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Letter to the Editor

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High glucose distinctively regulates Ca^{2+} influx in cytotoxic T lymphocytes upon target recognition and thapsigargin stimulation

The killing processes of $CD8^{+}$ cytotoxic T lymphocytes (CTLs) are highly dependent on calcium, both extracellular and cytosolic [1]. Engagement of TCRs triggers store-operated calcium entry (SOCE) mediated by stromal interaction molecules (STIM) to trap and activate ORAI channels [2]. Glucose is essential for effector functions of CTLs [3]. Elevated level of blood glucose is one of the leading symptoms of diabetes. Yet, the impact of high glucose (HG) on CTL function is not well understood.

To investigate the impact of HG on Ca^{2+} influx in CTLs, we used primary human $CD8^{+}$ T cells, stimulated with anti-CD3/anti-CD28 antibody-coated beads (CD3/CD28 beads) and cultured in normal glucose medium (NG, 5.6 mM) or HG medium (25 mM). To elicit SOCE, we used thapsigargin (Tg) to deplete Ca^{2+} from the ER [4]. For Tg-induced SOCE, we found no difference between NG- and HG-condition for unstimulated $CD8^{+}$ T cells (Fig. 1A-D) or at day 1.5 poststimulation (Fig. 1E-H). Remarkably, at days 3 and 7 after bead stimulation, Tg-induced SOCE was higher in HG-cultured CTLs compared to their NG counterparts (Fig. 1I-P). Upon bead stimulation, CTLs exhibited a significantly enhanced SOCE, which started to decline in NG condition but not in HG condition from day 3 (Fig. 1Q, R). These results suggest that Tg-elicited SOCE in CTLs is elevated by prolonged treatment of HG.

Target cell recognition, a more physiological stimulus than Tg, also elicits SOCE in CTLs. We used staphylococcal enterotoxin A and B (SEA/SEB)-pulsed Raji cells as target cells, which induce CTL-mediated cytotoxicity [1, 5]. We observed that at day 3 poststimulation, in HG-cultured CTLs, target recognition-induced Ca^{2+} influx was significantly reduced relative to their NG counterparts, despite of the same basal level of intracellular Ca^{2+} (Fig. 2A-D). At day 7, upon target cell engagement, only a moderate difference in the Ca^{2+} influx rate and the Ca^{2+} peak was identified between NG- and HG-cultured CTLs, whereas no difference in Ca^{2+} plateau (Fig. 2E-H). Furthermore, the Raji cells without SEA/SEB-pulsing did not elicit Ca^{2+} influx in CTLs (Fig. 2I). Together, we conclude that in CTLs, target recognition-induced Ca^{2+} influx is reduced by HG-culture.

Next, we investigated how HG could differently regulate SOCE induced by Tg or target recognition in CTLs. We first examined glucose uptake using a fluorescent analog of glucose 2-NBDG, and found no difference between HG- and NG-cultured CTLs (Fig. 2J, K). Subsequently, we analyzed the expression of STIMs and ORAIs. At mRNA level, in HG-cultured CTLs, *ORAI1* stayed unchanged, while *ORAI2* and *ORAI3* were slightly upregulated; both *STIM1* and *STIM2* were downregulated (Fig. 2L, M). Interestingly, at protein level, STIM1, STIM2, and ORAI1 in HG-CTLs were upregulated in Donor 1 and 4, but remained unchanged in Donor 2 and 3 (Fig. 2N, O). Due to lack of reliable antibody against ORAI2 and ORAI3, their protein levels could not be determined. Our findings suggest that the increase in Tg-induced SOCE in CTLs by HG could be mediated by upregulation of STIM1 and STIM2. Since ORAI2 and ORAI3 mainly function as negative regulators of Ca^{2+} entry in T cells [6, 7], upregulation of ORAI2 and ORAI3 by HG could contribute to the reduction of target recognition-induced Ca^{2+} influx. Inhibitory splice variants of STIMs, for example, STIM2.1 or phosphorylation of STIMs could also play a role.

After glucose loading, proportion of CTLs is reduced in humans [8]. Interestingly, we found that after killing started, the fraction of necrotic CTLs was higher in HG-condition after 16 h but not after 2 h (Fig. 2P, Q), suggesting that in a time scale of several days in HG, CTLs are more prone toward necrosis after execution of their killing function.

One main difference between Tg and target-recognition is that engagement of TCRs upon target-recognition activates many signaling cascades, whereas Tg only blocks the sarco/endoplasmic reticulum Ca^{2+}-ATPase, leading to passive depletion. In addition, CTLs are stimulated globally with Tg and locally with target cells at the immunological synapse (IS). Along this line, other factors could also be involved, for example, mitochondria or PMCA (plasma membrane Ca^{2+} ATPase). Both are enriched at the IS [9, 10] but scarcely distributed in Tg-stimulation. Considering that decreased intracellular

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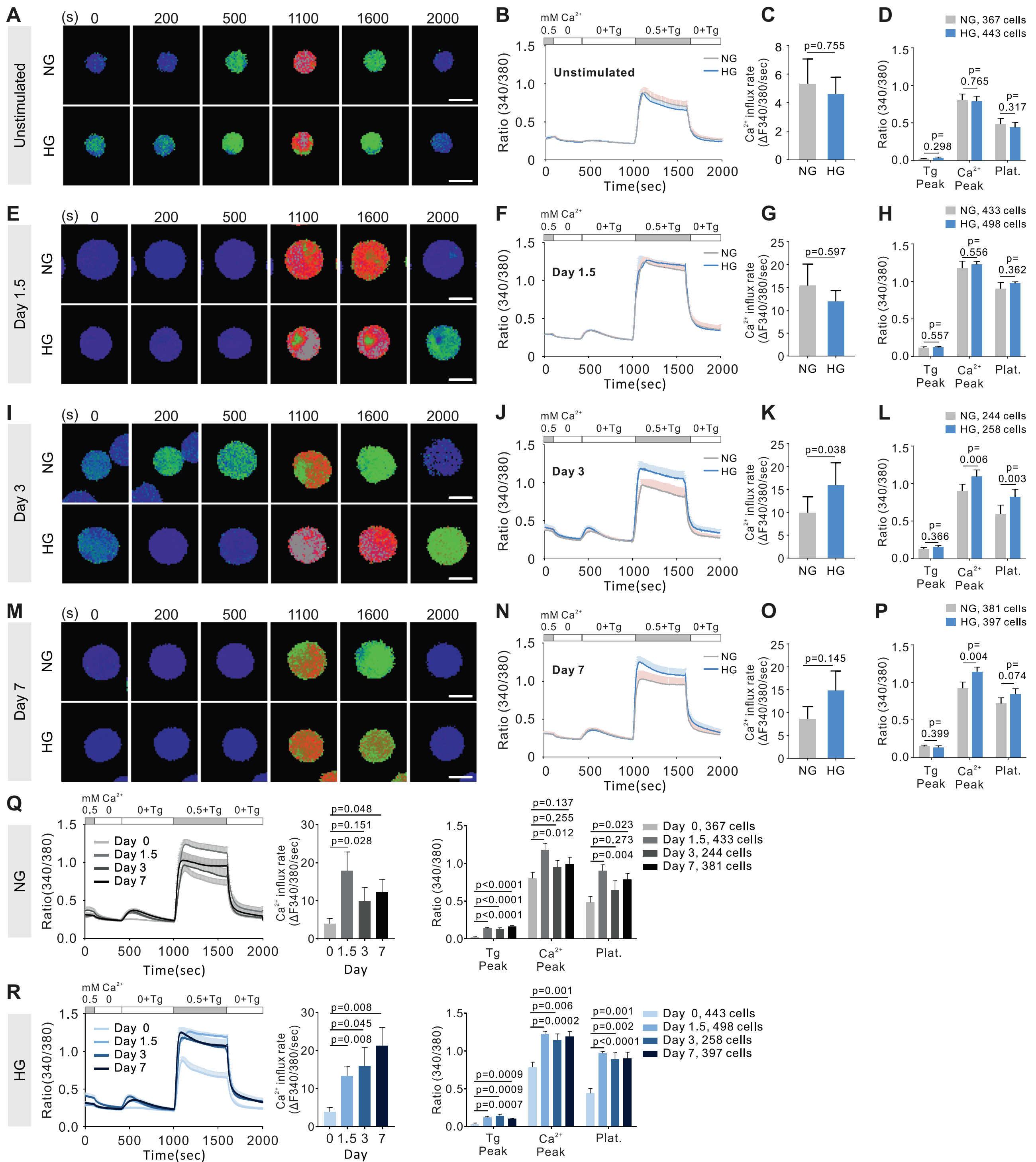

Figure 1. SOCE in CTLs is enhanced by HG-culture. Ca^{2+} imaging was conducted (20 × objective). Primary human $CD8^+$ T cells were either unstimulated or stimulated with CD3/CD28 beads in NG medium (5.6 mM) or HG (25 mM) medium and then loaded with Fura-2-AM. Exemplary cells are shown in A, E, I, and M. Calcium influx curves are shown in B, F, J, N (n [independent experiments/donors] = 4/6 [Day 0/Day 1.5], 5/7 [Day3], or 5/8 [Day7]). Paired t-test was used except for Q and R (unpaired t-test). Results are presented as mean ± SEM. Scale bars are 5 μm.

Ca^{2+} concentration in CTLs is correlated with enhanced CTL-mediated cytotoxicity [1], our findings suggest that increase the environmental glucose for a short period of time might be beneficial for CTLs to fight aberrant cells more efficiently. However, considering our result that HG-treated CTLs tend to die more after killing, in diabetic patients, it might lead to a reduction in CTL numbers, resulting in a comprised immune response against pathogens in a long run.

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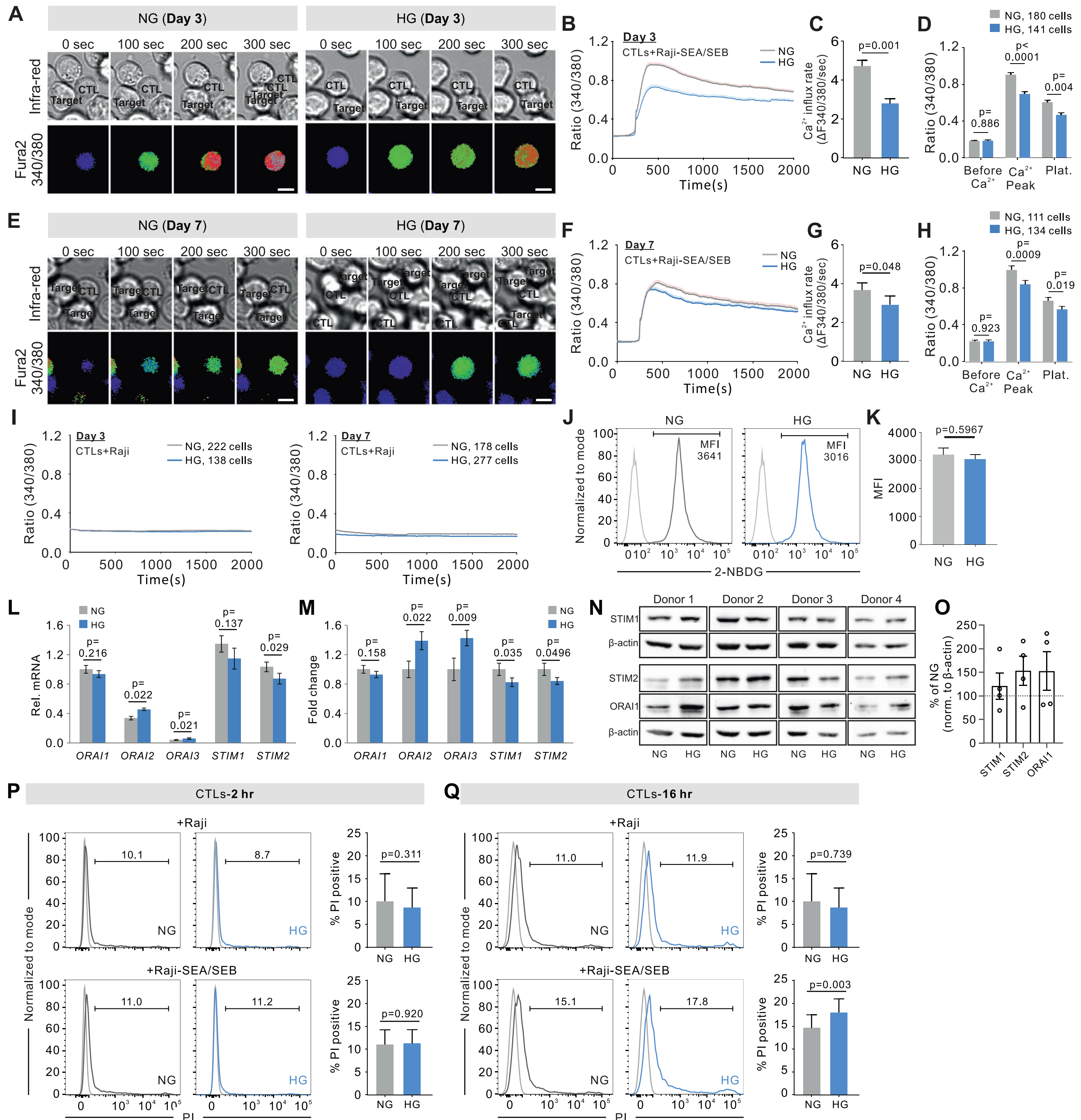

Figure 2. HG-culture reduces Ca^{2+} influx in CTLs upon target recognition and impairs CTL viability after killing. (A-I) Ca^{2+} imaging was conducted. CD3/CD28 bead-stimulated CTLs were loaded with Fura-2-AM (20 × objective, Scale bars: 5 μm). Exemplary cells are shown in A and E. Raji cells pulsed with (A-H) or without (I) SEA/SEB. n (independent experiments/donors) = 5/7 (A-D), 3/6 (E-H), 3/5 (I, Day3), or 3/6 (I, Day7) (J, K) Glucose uptake in CTLs. Day 3 CD3/CD28 bead-stimulated CTLs were loaded with 2-NBDG without target cells. Light gray: unstained cells. n = 5 independent experiments/8 donors. (L, M) Expression of *ORAIs* and *STIMs* in CTLs. The mRNA level was determined using qRT-PCR and either normalized to *ORAI1* in NG-cultured CTLs (L) or normalized to NG condition as fold change (M). n = 2 independent experiments/6 donors. (N, O) Protein levels of STIMs and ORAI1. Quantification by densitometry analysis following Western blotting (N) and normalization to β-actin (Q). n = 4 donors. (P, Q) Necrosis of CTLs after killing. Bead-stimulated CTLs were co-incubated with indicated target cells for 2 h (P, Raji n = 2 independent experiments/3 donors, Raji-SEA/SEB n = 4 independent experiments/5 donors.) or 16 h (Q, Raji n = 3 independent experiments/4 donors, Raji-SEA/SEB n = 5 independent experiments/6 donors). Propidium iodide (PI) was used to determine necrotic CTLs death. Gating strategy shown in Supporting information Fig. 1. Gray: no PI-staning. Paired *t*-test was used for all statistical analysis. Data shown as mean ± SEM.

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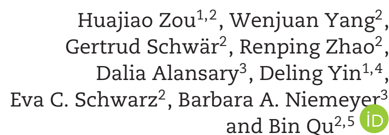

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Ethical Considerations: Research carried out for this study with human material (leukocyte reduction system chambers from human blood donors) is authorized by the local ethic committee (declaration from 16.4.2015 [84/15; Prof. Dr. Rettig-Stürmer]).

Conflict of Interest: The authors declare no commercial or financial conflict of interest.

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