that endogenous Treg suppress postischemic neuroinflammation and thereby prevent delayed brain infarct growth. This finding was originally made in an experimental paradigm using depletion of Treg cells by CD25 antibody as well as in adoptive lymphocyte transfer without Treg into lymphocyte-deficient Rag2 knockout mice. More recently, we also depleted Treg in transgenic mice expressing the diphtheria toxin receptor under the FoxP3 promoter using diphtheria toxin, confirming our previous findings. The increase of infarct volume in Treg-depleted mice was attributed to an exacerbation of the neuroinflammatory response, which was recently confirmed. These findings were made in models causing moderately extensive infarcts (ie, 30 fMCAO or distal pMCAO). On the other hand, although we and other investigators did not find a negative effect of Treg depletion after extensive cerebral ischemia, Kleinschnitz and coworkers reported a deleterious effect of Treg after prolonged fMCAO. The reason for the discrepancy of the Treg effects among models is not resolved, but may be related to the different intensity of the inflammatory response in the brain in different ischemia models.

The putatively protective effect of endogenous Treg in experimental stroke has prompted the exploration of the question whether therapeutic augmentation of Tregs may represent a promising strategy to protect the ischemic brain. Hu and coworkers reported that adoptive transfer of CD4CD25+ Treg improved infarct volume and long-term behavioral outcome. However, adoptive cell transfer of autologous Treg is hardly a viable strategy for patients in the hyperacute stroke phase. Alternatively, pharmacological amplification of Treg may be more feasible from a translational perspective. Using a paradigm of Treg amplification with histone decarboxylase inhibitors introduced by Hancock and colleagues, we found a beneficial effect of boosting endogenous Treg. However, treatment had to be initiated before ischemia onset to be effective. In the present study, we tested a more powerful and less pleiotropic approach of boosting Treg compared with histone deacetylase inhibition–stimulation with the CD28SA. Similar to previous studies in healthy mice and various disease models, CD28SA substantially increased the number of Treg in the periphery as well as in the brain.

Figure 5. Brain infiltrating macrophages are less activated in CD28 superagonistic monoclonal antibody (CD28SA)–treated mice. 
A. Representative gating strategy of leukocytes isolated from the ischemic hemisphere 7 days after pMCAO. Surface expression of major histocompatibility complex class II (MHCII) and CD11b was used as activation marker for macrophages/microglia and Ly6G+ granulocytes, respectively. B. Proportion of MHCII-positive cells that are gated on CD11b+CD45high macrophages or CD11b+CD45low resident microglia (*P<0.05, data are presented as mean±SD, n=8; 4 individual experiments). C. Proportion of Ly6G+ cells that are gated on CD45high-infiltrated cells and the mean fluorescence intensity (MFI) of CD11b expression. FCS indicates forward scatter; and SSC, side scatter.
treatment had a protective effect as measured by infarct size as well as by sensory-motor deficit. Moreover, the time window of ≥3 to 6 hours after ischemia onset supports potential therapeutic relevance, whereas histone deacetylase inhibition in our previous study had to be administered before ischemia onset to be effective. Importantly, the protective effect of CD28SA was robust across 2 experimental models of cerebral ischemia in our present study. Similar to several earlier studies addressing adaptive immunologic mechanisms, the effect of CD28SA was not evident 24 hours after MCAO, but became apparent 3 to 7 days after ischemia onset, suggesting interference with delayed mechanisms of brain damage.

The present findings are consistent with previously identified mechanisms of action of Treg in cerebral ischemia. Treg invade the brain and accumulate in the infarct border zone where they proliferate. This accumulation of Treg was augmented by CD28SA. Moreover, 90% of Treg expressed Ki-67, indicating that most Treg cells were activated and proliferating. CD28SA also boosted the in vivo and in vitro production of IL-10, a key mediator of Treg in various conditions, including brain ischemia. After CD28SA treatment in the present study, Treg cells were strongly activated and represented an impressive proportion of all IL-10-producing suppressor cells. This is in accordance with observations in other inflammatory disease models where CD28SA treatment boosted the number of IL-10-producing Treg that migrate into inflamed skin tissue to limit the inflammatory milieu. Indeed, we found a drastic increase of IL-10-producing Treg not only in the peripheral...
immune system but even more pronounced in the ischemic hemisphere. About 30% of brain-infiltrating Treg produced IL-10 in isotype-treated ischemic animals, which exceeds the percentage of IL-10-producing splenic Treg in the periphery. We also observed less activated macrophages in the brain after CD28SA, which corresponds to our previous finding that Treg affect neuroinflammation even before invading the brain.7

The primary aim of our study using CD28SA was to provide a proof of principle that augmentation of Treg is beneficial in experimental ischemic stroke. Any consideration of translation to the treatment of stroke patients must take previous severe adverse effects of a human CD28SA antibody into healthy subjects into account as human CD28SA induced a massive cytokine release syndrome in a phase I clinical trial.10,31 This serious adverse effect was probably caused by the stimulation of effector memory CD4 T-cells.32 We only found a mild increase of serum cytokine in the highest dose of CD28SA in the present study. However, from a safety perspective, the translation of our findings in mice to patients has to take into account the differences between species that have been specifically elucidated in the context of CD28SA19 subsequent to the disastrous cytokine release syndrome observed in a phase I study.20

Interestingly, recent data suggest that CD28SA-activated and -expanded human Treg are very potent suppressors of inflammatory cytokine release in PBMC cultures from patients with rheumatoid arthritis.34 The same study also showed that slow infusion of much lower doses (≤7 μg/kg) than used in the ill-fated HV trial of 2006 (bolus injection of 100 μg/kg) is well tolerated and leads to systemic release of IL-10 but not of proinflammatory cytokines. Further studies in different indications are needed to determine whether transient polyclonal Treg activation could be a safe and effective approach for stroke and other indications.

Inflammation after stroke encompasses immunosuppression and resulting increased susceptibility to bacterial infection. A limitation of our study is that we did not examine potential microbiological complications of CD28SA. Although Treg are rather conceived as immune modulators than as immunosuppressors,35 the effect of polyclonal activated Treg on long-term immunologic outcome requires further investigation.

In conclusion, our study suggests that CD28SA expands the number of Treg cells that produce IL-10 and thereby protects against brain damage in experimental murine stroke.

Acknowledgments
We thank Sabrina Heide for excellent technical assistance.

Sources of Funding
This work was supported by the Deutsche Forschungsgemeinschaft (DFG VE196/3-1) and the Else Kröner-Fresenius Foundation (2012/A118).

Disclosures
Dr Hünig is a consultant to TheraMAB LLC.

References
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*Stroke*. 2015;46:212-220; originally published online November 6, 2014;
doi: 10.1161/STROKEAHA.114.007756

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/46/1/212

Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL

Amplification of regulatory T cells using a CD28 superagonist reduces brain damage after ischemic stroke in mice

Supplemental Methods

Experimental stroke models

In most experiments, experimental brain ischemia was induced by permanent transcranial coagulation of the middle cerebral artery (MCA) distal from the lenticulo striatal arteries as described previously\(^1\). Briefly, mice were anesthetized with 1.0–2.0% isoflurane in O\(_2\)/N\(_2\)O. After a 1cm long skin incision between the left eye and ear, a burr hole was drilled, the dura mater was removed and the MCA was occluded permanently (pMCAO) using a bipolar electrocoagulation forceps (ERBOTOM, ERBE). For transient focal ischemia, mice were anesthetized with 1.0–2.0% isoflurane in O\(_2\)/N\(_2\)O. After neck dissection an incision was made into the external carotid artery between 2 ligations, and a silicon-covered 8–0 nylon monofilament (Doccol Corporation) was advanced via the internal carotid artery until a resistance was felt. Successful occlusion was verified by laser doppler flowmetry. Only animals with a reduction of relative cerebral blood flow to < 25% of baseline were included in the study. 60 minutes after filament insertion, mice were anesthetized again and the filament was removed.

Allocation of animals to treatment groups was randomized. Surgeons were blinded to treatment assignment. We excluded animals in which surgery lasted for more than 25min, or in which mice lost more than 25% of their body weight during the 7d observation period.

Measurement of Infarct Volume
Mice were anesthetized with an i.p. injection of Ketamin/Xylazin (100mg/kg and 10mg/kg, respectively) and transcardially perfused with saline. Brains were removed, immediately frozen and 20µm thick coronal cryosections were cut every 400µm. Stained sections were scanned at 300dpi and the infarct area was analyzed using a public domain image analysis program (ImageJ) program. The total infarct volume was obtained by multiplying measured areas and distance between sections. Correction for brain edema was applied by subtraction of the ipsilateral minus contralateral hemisphere volume from the directly measured infarct volume. Evaluators were blinded to treatment assignment.

**Functional outcome test**

Mice were placed between two boards each with a dimension of 30 x 20 x 1cm set at a 30° angle with a small opening along the joint to encourage the mouse entering the corner. Left and right turns with rearing movement were counted. We scored 12 turns for each test and calculated the ratio of right turns of all turns and normalized on the performance prior surgery of each individual mouse. For the cylinder test, mice were placed in a glass cylinder (8 cm in diameter) and recorded for 10 min. We scored contact of the cylinder wall with one forelimb during a full rear and landing with one forelimb at the cylinder bottom. At least 20 independent contacts were counted for one forelimb.

**Flow cytometry analysis**

Cells were stained for FACS using the following antibodies: CD4-PerCP-Cy5.5, CD4 Alexa 647, MHCII-FITC, CD45.2-APC, CD11c-PE, CD3-PE-Cy7, CD8a-APC, CD69-PerCP-Cy5.5, IL-10-PE, CD11b-APC-Cy7, Ly6G-FITC (BD Biosciences) and Foxp3-PE or FITC, IFN-γ-PE, TNF-α-PE, Ki-67 Alexa 700 from eBioscience. For intracellular staining, cells were restimulated with 100ng/mL phorbol myristate acetate (Sigma-Aldrich) and 600ng/mL ionomycin (Sigma-Aldrich) in the presence of protein transport inhibitor (BD GolgiStop or
GolgiPlug, BD Biosciences) in RPMI containing 10% fetal calf serum for 4 hours and stained with CD3, CD45, CD4, IL-10, CD11b, IFN-γ-PE, TNF-α and Foxp3 according to the manufacturer’s instructions (eBioscience). Data were collected on a LSRII flow cytometer (Becton Dickinson, Heidelberg, Germany) and analyzed by FlowJo software (Tree Star, Ashland, OR).

Reference