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Letter to the Editor

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Superagonistic CD28 stimulation induces IFN-γ release from mouse T helper 1 cells in vitro and in vivo

We have recently shown that mouse and human T helper 1 (Th1) cells require T cell receptor (TCR) and CD28 costimulation to efficiently release IFN-γ in vitro and in vivo [1]. Whether isolated CD28 stimulation, as mediated by superagonistic anti-CD28 monoclonal antibodies (CD28-SA), will also trigger IFN-γ release from mouse Th1 cells is, however, still unknown. For humans, it has been shown that the CD28-SA TGN1412/TAB08 induces IFN-γ secretion by Th1 cells and also the cytokine release syndrome (CRS) caused by saturating amounts of TGN1412/TAB08 is marked by high concentrations of IFN-γ in the circulation [2]. This severe adverse reaction had not been anticipated, which was in part due to the failure of rodent models to indicate a risk for CRS induction in humans [3]. Lack of *bona fide* effector/memory T cells in cleanly kept animals [4] would explain why mice and rats did not develop a CRS due to cytokine release from differentiated T helper cells. Moreover, $CD4^+$ $Foxp3^+$ regulatory T cells (Treg) are preferentially activated and expanded over $CD4^+$ $Foxp3^-$ conventional T cells after CD28-SA injection into rats and mice even leading to protection from, for example, EAE [5] and to efficient control of cytokine release by CD28-SA-stimulated Th cells [6].

To test the responsiveness of Th1 cells to isolated CD28 stimulation, we took an adoptive transfer approach using OT-II TCR-transgenic Th1 cells (Fig. 1A; Supporting Information Fig. S1A). All recipient mice received diphtheria toxin, but only DEleter of REGulatory T cells (DEREG) [7] and not WT littermate mice showed a strongly reduced proportion of Treg among $CD4^+$ T cells (Supporting Information Fig. S1B and C). We used DEREG animals as recipients as mouse Treg have already been shown to very efficiently block cytokine secretion after CD28-SA treatment in vivo [6].

IFN-γ concentrations in the serum were strongly increased in CD28-SA-treated mice lacking Treg (Fig. 1B) but also TNF, IL-2, IL-6, IL-17A, IL-4, IL-5, IL-13, and IL-10 were released systemically in these animals (Supporting Information Fig. S2). Particularly with regard to IFN-γ secretion responses were far more homogeneous and stronger among recipients of Th1 cells than in mice without Th1 cell transfer (Supporting Information Fig. S3). On a per-cell basis, CD28-SA treatment induced IFN-γ secretion by OT-II Th1 cells, but not by host-derived $CD4^+$ and $CD8^+$ T cells (Fig. 1C and D; Supporting Information Fig. S4). Depletion of Treg from recipients tripled the proportion of IFN-γ^+ cells among transferred Th1 cells, while not generally increasing the number of recovered Th1 cells (Supporting Information Fig. S1D). Our data, thus, indicate that CD28-SA stimulation induces IFN-γ secretion by *bona fide* mouse Th1 cells in vivo.

For further analyses into the molecular mechanisms behind IFN-γ release from Th1 cells upon CD28-SA stimulation, we established an in vitro cell culture system (Fig. 2A). In these cultures, we either stimulated the Th1 cells exclusively with CD28-SA or in the presence of total splenocytes and/ or Dynabeads Pan Mouse IgG® to provide optimal crosslinking of the CD28-SA (Fig. 2B). Total splenocytes were far less efficient than Dynabeads Pan Mouse IgG® in crosslinking the CD28-SA (Fig. 2B and C), but greatly increased IFN-γ secretion by Th1 cells in the presence of Dynabeads Pan Mouse IgG® with as little as 0.01 μg/mL already inducing a response (Fig. 2C; Supporting Information Fig. S5). Direct stochastic optical reconstruction microscopy (*d*STORM) revealed that CD28 is expressed in microclusters on the surface of mouse Th1 cells (Fig. 2D). Compared to the non-superagonistic anti-CD28 mAb E18 the CD28-SA stained less than half of the CD28 microclusters corroborating that it is the quality of binding, particularly the capacity to bind CD28 bivalently [8], which is key to superagonistic CD28 stimulation.

Analyzing cytokine concentrations in the culture supernatants showed that bystander splenocytes selectively enhanced IFN-γ secretion from Th1 cells upon CD28-SA stimulation (Fig. 2E; Supporting Information Fig. S6). Moreover, cell sorting experiments showed that it was antigen-presenting cells, but not T cells that enhanced IFN-γ secretion by the Th1 cells (Supporting Information Fig. S7). CD28-SA stimulation as well as OVA antigen not only induced CD40L expression on Th1 cells, but also CD40 expression on B cells (Supporting Information Fig. S8). In line with published data [9] blocking CD40L-CD40 interaction

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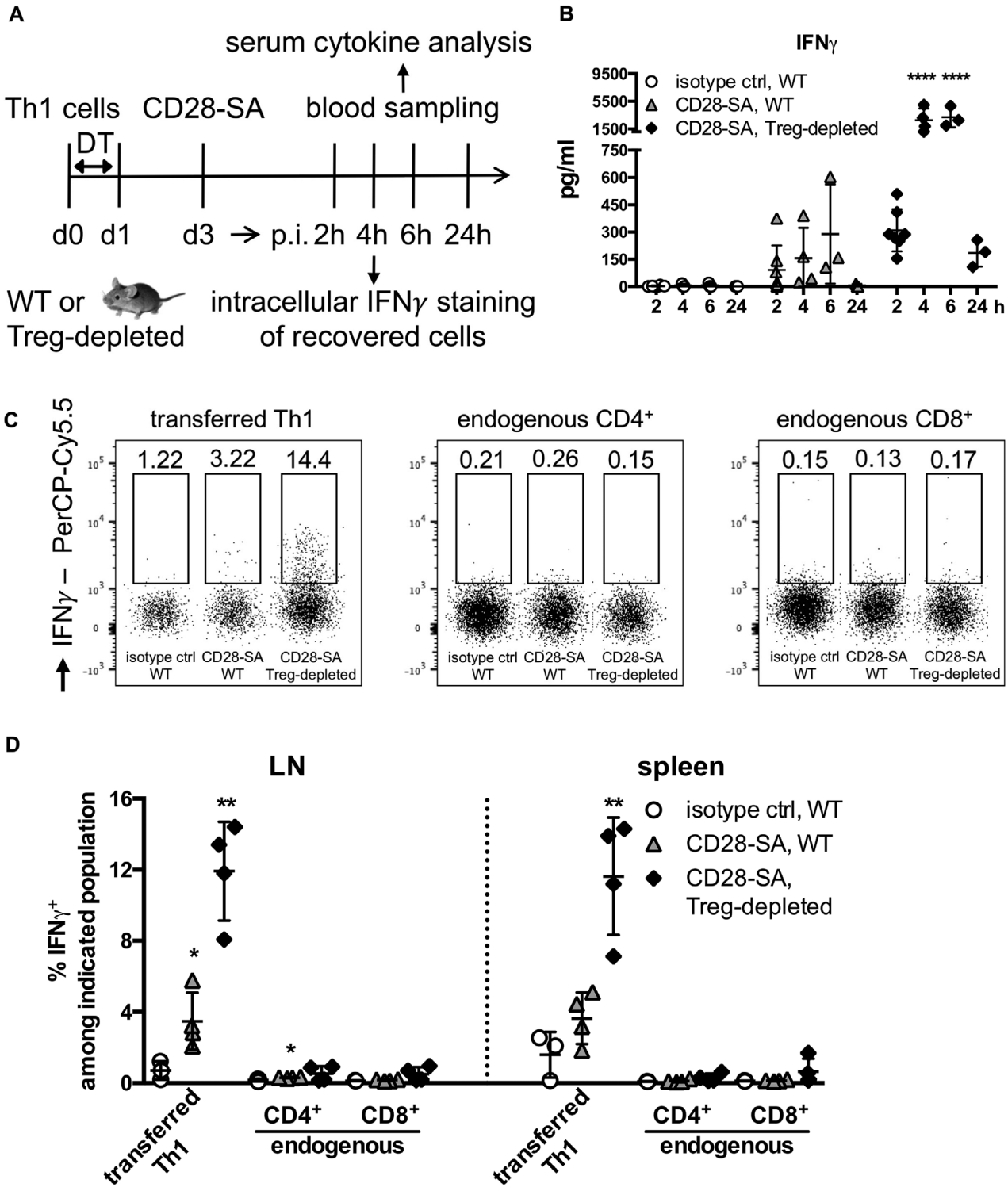

Figure 1. IFNγ secretion by Th1 cells in response to CD28-SA treatment in vivo. (A) Scheme summarizing experimental procedures. For FACS analysis lymph node and spleen cells were isolated. (B) Serum cytokine concentrations of IFNγ measured by bead-based immunassay. Data for individual mice, are shown together with means ± SD. Results were pooled from two to four independent experiments with a total of n = 3 to 7 mice per group. Two-way ANOVA followed by Dunnett test (comparison isotype controls versus CD28-SA at different time points) (**** $p<0.0001$). (C) IFNγ production by endogenous $CD4^+$ and $CD8^+$ T cells as well as transferred $Thy1.1^-$ $Thy1.2^+$ OT-II Th1 cells recovered from lymph nodes was assessed by intracellular staining after two-hour Brefeldin A treatment *ex vivo* (pregating shown in Suppl. Fig. 4) and were analyzed by flow cytometry and displayed using concatenated dot plots. (D) Frequencies of $IFN\gamma^+$ cells among transferred OT-II Th1 and endogenous $CD4^+$ and $CD8^+$ T cells. Data for individual mice are shown together with means ± SD. Results were pooled from two independent experiments with a total of $n = 3$ to 4 mice per group. Two-tailed unpaired *t*-test comparing CD28-SA with isotype control treatment ($^*p < 0.05$; $^{**}p < 0.01$).

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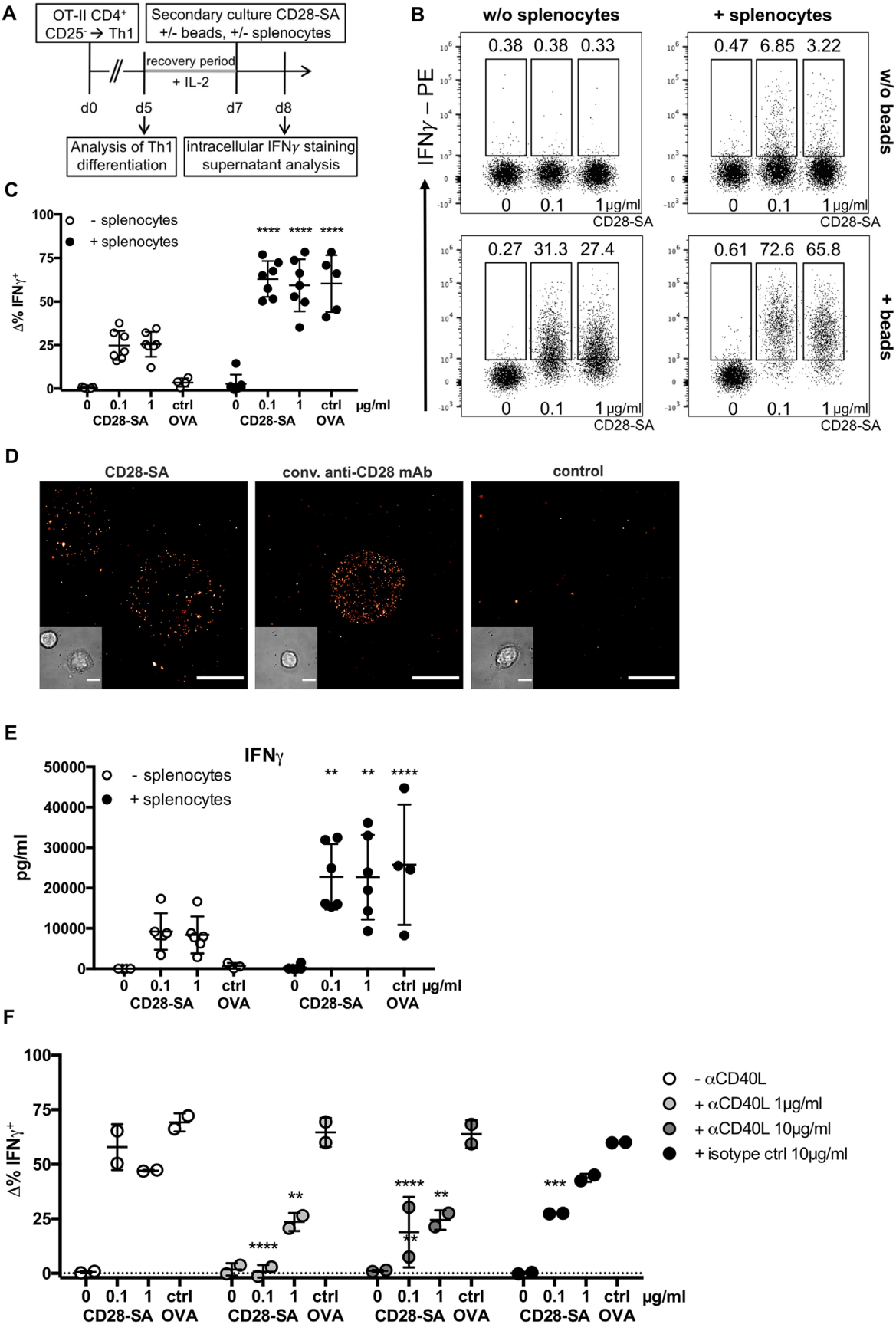

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nullified the enhancing effect of antigen-presenting cells on IFN-γ secretion by CD28-SA-stimulated Th1 cells (Fig. 2F).

We, thus, report for the first time that mouse Th1 cells are fully susceptible to superagonistic CD28 stimulation. The remaining challenges in the mouse model are to approximate the dose-response relationship to the human situation [2] (Supporting Information Fig. S9) and to further define the missing elements required to not only induce strong systemic cytokine release, but also clinically overt CRS [10]. For both aspects FcR binding of the CD28-SA and responsiveness of the monocyte compartment might be critical.

Stephanie Haack[1], Sarah Baiker[1], Jan Schlegel[2], Markus Sauer[2], Tim Sparwasser[3], Daniela Langenhorst[1] and Niklas Beyersdorf[1]

[1] Institute for Virology and Immunobiology, University of Würzburg, Würzburg, Germany
[2] University of Würzburg, Biotechnology and Biophysics, Würzburg, Germany
[3] Johannes Gutenberg University Mainz, Institute for Medical Microbiology and Hygiene, Mainz, Germany

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References

1 **Langenhorst, D. et al.**, *Front. Immunol.* 2018. **9**.
2 **Suntharalingam, G. et al.**, *N. Engl. J. Med.* 2006. **355**: 1018–1028.
3 **Hünig, T.**, *Nat. Rev. Immunol.* 2012. **12**: 317–318.
4 **Beura, L. K. et al.**, *Nature*. 2016. **532**: 512–516.
5 **Beyersdorf, N. et al.**, *J. Exp. Med.* 2005. **202**: 445–455.
6 **Gogishvili, T. et al.**, *PLoS One*. 2009. **4**: e4643.
7 **Lahl, K. et al.**, *J. Exp. Med.* 2007. **204**: 57–63.
8 **Evans, E. J. et al.**, *Nat. Immunol.* 2005. **6**: 271–279.
9 **Blair, P. J. et al.**, *J. Exp. Med.* 2000. **191**: 651–660.
10 **Rosshart, S. P. et al.**, *Science*. 2019. **365**.

Abbreviations: **CD28-SA**: superagonistic anti-CD28 monoclonal antibody · **CRS**: cytokine release syndrome · **DEREG**: DEleter of REGulatory T cells · **SPF**: specific pathogen-free · **TCR**: T cell receptor · **Th**: T helper · **Treg**: $CD4^+$ $Foxp3^+$ regulatory T cell

Keywords: CD28 • Th1 cells • cytokine release • interferon γ • Superagonistic antibody

*Full correspondence:*PD Dr. med. Niklas Beyersdorf, Institute for Virology and Immunobiology, Versbacher Str. 7, 97078 Würzburg; e-mail: niklas.beyersdorf@vim.uni-wuerzburg.de

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Figure 2. Response of Th1 cells to CD28-SA stimulation in vitro. (A) Setup of in vitro CD28-SA stimulation. (B) Concatenated dot plots depict IFNγ expression by OT-II Th1 cells cultured as indicated and measured by flow cytometry. (C) Net frequencies of $IFN\gamma^+$ cells among OT-II Th1 cells after stimulation with CD28-SA or OVA peptide aa329-337 in the presence of Dynabeads Pan Mouse IgG® and with or without total splenocytes. Results were pooled summarizing means of duplicate analyses from seven individual experiments with OT-II T cells originating from two mice and $Thy1.1^+$ splenocytes from one mouse per experiment. $\Delta\%$ $IFN\gamma^+$ = $\%IFN\gamma^+$ (IFNγ staining) - $\%IFN\gamma^+$ (isoclonic control); Graphs show means ± SD. Two-way ANOVA and Sidak test. $^{****}p < 0.0001$. (D) *d*STORM analysis of Alexa Fluor®-647-conjugated CD28-SA (left) and conventional anti-CD28 mAb (center, clone: E18) binding to Th1 cells. As a control Alexa Fluor®-647-conjugated mAb MOPC-21 was used (right). Scale bar: 3μm; original magnification: 160-fold. (E) Concentration of IFN-γ in culture supernatants as measured by bead-based immunassay. Results were pooled from up to six experiments with OT-II T cells originating from two mice and $Thy1.1^+$ splenocytes from one mouse per experiment. Graphs show means ± SD. Two-way ANOVA and Sidak test (comparison without/ with splenocytes). $^{**}p < 0.01$; $^{****}p < 0.0001$. (F) An anti-CD40L mAb (1 μg/mL, light grey, or 10 μg/mL, dark grey) or an isotype control mAb (10 μg/mL, black) were added to co-cultures of Th1 cells and splenocytes and IFN-γ was measured by flow cytometry. Results of duplicate cultures as well as means ± SD are shown. The experiment was repeated at least once with similar result with OT-II T cells originating from two mice and $Thy1.1^+$ splenocytes from one mouse. Two-way ANOVA and Dunnett test (comparison without/ with anti-CD40L/isotype for every stimulus/concentration analyzed). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

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