Glycogen synthase kinase 3 (GSK-3) controls T-cell motility andinteractions with antigen presenting cells

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Research note

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Abstract

Objective:The threonine/serine kinase glycogen synthase kinase 3 (GSK-3) targets multiple substrates in T-cells and regulates the expression of Tbet and PD-1. However, it has been unclear whether GSK-3 has any effect on T-cell motility or their interactions with antigen presenting cells. Results: Here, we show that GSK-3 controls T-cell motilityand interactions with other cells. Inhibition of GSK-3, using structurally distinct inhibitors, reduced T-cell motility in terms of speed and distance travelled. Furthermore, SB415286 reduced the number of cell to cell contacts, however the duration of these established contacts with other cells did not differ in the presence of SB415286. This inhibition of motility did not affect the ability of GSK-3 inhibitors to enhance cytolytic T-cell (CTL) function in killing tumor targets. These data show that the inhibition of GSK-3 has differential effects on T-cell motility and CTL function where the negative effects on cell-cell interactions is overridden by the increased cytolytic potential of CTLs.

Introduction

T-cells are activated via a tyrosine kinase phosphorylation cascade that is initiated when the T-cell receptor (TCR) recognises foreign antigens, or tumor neoantigens, as presented by major histocompatibility (MHC) antigens. The cascade is initiated by the immune cell *src* kinase $p56^{lck}$ which we have shown binds to the cytoplasmic tails of co-receptors CD4 and CD8 (1-3). Co-recognition of MHC-antigen by the TCR, and CD4 or CD8, brings $p56^{lck}$ into proximity of the TCR for the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of CD3 and the z-subunits of the TCR-CD3 complex (2). Phospho-ITAMs bind to a second tyrosine kinase, Zeta-chain-associated protein kinase 70 (ZAP-70) which is further activated by $p56^{lck}$ (4). $p56^{lck}$ and ZAP-70 phosphorylate downstream substrates such as adaptors or scaffolds which form multimeric complexes that integrate signals for T-cell effector functions. Examples of key adaptors include the linker for activation of T-cells (LAT)(5) and Src Homology 2 (SH2) domain-containing leukocyte protein-76 (SLP-76) (6) which regulate intracellular calcium, or adhesion and degranulation-promoting *adapter* protein (ADAP) and Src kinase-associated phosphoprotein 1 (SKAP1) which activate T-cell adhesion(7,8) (9).

By contrast, glycogen synthase kinase 3 (GSK-3) is a serine/threonine kinase that is active in resting T-cells and is inactivated upon T-cell activation (10,11). Isoforms of GSK-3; α and β differ in their N- and C-terminal sequences. TCR ligation induces GSK-3 phosphorylation (12-14), and the expression of active GSK-3β (GSK-3βA9) inhibits the proliferation of T-cells (12). GSK-3 phosphorylation also regulates cellular metabolism (15) and microtubule-associated protein 2C (MAP2C) regulation of microtubule re-modelling (16,17). Protein kinase B (PKB/AKT) and its downstream target GSK-3 in T-cells appear to operate independently of guanine nucleotide exchange factor VAV-1 (13). In $CD4^+$ T-cells, GSK-3 promotes the exit of nuclear factor of activated T-cells (NFAT) (18,19). Clinical trials using GSK-3 inhibitors have been undertaken in the treatment of type II diabetes and various neurological disorders (11,20,21). Recently, we reported that the inactivation of GSK-3α/β specifically down-regulates PD-1 expression for enhanced cytolytic T-cell (CTL) function and clearance of infections by Murid herpes virus-

4 and lymphocytic choriomeningitis virus (LCMV) Clone (Cl) 13 (22). Further, GSK-3 inactivation was as effective as anti-PD-1 blockade in the regression of melanoma and lymphoma tumors (23,24).

In this study, we assessed whether GSK-3 inhibition affects T-cell movement and interactions with other cells. Structurally distinct inhibitors of GSK-3 reduced T-cell motility as measured by velocity, distance and displacement. As consequence of this, the number of cell to cell contacts was reduced. Further, as previously demonstrated (22,23) GSK-3 inhibition increased CTL function in killing tumor targets and this was not impeded by the inhibitory effect of GSK-3 inhibition on T-cell motility.

Methods

Mice and cells: Primary mouse T-cells (OT-1, C57BL/6, 6-8 weeks old) were isolated from spleens and cultured *in vitro* in RPMI 1640 medium supplemented with 10% FCS, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin (GIBCO). Spleen cells were treated with a hypotonic buffer with 0.15M NH4CL, 10mM KHCO3 and 0.1mM EDTA, pH 7.2 to eliminate red blood cells before suspension in supplemented RPMI 1640 medium. A T-cell enriched population was purified by use of T-cell purification columns (R&D Systems, Minneapolis, MN). All mouse experiments were approved by the Home Office UK (PPL No. 70/7544). EL4 lymphoma cells were cultured in RPMI medium that was supplemented as above.

GSK-3 Inhibitors: SB415286, SB216763 (Abcam plc) and L803-mts (Tocris) were reconstituted in DMSO to give a stock solution of 25mM and diluted to a concentration of 10uM for *in vitro* use.

***In vitro* CTL generation:** OVA specific $CD8^+$ CTLs were generated by incubating isolated splenocytes from OT-1 Tg mice with SIINFEKL peptide of OVA ($OVA_{257-264}$) at 10ng/mL for 5-7 days. CTLs were generated in the presence or absence of GSK-3 inhibitor (at the concentration described above) for 7 days prior to co-culture.

***In vitro* Cytotoxicity assays:** T-cells were plated in 96-well plates at the start of culture with activating EL4 cells (EL4-OVA) pulsed with $OVA_{257-264}$ peptide (Bachem). EL4 cells were incubated with 10nM $OVA_{257-264}$ peptide (Bachem) for 1 hour at 37°C prior to co-culture at a ratio of 1:5 of EL4:T-cell.

. Cytotoxicity was assayed using a Cytotox 96 nonradioactive kit (Promega) following the instructions provided.

Live cell imaging: T-cells were labelled using Carboxyfluorescein succinimidyl ester (CFSE, Biolegend) and EL4-OVA target cells labelled with CellTracker™ Red CMTPX Dye (Thermo Fisher Scientific). Imaging was performed using co-cultures on Poly-L-lysine-treated chambered glass culture slides (Lab-tek). Cells were imaged at the interface using a Zeiss LSM 510 confocal microscope using excitation wavelengths of 492 nm for CFSE and 577 nm for CellTracker™ Red and a ×63 oil immersion objective. Images were collected at 10 second intervals. All images were processed by Volocity software (Improvision).

Statistical analysis: Statistical significance was tested using one-way analysis of variance (ANOVA) between groups followed by a series of t-tests. Performed using GraphPad Prism version 3.02 (GraphPad Software, San Diego, California, U.S.A.), $p < 0.05$ was considered as significant.

Results

Inhibition of GSK-3 slows T-cell motility

In order to assess the role of GSK-3 in T-cell motility, T-cells (OT-1 Tg) were initially imaged over a period of 5 min in the presence or absence of GSK-3 inhibitors, SB415286 (Left panel), SB216763 (middle panel) or L803-mts (right panle) (**Fig. 1A**). In the absence of SB415286, T-cells moved with a mean velocity of 3.9um/min with a wide range of motilities from 7um/min to 2um/min. The presence of SB415286 slowed cells with an average velocity of 1um/min (i.e. 75% reduction). Similar profiles were observed when other parameters were used to assess movement (**Fig. 1B, C**). The displacement showed a reduction from 41um to .25um (**Fig. 1B**) and the length was also reduced, from 104um to 30um(**Fig. 1C**). Spider graphs also illustrated the reduced distance travelled over time (**Fig. 1D**). Similar results were obtained using other structurally distinct inhibitors of GSK-3, all of which have been previously shown to decrease PD-1 expression and potentiate OT-I killing of targets (22,23). These included ATP-competitive inhibitors, L803-mts (**Fig. 1A-D right panel**) and SB216763 (**Fig. 1A-D, middle panel**). The peptide L803-mts (11 residues) is a cell-permeable phosphorylated peptide that is derived from the GSK-3 substrate; heat shock factor-1 (HSF-1) and is structurally unrelated to SB415286 and SB216763 (25). SB216763 has a preference for the GSK-3alpha isoform, while L803-mts preferentially inhibits GSK-3beta. These data therefore collectively show that GSK-3 kinase activity is needed for the optimal migration of T-cells.

The result of reduced motility could lead to an increase or decrease in contacts with other cell types. For example, disruption of adaptors needed for integrin binding reduces contacts with antigen presenting cells(26,27). Interestingly, the presence of SB415286 reduced the total number of contacts of OT-1 Tg T-cells with antigen-presenting cells by approx. 50% (**Fig. 2A**), with a mean of 73 contacts in the untreated cells verses 40 contacts in the inhibitor treated cells (SD= 4.3 and 2.9, respectively); however, GSK-3 inhibition had no significant effect on the duration of contact which occurred (**Fig. 2B**). This reduction of contacts can be seen further when looking at individual target cells (**Fig. 2C**) Fig. 2C left panel shows 20 individual target cells (EL4-OVA) and the number of contacts made. In the absence of SB415286 the mean number of contacts was 3.25 (SD± 0.27). This was reduced to a mean of 1.75 contacts (SD±0.22) in the presence of SB415286. The right panel of Fig.2C depicts the mean number of contacts from 3 independent experiments giving an overall mean of 3.8±0.15 in the absence of SB415286 and 1.7±0.1 in the presence of SB415286. These data show for the first time that GSK-3 activity is needed for optimal interactions with other cells.

To address whether the effects of GSK-3 inactivation on T-cell motility and number of contacts was reflected in an increase in target killing, we next cultured CTLs with SB415286 for various times and assessed levels of killing (Fig. 3). We previously reported that long term exposure of primary T-cells to

SB415286 increased the potency of killing by resultant CTLs (22-24). Indeed, the culturing of T-cells for 7 days in the presence of SB415286 potentiated the killing of EL4-OVA targets. The increase in killing efficiency seen was 3- to 5-fold for Effector:Target ratios of 2:1 through to 25:1. An Effector:Target ratio of 2:1 with inhibitor showed the same efficiency of killing as seen at a ratio of 25:1 for non-inhibitor treated cells (**Fig. 3A**). By contrast, following the generation of CTLs, the same cells were exposed to SB415286 for 24 hours **(Fig. 3B)** or 4 hours **(Fig. 3C)**. **Fig. 3D** shows an example of the killing of a tumor target with the bubbling of membranes characteristic of cell death. Under these conditions, over the time frame where SB416286 could affect motility, no effect on the killing of tumor targets was evident.

Discussion

Overall, the rationale of our study is to assess whether GSK-3 inhibition effects are seen at the level of T-cell velocity and interactions with other cells. Our study shows that GSK-3 plays a clear role in regulating the movement of T-cells and interactions with other cells. Indeed, the inhibition of GSK-3 reduced the velocity of T-cells as measured *in vitro* on plates coated with ICAM1 for adhesion. The net result of this reduced T-cell velocity also reduced the distance needed to interact with other cells. However, the actual duration of cell to cell interactions or dwell times was not affected by GSK-3 inhibition. This is a logical expectation given that movement is needed for T-cells to stochastically encounter other cell types or to respond to chemo-attractants such as chemokines.

The fact that GSK-3 inhibition does not have an effect within minutes of exposure but rather requires longer periods of exposure following activation suggests that its effect on motility is due indirectly to more long-term effects on T-cell activation or differentiation. Naive murine T-cells become effector T-cells followed by the generation of central memory T-cells (28). We previously showed that GSK-3 regulates this event (22,23,29-31). It is therefore most likely that the targeting of GSK-3 affects motility indirectly due to effects on the activation or differentiation status of the T-cell. In this instance, the potential disadvantage of reduced motility and interactions with other cells is overridden by the positive intracellular effects on CTL killing of targets (22,23,29,30).

Further, it is important to note that different inhibitors of GSK-3 had the same effect on T-cell motility. L803-mts is structurally unrelated to SB415286 and SB216763 (25). Further, SB216763 has a preference for the GSK-3alpha isoform, while L803-mts preferentially inhibits the GSK-3beta isoforms. Despite different structures and isoform specificities, the exposure of cells to all drugs overtime resulted in a population of cells with reduced motility after long-term exposure. Lastly, the short exposure of CTLs to SB415286 did not alter killing suggesting that these effects on T-cell motility do not substantially alter the ability of T-cells to kill tumor targets.

The underlying mechanism is not clear. As mentioned, the effects require long-term incubation with T-cells and are therefore most likely related to effects on the activation or differentiation of T-cells. However, effects on more proximal events are also possible since GSK-3 can phosphorylate microtubule-associated protein 2C (MAP2C) which prevents microtubule remodelling (16,17). It is also possible that

GSK-3 interfaces with adaptor proteins such as SKAP1 which regulate T-cell motility (32). The N-terminal region of SKAP1 binds to RapL such that a RapL mutation (L224A) abrogates SKAP1 binding and arrests T-cells even in the absence of antigen (17). Lastly, it is possible that GSK-3 may influence cell motility and chemotaxis by regulating Phosphoinositide 3-kinase (PI 3K) membrane localization in *Dictyostelium (33) or due to* effects on phosphatidylinositol-3,4,5-triphosphate (PIP3) metabolism, The target of rapamycin kinase (TORC)2 signaling, and remodeling of F-Actin (34,35). Teo *et al* have reported that *gsk3*$^{-}$ cells respond to stimuli with a reduced increase of PIP3 and no TORC2 activation (35), decreased adenylyl cyclase, while others have obtained different results (34). Future work will be needed to assess the full range of effects of GSK-3 on aspects of T-cell function linked to motility and migration.

Limitations: Work restricted to non- lymphoid cells

Abbreviations

CTL: Cytolytic T-cell

TCR: T-cell receptor

MHC: Major histocompatibility complex

ITAM: Immunoreceptor tyrosine-based activation motif

PKB/AKT: Protein kinase B

LCMV: Lymphocytic choriomeningitis virus

CFSE: Carboxyfluorescein succinimidyl ester

PI 3K: Phosphoinositide 3-kinase

TORC2: The target of rapamycin kinase

NFAT: Nuclear factor of activated T-cells

SLP-76: SH2 domain containing leukocyte protein of 76 kDa

ADAP: Adhesion and degranulation-promoting adapter protein

GSK-3: Glycogen synthase kinase-3

LAT: Linker for activation of T-cells

SKAP1 (aka SKAP55); Src kinase-associated phosphoprotein1 or 55 KDa)

PIP3: Phosphatidylinositol-3,4,5-triphosphate

MAP2C: Microtubule-associated protein 2C

HSF-1: Heat shock factor-1

ZAP-70: Zeta-chain-associated protein kinase 70

Declarations

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Author contributions: A.T. and C.R. designed different aspects of the research. A.T. conducted the experiments. A.T. and C.R. drafted the manuscript. All authors have read and approved the manuscript.

Notes

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References

1. Rudd, C. E., Trevillyan, J. M., Dasgupta, J. D., Wong, L. L., and Schlossman, S. F. (1988) The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc Natl Acad Sci U S A* **85**, 5190-5194
2. Barber, E. K., Dasgupta, J. D., Schlossman, S. F., Trevillyan, J. M., and Rudd, C. E. (1989) The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. *Proc Natl Acad Sci U S A* **86**, 3277-3281
3. Veillette, A., Bookman, M. A., Horak, E. M., and Bolen, J. B. (1988) The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell* **55**, 301-308
4. Chan, A. C., Iwashima, M., Turck, C. W., and Weiss, A. (1992) ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* **71**, 649-662

5. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998) LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* **92**, 83-92
6. Jackman, J. K., Motto, D. G., Sun, Q., Tanemoto, M., Turck, C. W., Peltz, G. A., Koretzky, G. A., and Findell, P. R. (1995) Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells. *J Biol Chem* **270**, 7029-7032
7. Wang, H., Moon, E. Y., Azouz, A., Wu, X., Smith, A., Schneider, H., Hogg, N., and Rudd, C. E. (2003) SKAP-55 regulates integrin adhesion and formation of T cell-APC conjugates. *Nat Immunol* **4**, 366-374
8. Raab, M., Wang, H., Lu, Y., Smith, X., Wu, Z., Strebhardt, K., Ladbury, J. E., and Rudd, C. E. (2010) T cell receptor "inside-out" pathway via signaling module SKAP1-RapL regulates T cell motility and interactions in lymph nodes. *Immunity* **32**, 541-556
9. Kliche, S., Breitling, D., Togni, M., Pusch, R., Heuer, K., Wang, X., Freund, C., Kasirer-Friede, A., Menasche, G., Koretzky, G. A., and Schraven, B. (2006) The ADAP/SKAP55 signaling module regulates T-cell receptor-mediated integrin activation through plasma membrane targeting of Rap1. *Mol Cell Biol* **26**, 7130-7144
10. Woodgett, J. R. (1990) Molecular cloning and expression of glycogen synthase kinase-3/factor A. *The EMBO journal* **9**, 2431-2438
11. Cohen, P., and Frame, S. (2001) The renaissance of GSK3. *Nat Rev Mol Cell Biol* **2**, 769-776
12. Ohteki, T., Parsons, M., Zakarian, A., Jones, R. G., Nguyen, L. T., Woodgett, J. R., and Ohashi, P. S. (2000) Negative regulation of T cell proliferation and interleukin 2 production by the serine threonine kinase GSK-3. *J Exp Med* **192**, 99-104
13. Wood, J. E., Schneider, H., and Rudd, C. E. (2006) TcR and TcR-CD28 engagement of protein kinase B (PKB/AKT) and glycogen synthase kinase-3 (GSK-3) operates independently of guanine nucleotide exchange factor VAV-1. *J Biol Chem* **281**, 32385-32394
14. Appleman, L. J., van Puijenbroek, A. A., Shu, K. M., Nadler, L. M., and Boussiotis, V. A. (2002) CD28 costimulation mediates down-regulation of p27kip1 and cell cycle progression by activation of the PI3K/PKB signaling pathway in primary human T cells. *J Immunol* **168**, 2729-2736
15. Hur, E. M., and Zhou, F. Q. (2010) GSK3 signalling in neural development. *Nat Rev Neurosci* **11**, 539-551
16. Sanchez, C., Perez, M., and Avila, J. (2000) GSK3beta-mediated phosphorylation of the microtubule-associated protein 2C (MAP2C) prevents microtubule bundling. *Eur J Cell Biol* **79**, 252-260
17. Goold, R. G., and Gordon-Weeks, P. R. (2001) Microtubule-associated protein 1B phosphorylation by glycogen synthase kinase 3beta is induced during PC12 cell differentiation. *J Cell Sci* **114**, 4273-4284
18. Beals, C. R., Sheridan, C. M., Turck, C. W., Gardner, P., and Crabtree, G. R. (1997) Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* **275**, 1930-1934
19. Neal, J. W., and Clipstone, N. A. (2001) Glycogen synthase kinase-3 inhibits the DNA binding activity of NFATc. *J Biol Chem*, 3666-3673

20. Jope, R. S., and Roh, M. S. (2006) Glycogen synthase kinase-3 (GSK3) in psychiatric diseases and therapeutic interventions. *Curr Drug Targets* **7**, 1421-1434
21. Mazanetz, M. P., and Fischer, P. M. (2007) Untangling tau hyperphosphorylation in drug design for neurodegenerative diseases. *Nat Rev Drug Discov* **6**, 464-479
22. Taylor, A., Harker, J. A., Chanthong, K., Stevenson, P. G., Zuniga, E. I., and Rudd, C. E. (2016) Glycogen synthase kinase 3 inactivation drives T-bet-mediated downregulation of co-receptor PD-1 to enhance CD8(+) cytolytic T cell responses. *Immunity* **44**, 274-286
23. Taylor, A., Rothstein, D., and Rudd, C. E. (2018) Small-molecule inhibition of PD-1 transcription Is an effective alternative to antibody blockade in cancer therapy. *Cancer Res* **78**, 706-717
24. Taylor, A., and Rudd, C. E. (2017) Glycogen Synthase Kinase 3 inactivation compensates for the lack of CD28 in the priming of CD8+ cytotoxic T-Cells: Implications for anti-PD-1 Immunotherapy. *Front Immunol.* **8**, 1653.
25. Kaidanovich-Beilin, O., and Eldar-Finkelman, H. (2006) Peptides targeting protein kinases: strategies and implications. *Physiology* **21**, 411-418
26. Wang, H., McCann, F. E., Gordan, J. D., Wu, X., Raab, M., Malik, T. H., Davis, D. M., and Rudd, C. E. (2004) ADAP-SLP-76 binding differentially regulates supramolecular activation cluster (SMAC) formation relative to T cell-APC conjugation. *J Exp Med* **200**, 1063-1074
27. Wei, B., Han, L., Abbink, T. E., Groppelli, E., Lim, D., Thaker, Y. R., Gao, W., Zhai, R., Wang, J., Lever, A., Jolly, C., Wang, H., and Rudd, C. E. (2013) Immune adaptor ADAP in T cells regulates HIV-1 transcription and cell-cell viral spread via different co-receptors. *Retrovirology* **10**, 101
28. Youngblood, B., Hale, J. S., Kissick, H. T., Ahn, E., Xu, X., Wieland, A., Araki, K., West, E. E., Ghoneim, H. E., Fan, Y., Dogra, P., Davis, C. W., Konieczny, B. T., Antia, R., Cheng, X., and Ahmed, R. (2017) Effector CD8 T cells dedifferentiate into long-lived memory cells. *Nature* **552**, 404-409
29. Krueger, J., Rudd, C. E., and Taylor, A. (2019) Glycogen synthase 3 (GSK-3) regulation of PD-1 expression and and its therapeutic implications. *Semin Immunol* **42**, 101295
30. Taylor, A., and Rudd, C. E. (2017) Glycogen Synthase Kinase 3 Inactivation Compensates for the Lack of CD28 in the Priming of CD8(+) Cytotoxic T-Cells: Implications for anti-PD-1 Immunotherapy. *Front Immunol* **8**, 1653
31. Krueger, J., and Rudd, C. E. (2017) Two Strings in One Bow: PD-1 Negatively Regulates via Co-receptor CD28 on T Cells. *Immunity* **46**, 529-531
32. Wang, H., and Rudd, C. E. (2008) SKAP-55, SKAP-55-related and ADAP adaptors modulate integrin-mediated immune-cell adhesion. *Trends Cell Biol* **18**, 486-493
33. Sun, T., Kim, B., and Kim, L. W. (2013) Glycogen Synthase Kinase 3 influences cell motility and chemotaxis by regulating PI3K membrane localization in Dictyostelium. *Dev Growth Differ* **55**, 723-734
34. Kolsch, V., Shen, Z., Lee, S., Plak, K., Lotfi, P., Chang, J., Charest, P. G., Romero, J. L., Jeon, T. J., Kortholt, A., Briggs, S. P., and Firtel, R. A. (2013) Daydreamer, a Ras effector and GSK-3 substrate, is important for directional sensing and cell motility. *Mol Biol Cell* **24**, 100-114

35. Teo, R., Lewis, K. J., Forde, J. E., Ryves, W. J., Reddy, J. V., Rogers, B. J., and Harwood, A. J. (2010) Glycogen synthase kinase-3 is required for efficient Dictyostelium chemotaxis. *Mol Biol Cell* **21**, 2788-2796

Figures

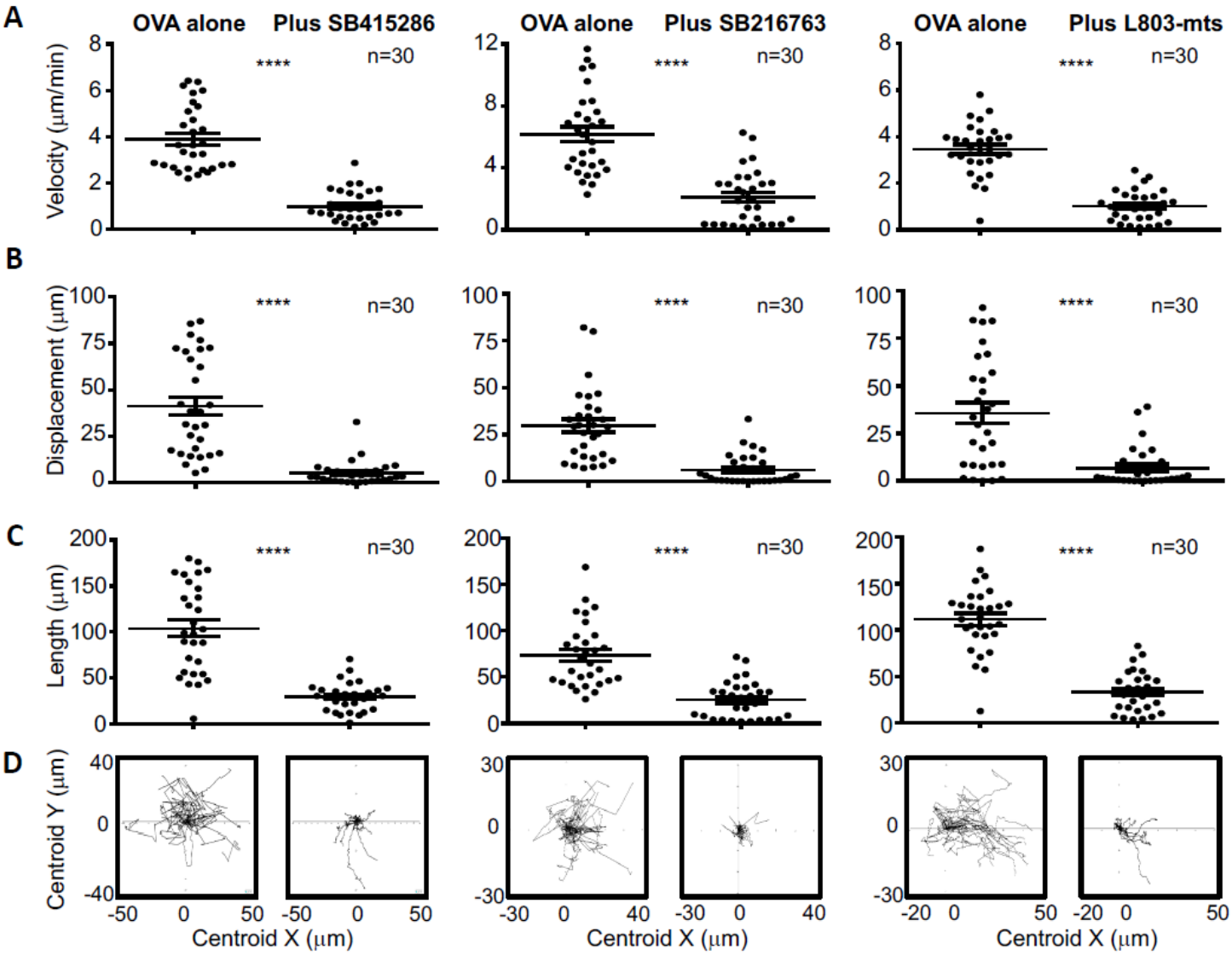

Figure 1

GSK-3 inhibition decreases T-cell motility. Cells treated with GSK-3 inhibitors (Left panel: SB415286, Middle panel: SB216763, right panel: L803-mts) for 7 days show reduced motility in the presence of target cells (EL4-OVA). Tracking of 30 individual cells showed differences in (A) velocity, (B) displacement and (C) length of track travelled. Spider plots (D) show the traced tracks of all cells in area imaged. Data shown representative of three independent experiments. **** = P value<0.0001. Mean shown ± standard deviation

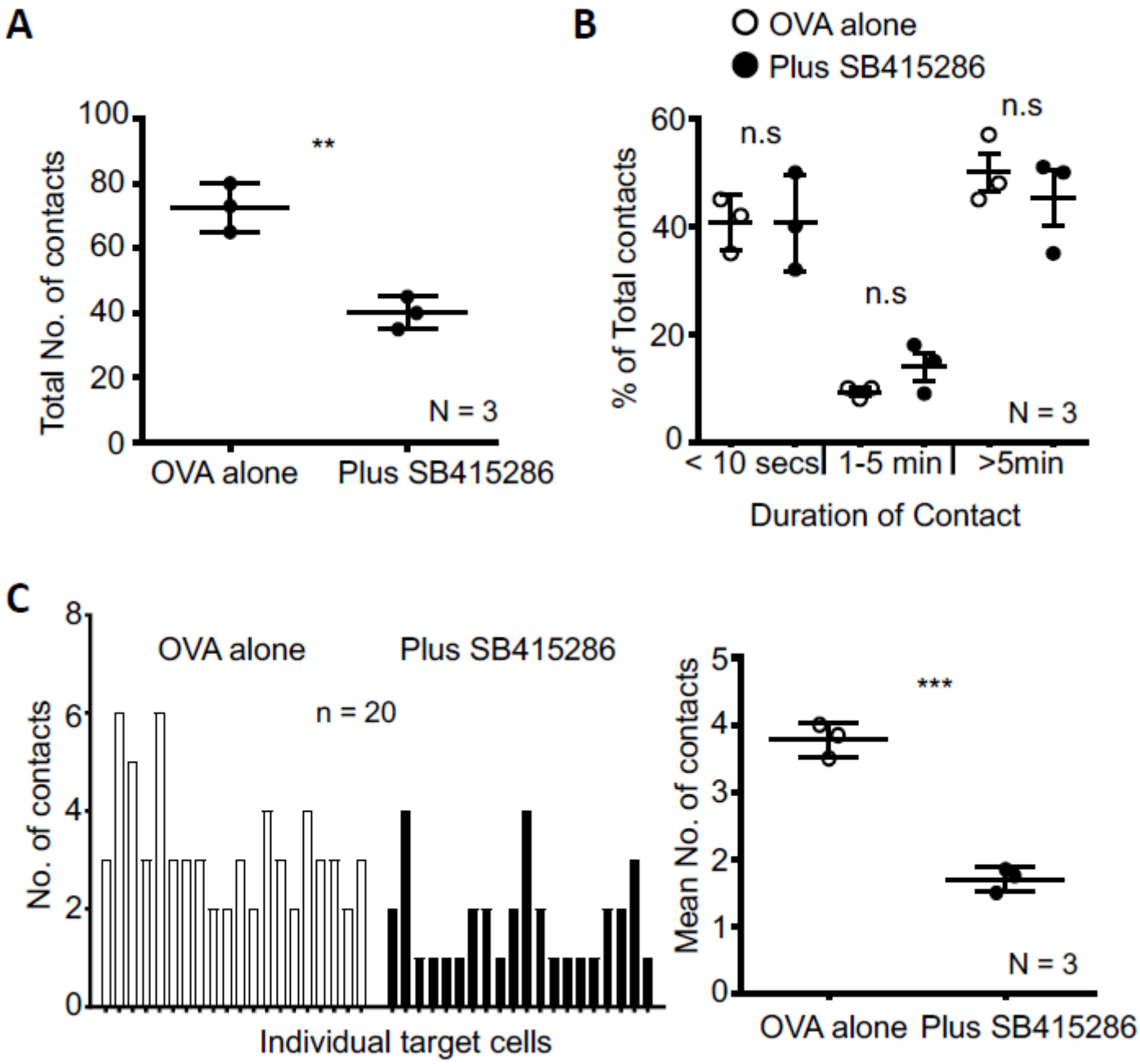

Figure 2

SB415286 decreases T-cell contacts with other cells. Inhibition of GSK-3 reduces the number of cell-to-cell contacts required to induce target killing. Quantification of contact times (A) total number of contacts for each condition (Ova alone Mean =72.67 ±4.3, Plus SB415286 Mean = 40.00 ±2.89). (B) % of contacts lasting the duration shown. Data shown is pooled from (N) = 3 independent experiments. n.s; no significant difference (C) Left panel, number of contacts by each individual target cell tracked (n=20 Target cells (EL4-OVA)). Right panel, Mean number of contacts by individual target cells from (N) = 3 independent experiments (Ova alone Mean =3.8 ±0.15, Plus SB415286 Mean = 1.7 ±0.10. *, P < 0.05; **, P < 0.005 ***, P<0.0005

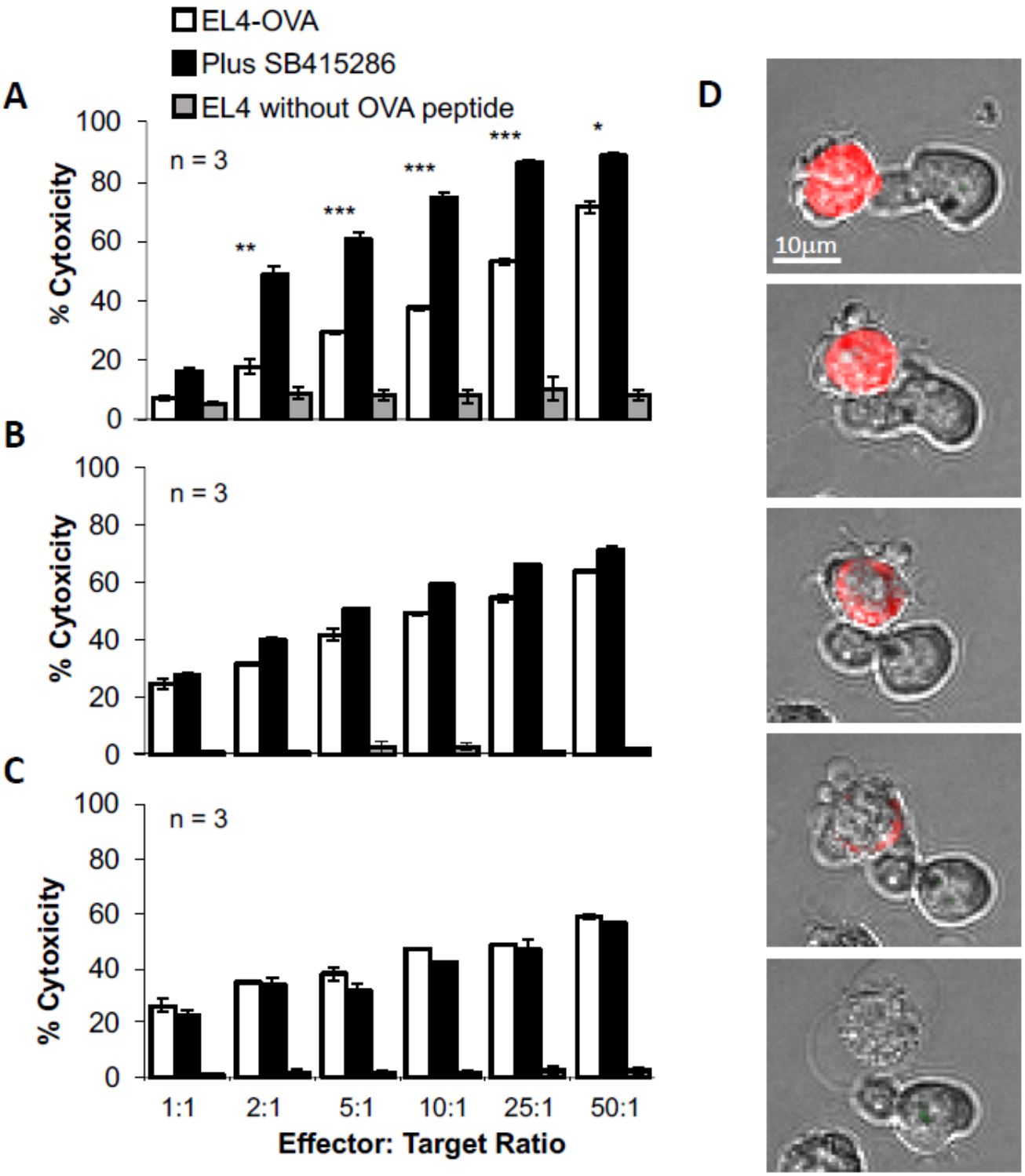

Figure 3

GSK-3 inactivation requires long-term incubation with T-cells to enhance CTLs killing of tumors. GSK inhibitor increases cytolytic killing of CTLs when present during over the cell culture period needed to induce differentiation. CTLS were generated by incubating splenocytes from OT-1 Tg mice with OVA-peptide for 7 days. SB415286 was added to cultures on (A) day 0 or (B) day 6. On day 7 T-cells were washed and a 4hr cytolytic assay performed using EL4 cells pulsed or non-pulsed with Ova-peptide as

target cells. Panel (C) depicts T-cells only treated with SB415286 after the wash step and prior to the 4hr CTL assay (In panels (A) and (B) any remaining SB415296 in culture was washed away). Error bars based on triplicate values in individual experiments, data shown representative of 3 independent experiments. *, $P < 0.05$; **, $P < 0.005$ ***, $P<0.0005$ (D) Figure shows examples of T-cells interacting and killing tumor targets (EL4-OVA cells labelled in red). The CTL then goes on to lyse the cell as can be seen from the characteristic bubbling of the cell cytoplasm and the clear vacuole. Data shown representative of three independent experiments.