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Running Title: A role for glycolysis in the induction of dendritic cell necroptosis

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Abstract:

γδ T lymphocytes are a poorly understood class of immune cells that accumulate at sites of inflammation due to infection or autoimmunity. Part of the mystery of this T cell subset derives from lack of knowledge of ligands that bind to the T cell receptor (TCR) of γδ T cells. The conditions under which these ligands are induced remain ill-defined, but the Budd laboratory has recently reported that dendritic cell (DC) necroptosis exposes a previously unreported ligand for the γδ TCR, leading to activation of γδ T cells. Necroptosis is caspase-independent and can be induced via inhibition of caspase-8, leading to formation of a complex containing Receptor Interacting Protein Kinase 1 (RIPK1) known as the ripoptosome. In this study, we describe a new connection between glycolysis of DC and their susceptibility to necroptosis. DC grown with GM-CSF are highly glycolytic and susceptible to necroptosis induced by the pan-caspase blocker zVAD. However we demonstrate that GM-CSF-generated DCs treated with the glycolysis inhibitor 2-deoxyglucose are protected against necroptosis by zVAD. We propose that aerobic glycolysis is a prerequisite for DC necroptosis and accordingly their ability to activate γδ T cells. Inhibition of glycolysis does not impact caspase activity levels, production of essential cytokines, or production of proteins essential for the ripoptosome. Instead, non-glycolytic DC have increased levels of cleaved RIPK, which serves as an inhibitor of Ripoptosome formation. It has been established that activation of DC leads to increased cellular rates of aerobic glycolysis. It may be that naïve dendritic cells are protected from necroptosis by increased cleavage of RIPK in order to prevent unnecessary cell death and the triggered activation of a downstream immune response.

Introduction

Lyme disease is the most common vector-borne illness in North America. In the United States, the Center for Disease Control (CDC) reports the incidence of Lyme disease to be 300,000 annual cases. This disease has reached epidemic magnitudes in the northeastern United States and it is also prevalent in the upper Midwest. However, Lyme disease incidence is gradually spreading to southern and western states. Experts attribute this change to forest regrowth, climate change, and spread of deer [1]. The causative agent of Lyme disease is the spirochetal pathogen *Borrelia burgdorferi,* which is transmitted to humans via the *Ixodes scapularis* tick, more commonly known as the deer tick [2, 3].

γδ T cells are a unique cellular lineage functioning at the interface between the adaptive and innate immune responses and are often highly lytic against infected cells, transformed cells, and infiltrating CD4+ T cells in inflammatory arthritis [4, 5]. Within organs or tissues inflamed by the presence of *Borrelia burgdorferi,* an unusual accumulation of γδ T lymphocytes occurs [6]. This phenomenon has been similarly reported for certain infections of other bacteria, viruses, protozoans and tumors [7, 8], and in autoimmune disorders including rheumatoid arthritis [9-12]. Mice lacking γδ T cells exhibit greater bacterial burden, enhanced cardiac inflammation, and an overall reduced adaptive immune response to infection with *B. burgdorferi* [13]. *These collective studies indicate that the main function of γδ T cells is likely to protect host tissues from injuries secondary to inflammation of various causes.* Thus, it is not surprising that γδ T cells are reported to be reactive to host proteins upregulated or exposed during inflammation [14]. Our previous work has further demonstrated that γδ T cells respond indirectly to *B. burgdorferi* via Toll-like receptor (TLR) 2 activation on monocytes or dendritic cells (DC) [15]. The γδ T cells can subsequently promote full activation of DC [16]. *B. burgdorferi* can also promote cell death of macrophages in a TLR2-dependent manner [17]. These findings prompted further research into

whether activation of γδ T lymphocytes by *B. burgdorferi* either requires or is enhanced by cell death of DC.

Caspase-8 is critical for the survival and proliferation of T lymphocytes [18-22] and specifically promotes cell survival by cleaving Receptor Interacting Protein Kinase 1 (RIPK1). Cleaved RIPK1 inhibits full-length RIPK1 from complexing with FADD and caspase-8 to form the ripoptosome, which induces necroptosis [23, 24]. Necroptosis is a recently discovered form of pro-inflammatory, programmed cell death that, unlike all other known types of cell death, does not depend on death effector caspase activity [25]. The proteins RIPK1 and Mixed Lineage Kinase Domain-Like Protein (MLKL) are required for the induction of necroptosis, which can be triggered by interferons, TLR signaling, death receptors, intracellular RNA and DNA sensors, and likely other mediators [26]. Recently, our group examined the role of caspase activity in the survival of DC and in their ability to activate γδ T cells [27]. We demonstrated that inhibition of caspases induced necroptosis in murine bone marrow-derived DC (BMDC) generated with GM-CSF, yet these cells manifested an enhanced ability to activate γδ T cells, even in the absence of *B. burgdorferi.* Conversely, BMDC generated with GM-CSF + Interleukin(IL)-4 strongly upregulated expression of c-FLIP, a known stabilizer of caspase-8, which promoted increased caspase-8 activity and decreased necroptosis following caspase inhibition. These cells had high survival rates, due to the lack of necroptosis, yet demonstrated a considerably reduced ability to activate γδ T cells. The findings support a model under which γδ T cell ligands are induced or exposed in stressed or necroptotic DC (Collins *et al.* submitted for publication).

We initially attributed the lack of necroptosis induced by caspase inhibition in GM-CSF + IL-4-cultured BMDC to the elevated levels of c-FLIP. However, the results presented herein demonstrate that inhibition of glycolysis in GM-CSF-cultivated BMDC renders them resistant to caspase inhibition-induced necroptosis. Thus, a connection may exist between metabolism and necroptosis that has yet to be

elucidated. This is fascinating in light of recent studies demonstrating that activation of immune cells corresponds with increased aerobic glycolysis, which is necessary to make the precursors of nucleic acids and lipids necessary for cell division, and decreased oxidative phosphorylation [28-31]. Identification of such a link between metabolism and necroptosis would dramatically expand our knowledge of this form of cell death, which has only been characterized in recent years [26]. Furthermore, this study may provide new insight into a potential mechanism for exposure of a γδ T cell ligand by necroptotic DC. Both of these potential outcomes have great merit, as there exists limited information surrounding the prominent roles that γδ T cells and necroptotic DC appear to play in the response to infection/autoimmune-generated inflammation. *A more accurate and complete understanding of this immune mechanism could have an impact on the ways we approach a wide variety of diseases and autoimmune states. The connection between metabolism and immune response additionally has implications for our understanding of immunology and is currently of great interest in our field.*

Methods

Reagents.

Contamination with environmental LPS was minimized during the preparation of buffers and regents by using autoclaved and baked (180°C for 4 hours) glassware, disposable plastic ware, and pyrogen-free H_2O.

Mice.

C57BL/6J mice were housed and bred according to protocols approved by the UVM IACUC. Mice were used at 8 to 12 weeks of age for harvest of DC and γδ T cells from spleens, and harvest of

bone marrow cells. TNF-/- mice were used to assess the role, or lack thereof, that TNF plays in the initiation of necroptosis.

Bone marrow dendritic cells (BMDC).

The preparation of bone marrow-derived dendritic cells (BMDC) was done according to the method of *Lutz et. al.* [32] using GM-CSF (10 μg/ml BioSource International). Cells were used on day 7.

Splenic dendritic cell purification.

Dendritic cells were isolated and disrupted through nylon mesh mesh in RPMI 1640 with 25 mM Hepes (MediaTech, Herndon, VA) containing 5% (v/v) bovine calf serum (HyClone, Logan, UT). Differentiated dendritic cells were obtained via $CD14^+$ magnetic beads isolation (Miltenyi Biotech) and were immediately used for experimentation.

Splenic γδ T cell purification and culture.

The spleen of an C57BL/6 mouse was isolated and disrupted through nylon mesh in RPMI 1640 with 25 mM Hepes (MediaTech, Herndon, VA) containing 5% (v/v) bovine calf serum (HyClone, Logan, UT). Erythrocyte lysis of splenocytes was performed using Geys solution. Magnetic depletion was used to remove bead-bound cells. Isolated γδ T cells were resuspended in complete medium (RPMI 1640 (Mediatech, Inc., Herndon, VA, USA), 2.5 mg/ml glucose (Sigma, St. Louis, MO), 10 mg/ml folate (Invitrogen, Carlsbad, CA), 110 μg/ml pyruvate (Invitrogen), 5×10^{-5} M 2-mercaptoethanol (Sigma), 292.3 μg/ml glutamine (Invitrogen), 100 units/ml penicillin-streptomycin (Invitrogen), and 5% fetal calf serum). Purified γδ T cells were initially activated at a density of 1 x 10^6 cells/ml by plate-bound anti-TCR-γδ (10 μg/ml, clone GL-3), and recombinant human IL-2 (50 units/ml, Cetus). After 2 days, cells were removed from anti-TCR-γδ stimulation, supplied with fresh medium plus IL-2, and

returned to culture at a density of 0.5 x 10^6 cells/ml. Cells were counted, and daily supplied with fresh media containing 50 units/ml IL-2. At the time of experiments, typically day 7, cultures were routinely over 95% γδ T cells.

Co-incubation of Dendritic Cells and T lymphocytes.

Cultures of DC (5×10^5/ml) with αβ T cells (1×10^6/ml) were made in AIM-V medium with GM-CSF (800 U/ml) and 10% FBS. To some cultures was added 2-deoxyglucose or rapamycin (10ng/ml) at the doses indicated. 24 hours later, *B. burgdorferi* sonicate (10 μg/ml) (Sigma-Aldrich),caspase-8 inhibitor zVAD-fmk (MP Biomedical, Santa Ana CA), was added. Cells were incubated for 24 h at 37℃ with 5% CO_2 release. Supernatants were collected for cytokine analysis and cells were assessed for viability.

Cell Viability Assessment.

Dendritic cells were removed from experimental plates and percent viability was obtained via trypan blue staining and a hemocytometer on a light microscope. Results were confirmed via flow cytometry.

Measurement of Caspase Activity.

Pan-caspase activity was determined using the Apo-ONE Assay, which measures the cleavage of DEVD-rhodamine. Spectrophotometric readings were taken using a Fluorescence reader.

Measurement of Metabolic Activity.

Between 3 and 5×10^5 cells/well were seeded into Seahorse XF24 microplates and incubated at 37°C for approximately 24 hr. Subsequently, basal oxygen consumption rate (OCAR) and extracellular acidification rate (ECAR) were measured by the Seahorse XF24 Flux analyzer.

Cytokine Analysis.

A commercial bioplex assay (BD biosciences) was used to analyze supernatant concentration of cytokines IL-1β, TNF, IFNg, and IL-10 via the manufacturer's instructions. Briefly, samples were run undiluted or diluted 1:10 in RPMI complete media. 50 μl of the magnetic bead working solution was added to each well, then 50 μl of appropriate samples or standards were then added to wells and incubated at room temperature for 30 min at 300 rpm on an IKA MS 3 digital shaker. After three washes with 100 μl Bio-Plex wash buffer, incubation with 25 μl of detection antibody solution was done at room temperature for 30 min on the shaker. Following another set of three washes, 50 μl of streptavidin-phycoerythrin (PE) in assay buffer was added to each well and incubated as described for the previous step. After an additional three washes, 125 μl of Bio-Plex assay buffer was added. Sample data was analyzed with Bio-Plex Manager software.

Flow Cytometry.

A Live/Dead Fixable Dead Cell Stain kit (Invitrogen) was used as per the manufacturers' instructions to evaluate cell viability via flow cytometry. For direct staining, single cell suspensions (1 x 10^6 total cells per staining condition) were washed with cold (4°C) PBS containing 1% (w/v) BSA fraction V (Sigma) (PBS/1% BSA), incubated with unconjugated hamster IgG1 (50 μg/ml) for 15 min at 4°C, washed and then incubated with the appropriate antibodies in PBS/1% BSA. After washing, the cells were fixed with freshly made 1% (v/v) methanol-free formaldehyde (Ted Pella Inc., Redding, CA) in PBS/1% BSA. Flow cytometry was performed on an LSRII (BD Biosciences, San Jose, CA) calibrated with compensation beads (BD Biosciences). Analysis was performed using FlowJo software (Tree Star, Inc., Ashland, OR).

Western Blot.

Day 10 DC were lysed in Tris-buffer containing Complete Protease Inhibitor (Roche) and 0.5% NP-40 (Roche). Insoluble cell fragments were removed by centrifugation, and the protein concentration

of the resulting whole cell lysates was determined by Bradford assay (BioRad, Hercules, CA). Whole cell lysates (30 μg per lane) were separated by SDS-PAGE. After gel electrophoresis, proteins were transferred to PVDF membranes (0.2 μm pore size, Bio-Rad), and immunoblots were performed using antibodies (1μg/ml) against MLKL (Cell Signaling), RIPK1 (BD Biosciences), and β-actin (BD biosciences). Membranes were blocked with 4% BSA and developed via x-ray radiography.

Statistical Analysis.

Results are expressed as a mean ± SEM and are representative of at least 2 experiments each. GraphPad Prism was used for statistical analysis and graphing capabilities.

Results

Splenic DCs are less susceptible to caspase-inhibition-mediated necroptosis and accordingly display a reduced ability to activate γδ T cells.

Our group recently reported that BMDC exposed to the pan-caspase inhibitor zVAD undergo necroptosis due to inhibition of caspase-8. These necroptotic BMDC augment activation of γδ T cells. To extend these results using cultured DC, fresh DC were isolated from the mouse spleen in order to assess whether *in vivo*-differentiated DC respond to caspase inhibition in the same manner as *in vitro*-differentiated BMDC. Surprisingly, splenic DC did not undergo necroptosis following exposure to zVAD as did GM-CSF-differentiated BMDC **(Figure 1A).** Cytokine analysis of supernatants revealed that fresh DC also did not activate γδ T cells, as indicated by a lack of production of the γδ T cell cytokine IFNγ **(Figure 1B).** Of note is that splenic DC are visually much smaller than GM-CSF-cultivated BMDC. Interestingly, ongoing research in the Budd laboratory has revealed that IL-15-differentiated αβ T cells

are less glycolytic than IL-2-differentiated αβ T cells. In parallel with this, IL-15 T cells are resistant to cell death, whereas IL-2 T cells are quite susceptible to cell death. *In-vitro*-differentiated BMDC are quite glycolytic compared to freshly isolated DC, and as such we wondered whether the cultivated BMDC would also be more susceptible to cell death.

Decreased aerobic glycolysis protects cells BMDC from zVAD-mediated necroptosis.

To test our hypothesis that the glycolytic state affects the DC response to zVAD-mediated caspase inhibition, we used 2-deoxyglucose (2-DG) or rapamycin to decrease cellular glycolysis. 2-DG is a competitive inhibitor of glycolysis while rapamycin inhibits glycolysis by targeting mTOR, a master regulator of metabolism. In GM-CSF-differentiated BMDC, exposure to 2-DG or rapamycin had a protective effect against zVAD-mediated necroptosis (**Figure 2A, B)**. These inhibitors did not affect IL-4-differentiated BMDC's innate protection from zVAD-mediated necroptosis **(Figure 2A).** Exposure to 2-DG similarly reduced rates of zVAD-mediated necroptosis in human cells **(Figure 2C).**

Although our previous work attributes IL-4-differentiated BMDC's protection from zVAD-mediated necroptosis to the increased presence of caspase-8 homolog c-FLIP, we wondered if there might be an additional explanation. Accordingly, we measured the glycolytic activity of GM-CSF + IL-4-differentiated versus GM-CSF-differentiated BMDC by an extracellular flux analyzer. Surprisingly however, IL-4 differentiated BMDC had basal levels of glycolysis 5-6-fold higher than GM-CSF-differentiated BMDCs **(Figure 3B).** 2-DG-treatment of both types of BMDCS effectively reduced levels of glycolysis to near undetectable levels, indicating that the lack of change in necroptosis levels observed in 2-DG-treated IL-4 BMDC was not due to incomplete inhibition of glycolysis.

As such, 2-DG effectively decreases levels of aerobic glycolysis in both types of BMDC, and yet this change only impacts necroptosis in the GM-SCF-only-differentiated population. We previously

demonstrated that increased levels of caspase-8 homolog c-FLIP are present in IL-4-treated BMDC. c-FLIP can inhibit ripoptosome formation by maintaining activated caspase-8, and consequently cleaved RIPK1. Accordingly, we wished to further examine how 2-DG or rapamycin confers resistance of BMDC to zVAD-mediated necroptosis. A DVED caspase activity assay indicated that zVAD effectively reduced caspase activity in GM-CSF-treated BMDC, regardless of differentiation conditions or exposure to glycolysis inhibitor **(Figure 3A).** zVAD inhibited caspase activity in BMDC, regardless of treatment with or without glycolysis inhibitor. However, as previously stated, exposure to zVAD did not induce necroptosis in 2DG-treated BMDC **(Figure 2).** This suggests that a factor triggered by aerobic glycolysis is necessary for the induction of necroptosis in DC.

*TNF production is not the missing signal required for induction of necroptosis***.**

It has been demonstrated in many cell types that inhibiting caspases alone is sufficient to induce necroptosis. Our results suggest a "second signal," one perhaps intrinsically present in cells and linked to aerobic glycolysis, also plays a role in the regulation of necroptosis. In some immortalized cells, the cytokine TNF, in addition to caspase blockade, is required to induce necroptosis. Furthermore, activated DC are highly glycolytic and can produce large amounts of TNF, so we wondered whether inhibition of glycolysis might curb production of TNF and decrease necroptosis induction. To test this hypothesis, we used BMDC obtained from a TNF-knock out mouse. Addition of zVAD alone was still sufficient to induce necroptosis even in the absence of TNF in these BMDC, based on both trypan blue staining and analysis by flow cytometry **(Figure 4A, B).** To ensure that this was not simply the result of redundancy in the system, in that another cytokine might also induce necroptosis, we performed an add-back experiment in which WT BMDC incubated with 2DG were treated with exogenous TNF. BMDC did not undergo necroptosis when exposed to exogenous TNF, indicating that the "second signal" required for necroptosis is not TNF as has been reported for some immortalized cells.

Cleaved RIPK, an inhibitor for ripoptosome formation, is increased in 2DG-treated BMDC.

Aerobic glycolysis is important for synthesis of important metabolic intermediates that cells use to generate cellular biomass. To determine whether non-glycolytic BMDC were producing crucial ripoptosome proteins, we used western blotting to measure levels of RIPK1 and MLKL in BMDC treated with or without 2-DG. Levels of full-length RIPK1 and MLKL were similar in all BMDC, regardless of treatment with 2-DG. However, 2-DG-treated BMDC expressed increased levels of cleaved RIPK1, which acts as an inhibitor of ripoptosome formation (**Figure 5)**.

Discussion

As necroptosis is a recently discovered pathway of cell death, its mechanism of action and down-stream effects on the immune system have yet to be fully described. Studies in mouse-models, including those in the Budd laboratory, have linked necroptosis to the development of inflammation and implicated this cellular pathway in the pathogenesis of inflammatory diseases. Specifically, the Budd laboratory has demonstrated that necroptotic DC activate γδ T cells. These innate-like T cells operate at the intersection of the innate and adaptive immune responses and are upregulated during inflammatory diseases such as Lyme disease and Rheumatoid Arthritis. The work presented in this study indicates, for the first time, that the metabolic state of DC plays a role in the induction of necroptosis. This is especially interesting in light of recent reports demonstrating that a hallmark of immune cell activation is a "switch" from oxidative phosphorylation to aerobic glycolysis [28-31]. It has been demonstrated in αβ T lymphocytes [28, 29], macrophages and DC [30, 31] that prior to activation, immune cells primarily utilize oxidative phosphorylation and fatty acid breakdown to generate energy. Upon activation, they rapidly begin aerobic glycolysis in order to generate the metabolites and energy carriers necessary for production of inflammatory mediators and cellular biomass, similar to the Warburg Effect documented in cancer cells

[29, 30]. Activation of DC and the corresponding switch to aerobic glycolysis may be essential for DC necroptosis and subsequent activation of γδ T cells.

Observations of primary DC resistance to zVAD-mediated necroptosis triggered our initial interest in the role of metabolism in the initiation of necroptosis. Given that DC isolated directly from the murine spleen are less proliferative than GM-CSF-cultured BMDC, we wondered whether glycolysis levels might be the differing factor. When glycolysis was inhibited in primary human DC or murine BMDCs, these cells did not undergo necroptosis following caspase inhibition. These data suggest that DC must be glycolytic in order to undergo necroptosis.

Aerobic glycolysis as a pre-requisite for DC necroptosis could ensure that only activated DC undergo necroptosis and accordingly activate other members of the immune system, including γδ T cells. DC can become activated through signaling on TLRs, as in the case of infection with *B. burgdorferi*. Signaling through TLRs has been shown to cause an increase in lactic acid production and glucose consumption, both indicative of increased glycolysis [33-34]. This transition to glycolysis is absolutely essential for full activation of the DC [34, 35]. Necroptosis is a defense mechanism against viral caspase inhibitors, which aim to prevent host cells from undergoing caspase-dependent forms of cell death [36, 37]. Necroptosis also occurs within inflammatory diseases, but the mechanism of caspase-inhibition remains unclear [38]. Regardless of whether necroptosis is virally induced or triggered by an inflammatory state, it appears that DC must become activated, as characterized by a switch to aerobic glycolysis, in order to undergo necroptosis following caspase-inhibition.

Previously, we determined that the presence of caspase-8 homolog, c-FLIP, is present at much higher levels in IL-4-generated BMDC. Caspase-8 cleaves RIPK1, which inhibits formation of the ripoptosome. c-FLIP both stabilizes caspase-8 activation, as well as limits the substrates that can gain access to the enzymatic pocket of caspase-8, including the exclusion of zVAD [27]. Interestingly, GM-

CSF + IL-4-grown BMDC are much more glycolytic than GM-CSF-only-cultivated BMDC. These data suggest that IL4-grown BMDC may be primarily resistant to necroptosis due to c-FLIP upregulation, which appears separate from the mechanism of resistance conferred by low aerobic glycolysis. Current research efforts aimed at quantitating c-FLIP levels in 2-DG treated BMDCs will provide additional information in the near future.

The mechanism by which decreased aerobic glycolysis prevents induction of necroptosis remains elusive, but our data demonstrate that the connection exists in both human DC and murine BMDC. Low glycolysis does not impact the ability of zVAD to inhibit caspase activity, nor is low glycolysis preventing production of TNF. Addition of exogenous TNF cannot "rescue" the necroptotic capabilities of non-glycolytic BMDC, and $TNF^{-/-}$ BMDC undergo necroptosis by zVAD as readily as wild-type BMDC. Non-glycolytic DC also produce levels of ripoptosome proteins RIPK and MLKL comparable to those produced by glycolytic DC.

A crucial step in the formation of the ripoptosome, and subsequent induction of necroptosis, is dimerization of RIPK1 and RIPK3 [39]. Caspase-8 can prevent dimerization by cleaving RIPK1 in its intermediate domain, separating its active kinase domain and its death domain. Cleaved RIPK1 subsequently acts as an inhibitor of full-length RIPK1 [40]. Interestingly, even with inhibited levels of caspase-8, non-glycolytic BMDC still express increased cleaved RIPK1.

These data suggest that naïve, non-glycolytic DC might prevent unnecessary necroptosis and subsequent activation of downstream immune responses via cleaved RIPK1. Upon activation, glycolytic DC express lower levels of cleaved RIPK1, and accordingly necroptosis is induced following caspase inhibition. In the future, measurements of glycolysis and cleaved RIPK1 in naïve DC will provide a clearer picture of this phenomenon.

This study has already indicated an interesting and previously unreported connection between necroptosis and aerobic glycolysis, demonstrating that activation of DC may be driving a crucial signal for conferring susceptibility to necroptosis. Continued research on this topic will expand our currently limited understanding of how DC necroptosis is influenced by cell metabolism, and how cell death can lead to activation of the immune response. *Increased understanding of the connection between metabolism and immunology has the potential to drastically shape our future approaches to modern health challenges.*

References

1. Steere, A.C. 1994. Lyme disease: a growing threat to urban populations. *Proc Natl Acad Sci USA.* 91(7):2378-83.
2. Shadick, N., C. Phillips, W. Logigian, A. Steere, R. Kaplan, V. Berardi, P. Durray, M. Larson, E. Wright, K. Ginsburg, J. Katz, and M. Liang. 1994. The Long-Term Clinical Outcomes of Lyme Disease. *Annals of Internal Medicine*. 121.8: 560-67.
3. Radolf, J.D., J.C. Salazar, and R.J. Dattwyler. 2010. Lyme Disease in Humans. In *Borrelia: Molecular Biology, Host Interaction, and Pathogenesis*. Caister Academic , Norfolk, UK. P. 319-356.
4. Wilhelm, M., V. Kunzmann, S. Eckstein, P. Reimer, F. Weissinger, T. Ruediger, and H.P. Tony. 2003. Gamma Delta T cells for immune therapy of patients with lymphoid malignancies. *Blood* 102:200-206.
5. Costa, G., S. Loizon, M. Guenot, I. Mocan, F. Halary, G. de Saint-Basile, V. Pitard, J. Dechanet-Merville, J.F. Moreau, and M. Troye-Blomberg. 2011. Control of Plasmodium falciparum

erythrocytic cycle: gammadelta T cells target the red blood cell-invasive merozoites. *Blood* 118:6952-6962.

6. Collins C, C. Shi, Q. Russell, K.A. Fortner, and R.C. Budd. 2008. Activation of γδ T Cells by Borrelia Burgdorferi Is Indirect via a TLR- and Caspase-Dependent Pathway. *J. Immuno.* 181.4:2392-398.

7. Girardi, M., D.E. Oppenheim, C.R. Steele, J.M. Lewis, E. Glusac, R. Filler, P. Hobby, B. Sutton, R.E. Tigelaar, and A.C. Hayday. 2001. Regulation of cutaneous malignancy by gammadelta T cells. *Science* 294:605-609.

8. Born, W., C. Cady, J. Jones-Carson, A. Mukasa, M. Lahn, and R. O'Brien, 1999. Immunoregulatory functions of gamma delta T cells. *Advances in Immunology* 71:77-144.

9. Peterman, G.M., C. Spencer, A.I. Sperling, and J.A. Bluestone. 1993. Role of gamma delta T cells in murine collagen-induced arthritis. *J Immunol* 151:6546-6558.

10. Pelegri, C., P. Kuhnlein, E. Buchner, C.B. Schmidt, A. Franch, M. Castell, T. Hunig, F. Emmrich, and R.W. Kinne. 1996. Depletion of gamma/delta T cells does not prevent or ameliorate, but rather aggravates, rat adjuvant arthritis. *Arthritis Rheum* 39:204-215.

11. Peng, S.L., M.P. Madaio, A.C. Hayday, and J. Craft. 1996. Propagation and regulation of systemic autoimmunity by gamma delta T cells. *J Immunol* 157:5689-5698.

12. Mukasa, A., K. Hiromatsu, G. Matsuzaki, R. O'Brien, W. Born, and K. Nomoto. 1995. Bacterial infection of the testis leading to autoaggressive immunity triggers apparently opposed responses of alpha beta and gamma delta T cells. *J Immunol* 155:2047-2056.

13. Shi, C., B. Sahay, J. Russel, K. Fortner, N. Hardin, T.J. Sellati, and R.C. Budd. 2011. Reduced Immune Response to Borrelia Burgdorferi in the Absence of γδ T cells. *Infection and Immunity.* 79.19:3940-946.

14. Aydintug, MK., C.L. Roark, X. Yin, J.M. Wands, W.K. Born, and R.L. O'Brien. 2004. Detection of cell surface ligands for the gamma delta TCR using soluble TCRs. *J. Immunol.* 172:4167–4175.

15. Collins, C., C. Shi, J.Q. Russell, K.A. Fortner, and R.C. Budd. 2008. Activation of gammadelta T cells by Borrelia burgdorferi is indirect via a TLR- and caspase-dependent pathway. *J Immunol* 181:2392-2398.

16. Collins, C., J. Wolfe, K. Roessner, C. Shi, L.H. Sigal, and R.C. Budd. 2005. Lyme arthritis synovial gamma delta T cells instruct dendritic cells via fas ligand. *J Immunol* 175:5656-5665.

17. Cruz, A.R., M.W. Moore, C.J. La Vake, C.H. Eggers, J.C. Salazar, and J.D. Radolf. 2008. Phagocytosis of Borrelia burgdorferi, the Lyme disease spirochete, potentiates innate immune activation and induces apoptosis in human monocytes. *Infection & Immunity* 76:56-70.

18. Kennedy, N.J., T. Kataoka, J. Tschopp, and R.C. Budd. 1999. Caspase activation is required for T cell proliferation. *J Exp Med* 190:1891-1896.

19. Chun, H.J., L. Zheng, M. Ahmad, J. Wang, C.K. Speirs, R.M. Siegel, J.K. Dale, J. Puck, J. Davis, C.G. Hall, S. Skoda-Smith, T.P. Atkinson, S.E. Straus, and M.J. Lenardo. 2002. Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature* 419:395-399.

20. Salmena, L., B. Lemmers, A. Hakem, E. Matysiak-Zablocki, K. Murakami, P.Y. Au, D.M. Berry, L. Tamblyn, A. Shehabeldin, E. Migon, A. Wakeham, D. Bouchard, W.C. Yeh, J.C. McGlade, P.S. Ohashi, and R. Hakem. 2003. Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. *Genes & Development* 17:883-895.

21. Kang, T.B., T. Ben-Moshe, E.E. Varfolomeev, Y. Pewzner-Jung, N. Yogev, A. Jurewicz, A. Waisman, O. Brenner, R. Haffner, E. Gustafsson, P. Ramakrishnan, T. Lapidot, and D. Wallach. 2004. Caspase-8 serves both apoptotic and nonapoptotic roles. *J Immunol* 173:2976-2984.

22. Helfer, B., B.C. Boswell, D. Finlay, A. Cipres, K. Vuori, T. Bong Kang, D. Wallach, A. Dorfleutner, J.M. Lahti, D.C. Flynn, and S.M. Frisch. 2006. Caspase-8 promotes cell motility and calpain activity under nonapoptotic conditions. *Cancer Research* 66:4273-4278. doi:10.1158/0008-5472.CAN-05-4183

23. Feoktistova, M., P. Geserick, B. Kellert, D.P. Dimitrova, C. Langlais, M. Hupe, K. Cain, M. MacFarlane, G. Hacker, and M. Leverkus. 2011. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Molecular Cell* 43:449-463. doi:10.1016/j.molcel.2011.06.011

24. Tenev, T., K. Bianchi, M. Darding, M. Broemer, C. Langlais, F. Wallberg, A. Zachariou, J. Lopez, M. MacFarlane, K. Cain and P. Meier. 2011. The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Molecular Cell* 43:432-448. doi: 10.1016/j.molcel.2011.06.006

25. Berger, A.K., and P. Danthi. 2014. Reovirus Activates a Caspase-Independent Cell Death Pathway. *mBio*. 4(3):e00178-13. doi:10.1128/mBio.00178-13.

26. Pasparakis, M., and P. Vandenabeele. 2015. Necroptosis and its role in inflammation. *Nature.* 517:311-320. doi:10.1038/nature14191.

27. Collins C., K. Bashant, C. Erikson, P. Thwe, KA, Fortner, and R.C. Budd. Caspase inhibition of dendritic cells promotes cell death but enhances activation of γδ T cells. *Under review at the Journal of Innate Immunity*.

28. Chang, C-H., J.D. Curtis, L.B. Maggi, B. Faubert, A.V. Villarino, D. O'Sullivan, S.C. Huang, G.J. Van der Windt, J. Blagih, J. Qiu, J.D. Weber, E.J. Pearce, R.G. Jones, and E.L. Pearce. 2013. Posttranscriptional Control of T Cell Effector Function by Aerobic Glycolysis. *Cell*. 153(6):1239-1251. doi:10.1016/j.cell.2013.05.016.

29. Yang, Z., E.L. Matteson, J.J. Goronzy, and C.M. Weyand. 2015. T-cell metabolism in autoimmune disease. *Arthritis Research & Therapy*, *17*(1), 29. doi:10.1186/s13075-015-0542-4

30. O'Neill, LA., and E.J. Pearce. Immunometabolism governs dendritic cell and macrophage function. *The Journal of Experimental Medicine. 213*(1):15-23. doi:10.1083/jcb.2121OIA306

31. Pearce, EJ., and B. Everts. 2014. Dendritic Cell Metabolism. *Nature Reviews Immunology* 15:18-29. doi:10.1038/nri3771

32. Lutz, M. B., N. Kukutsch, A.J. Ogilvie, S. Robner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223: 77–92

33. Jantsch, J., D. Chakravortty, N. Turza, A.T. Prechtel, B. Buchholz, R.G. Gerlach, M. Volke, J. Gläsner, C. Warnecke, M.S. Wiesener, K.U. Eckardt, A. Steinkasserer, M. Hensel, and C. William. 2008. Hypoxia and hypoxia-inducible factor-1α modulate lipopolysaccharide-induced dendritic cell activation and function. *J. Immunol.*180:4697–4705. doi:10.4049/jimmunol.180.7.4697

34. Krawczyk, C.M., T. Holowka, J. Sun, J. Blagih, E. Amiel, R.J. DeBerardinis, J.R. Cross, E. Jung, C.B. Thompson, R.G. Jones, and E.J. Pearce. 2010. Toll-like receptor–induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood.* 115:4742–4749. doi:10.1182/blood-2009-10-249540

35. Everts, B., E. Amiel, G.J. van der Windt, T.C. Freitas, R. Chott, K.E. Yarasheski, E.L. Pearce, and E.J. Pearce. 2012. Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood.* 120:1422–1431. doi:10.1182/blood-2012-03-419747

36. Guo, H., W.J. Kaiser, and E.S. Mocarski. 2015. Manipulation of Apoptosis and Necroptosis Signaling by Herpesviruses. *Med Microbiol Immunol.* 204(3):439-48. doi: 10.1007/s00430-015-0410-5

37. Cho, Y., S. Challa, D. Moquin, R. Genga, T.D. Ray, M. Guilford, and F.K. Chan. 2009. Phosphorylation-Driven Assembly of RIP1-RIP3 Complex Regulates Programmed Necrosis and Virus-Induced Inflammation. *Cell*. 137(6):1112-1123. doi:10.1016/j.cell.2009.05.037.

38. Oberst, A. 2015. Death in the fast lane: what's next for necroptosis? *FEBS J.* doi:10.1111/febs.13520.

39. Orozco, A., N. Yatim, M.R. Werner, H. Tran, S.G. Gunja, L. Albert, D.R. Green, A. Oberst. 2014. RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. *Cell Death and Differentiation* 21:1511-1521. doi: 10.1038/cdd.2014.76

40. Ofengeim, D., and J. Yuan. 2013. Regulation of RIP1 kinase signaling at the crossroads of inflammation and cell death. *Nature reviews molecular cell biology.* 14:727-736. doi:10.1038/nrm3683

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

Figures

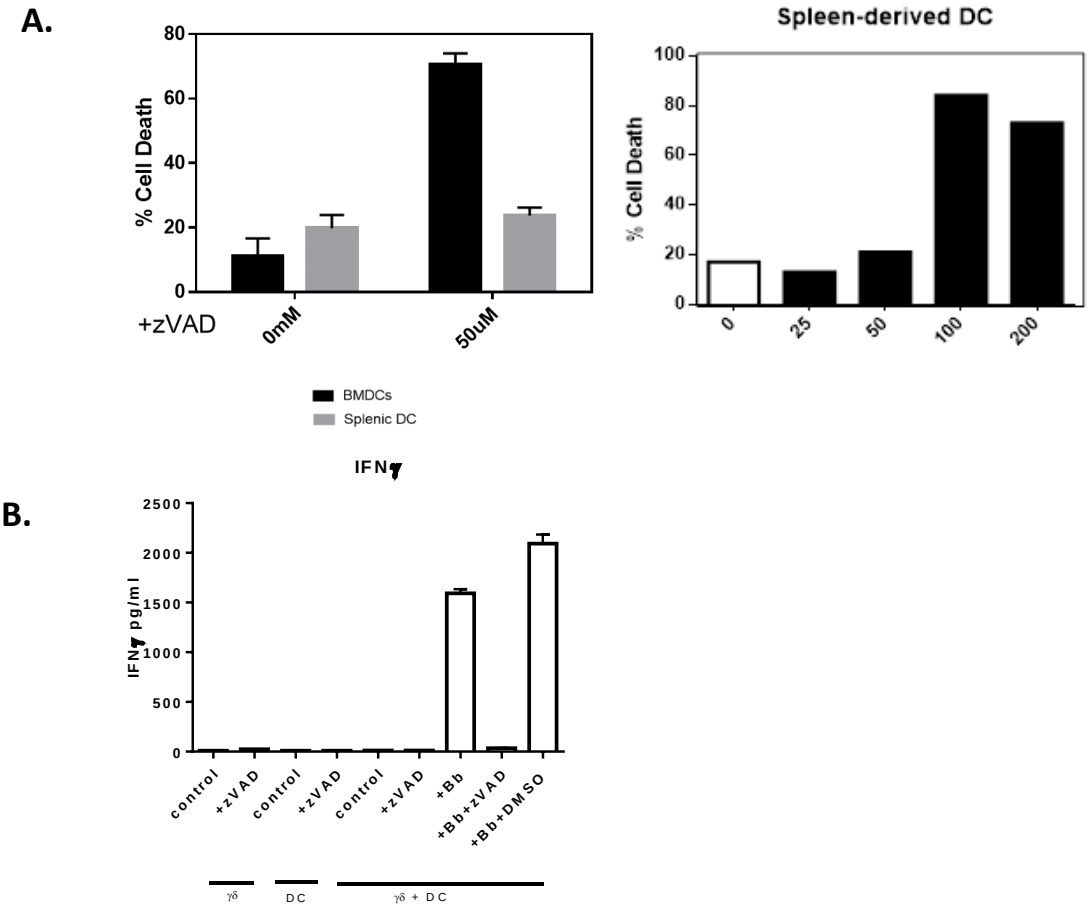

Figure 1. Freshly isolated splenic DC are less susceptible to caspase-inhibition-mediated necroptosis than cultured BMDC and accordingly display a reduce ability to activate γδ T cells. **(A)** Cell death assessed by trypan blue staining revealed that fresh splenic DC did not die following treatment with 50 μM caspase-inhibitor zVAD, unlike their BMDC counterparts that were differentiated with GMCSF *in vitro*. **(B)** Cytokine analysis of supernatants indicates that zVAD-treated splenic DCs do not successfully activate γδ T cells. IFNγ serves as an indicator of γδ T cell activation as it is not produced by DCs or inactive γδ T cells. Bars: Black: γδ T cells, Grey: DC; White: γδ/DC co-incubated. All graphs are representative of at least two independent experiments.

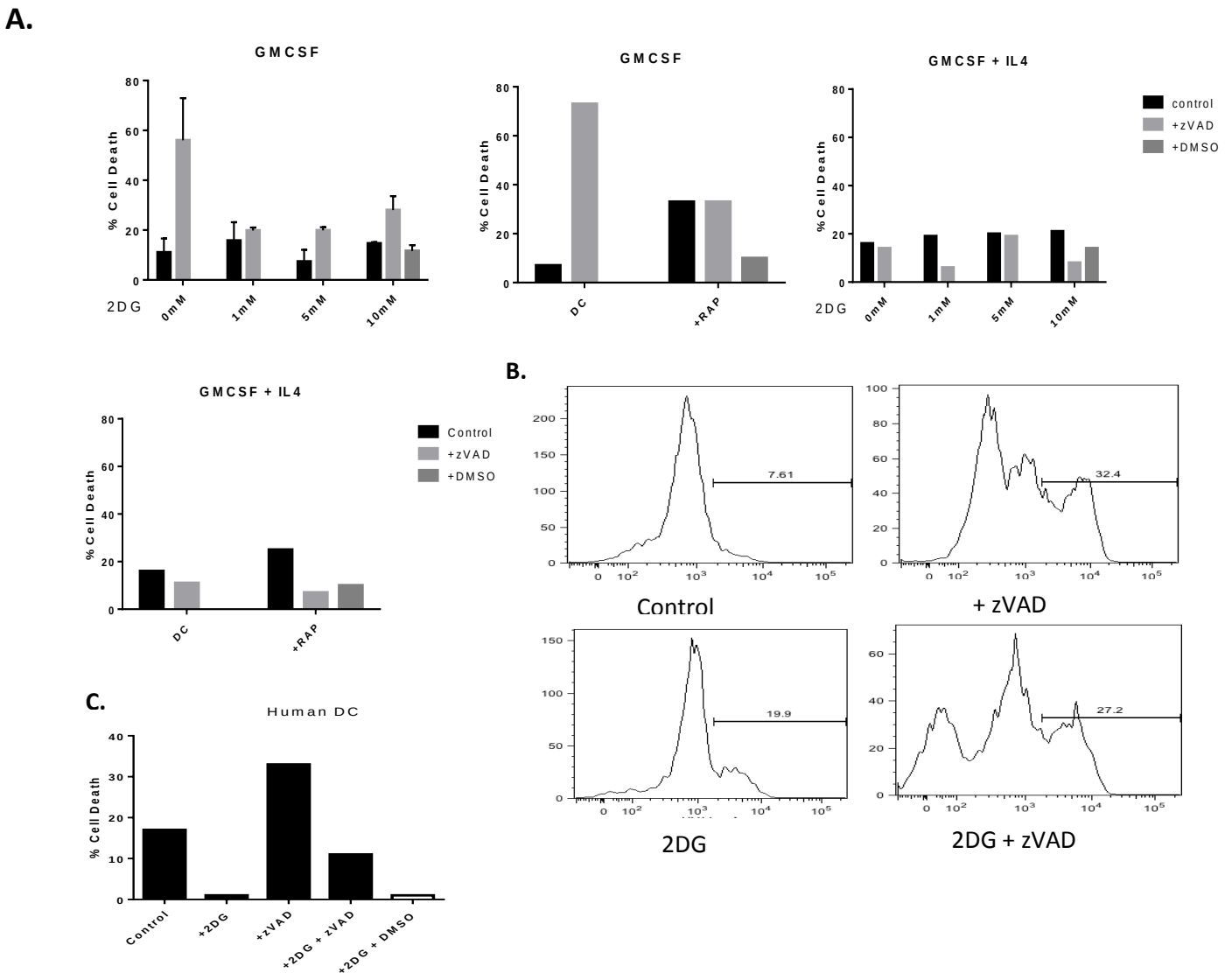

Figure 2. Reduction of glycolysis in GM-CSF-cultivated DC prevents zVAD-mediated necroptosis. ***(A)*** BMDC grown with GM-CSF alone or GM-CSF + IL-4 were exposed to 0-10mM 2DG or 10 ng/ml rapamycin for 24 hours prior to treatment with 50 μM zVAD. Cell death was assessed via trypan blue staining. **(B)** BMDC grown with GMCSF were exposed to 2DG as described in A. Cell death was assessed via flow cytometry. **(C)** Human DCs were isolated from human blood and prepared as described in A with exposure to 10 mM 2DG.

A. **B.**

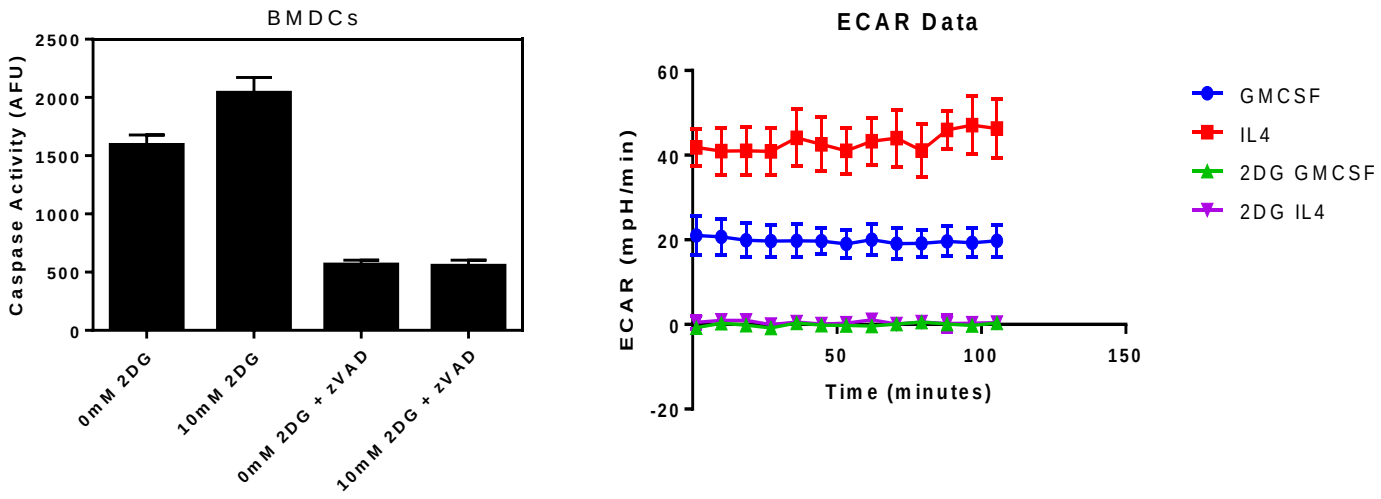

Figure 3. Reduced sensitivity to zVAD in DC treated with 2DG is not due to issues in zVAD or 2DG efficacy. **(A**) BMDCs were exposed to 2DG for 24 hours prior to treatment with zVAD. 24 hours later a DVED caspase activity assay measured total caspase activity present in equal numbers of viable cells from each sample. Results are representative of two independent trials. **(B)** Glycolysis as reflected in lactate production and extracellular acidification rate (ECAR). Measurements made in GM-CSF alone versus and GM-CSF + IL-4-differentiated BMDC based on an extracellular flux analyzer. Measurements taken between 0 and 50 minutes.

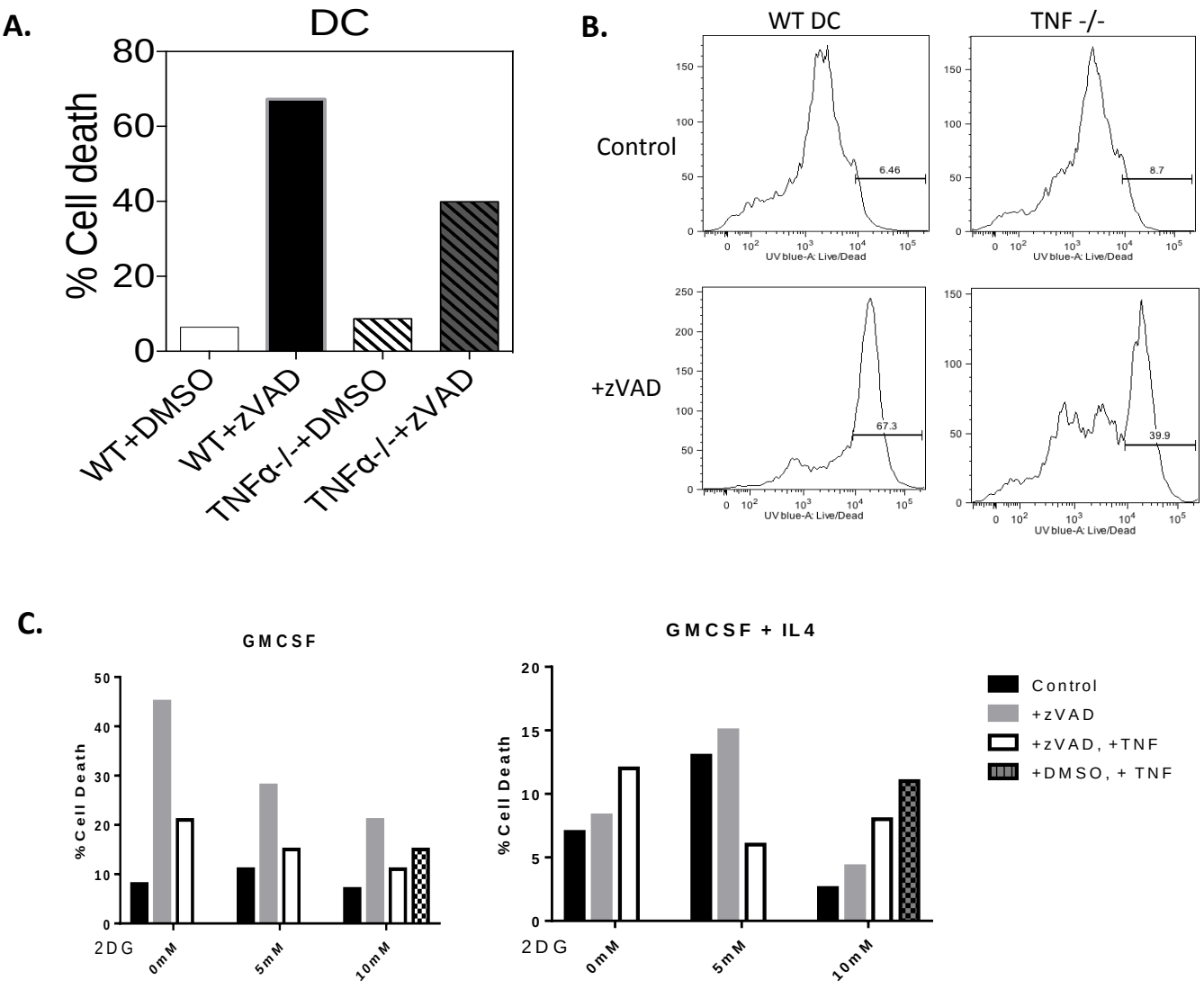

Figure 4. Reduced sensitivity to zVAD in DC treated with 2DG is not due to cells' inability to produce TNF, as it is in some immortalized cell lines. **(A)** BMDCs from a TNF knock-out mouse were differentiated from bone marrow precursors in vitro using GM-CSF alongside WT BMDCs. Both cell populations were exposed to 10mM 2DG for 24 hours prior to treatment with 50μM zVAD. 24 hours later, cell death was assessed using trypan blue staining or in **(B)** via flow cytometry. **(C)** BMDCs were prepared as described previously, but 0 or 10ng/ml exogenous TNF was added at the time of 50μM zVAD. Cell death was assessed via trypan blue staining. All results are indicative of three experiments.

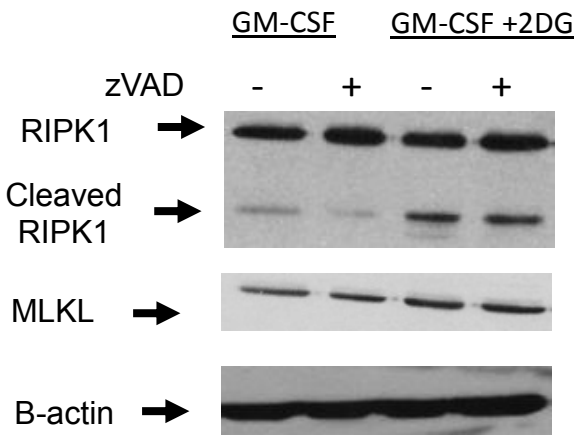

Figure 5. Levels of cleaved RIPK increase with 2DG-treatment of BMDCs. GM-CSF-differentiated BMDCs were treated with or without 2DG for 24 hours prior to treatment with or without zVAD. Ripoptosome proteins were quantitated by western blotting. Results are indicative of three independent experiments.