Diacylglycerol kinase ζ deficiency triggers early signs of aplastic anemia in mice

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Key points

- DGK ζ -deficiency in mice results in larger numbers of CD69-positive T cells in bone marrow, with enhanced expression of IFN γ and lytic enzymes.
- DGK ζ loss recapitulates many clinical aspects of human aplastic anemia, identifying a critical hub for immune system-dependent bone marrow failure.

Visual abstract

Abstract

Acquired aplastic anemia (AA) is a rare blood disorder that results from immune-mediated destruction of bone marrow (BM) progenitor cells. Improved understanding of the mechanisms that favor T cell attack in BM could help to improve early diagnosis and disease treatment. Diacylglycerol kinase ζ (DGK ζ) limits T cell responses through phosphorylation of diacylglycerol into phosphatidic acid. This reaction attenuates diacylglycerol-dependent activation of the Ras/ERK/CD69 and PKC θ /NF κ B pathways in response to antigen. Here we show that, in contrast to the lack of basal activation observed in peripheral lymphoid organs, DGK $\zeta^{-/-}$ mice showed increased numbers of activated T cells in BM, together with a significant increase in IFN γ as well as perforin and granzyme B and C levels. The enhanced presence of T cells in DGK $\zeta^{-/-}$ mouse BM correlates with reduced BM cellularity, impaired hematopoiesis, and lower frequency of circulating red cells, granulocytes, and platelets. Our studies coincide with the recent characterization of lower DGK ζ expression in T cells isolated from the BM of patients with acquired AA, and suggest that limited DGK ζ expression and/or functions predispose to T cell-mediated BM destruction. This study identifies the BM as a niche particularly sensitive to DGK ζ deficiency and indicates that this mouse model could be of interest for studying the mechanism that contributes to AA development.

Introduction

Aplastic anemia (AA), the prototypical bone marrow (BM) failure syndrome, is characterized by peripheral pancytopenia and hypoplastic BM.¹ Most AA cases are acquired, idiopathic, and result from aberrant activation in the BM of T helper type-1 CD4⁺ (Th1) and cytotoxic CD8⁺ T cells (CTL).² The first choice in AA treatment is allogeneic stem cell transplant using BM cells, or immunosuppressive therapies when transplant is not possible. There is an intensive search for recurrent cytogenetic abnormalities and/or somatic mutations that identify? predisposition to this disease. The identification of biological markers that indicate BM attack could assist in the selection of candidates for transplant, prognosis, and/or the predictive response to immunosuppressive therapies.

Diacylglycerol kinases (DGK) are a family of kinases that phosphorylate diacylglycerol (DAG) to phosphatidic acid (PA).³ DGK ζ is abundantly expressed in healthy naïve peripheral T cells, where regulates DAG-dependent activation of Ras/ERK and PKC θ /NF κ B signaling pathways.⁴ DGK ζ downmodulation in response to antigenic challenge facilitates the correct activation of T cell helper and cytotoxic functions. Germline-DGK ζ -deficient mice (DGK $\zeta^{-/-}$) have hyperproliferative T lymphocytes, which are resistant to anergy induction⁵ and show enhanced elimination of xenotransplanted tumors.⁶⁻⁸

No human studies to date have described autoimmune-related DGK ζ alterations. DGK $\zeta^{-/-}$ mice show no overt autoimmune symptoms, which has been attributed to enhanced development and function of regulatory T cells.⁹ Upregulation of the microRNA miR-34a during T cell activation facilitates DGK ζ suppression,¹⁰ whereas Egr2-dependent transcription triggers DGK ζ induction in tumor-induced T cell anergy.¹¹ Studies in AA patients have shown that mirR34a abundance in T cells is a marker of AA severity, and that higher mirR34a expression correlates with DGK ζ reduction.¹² The lesser DGK ζ abundance in AA patient BM T lymphocytes suggests a causal relationship between DGK ζ deficit, enhanced T cell

functions, and BM destruction. This observation ~~in AA patients~~ prompted us to investigate the direct consequences of DGK ζ genetic deletion in BM homeostasis. Our studies in DGK $\zeta^{-/-}$ mice indicate increased presence of activated T cells in the BM, enhanced erythrocyte destruction, diminished hematopoietic precursor, and peripheral cytopenia. All these abnormalities, characteristic of BM failure, mirrored many of the clinical symptoms described at early stages of AA. Our studies identify an autoimmune disorder as the result of DGK ζ deficiency and show the utility of DGK $\zeta^{-/-}$ mice as an experimental model for the study of AA onset and development.

Methods

Mice. $DGK\zeta^{-/-}$ and $DGK\alpha^{-/-}$ C57BL/6 mice were kindly donated by Drs. G Koreztky (Weill Cornell Medicine, New York, NY) and XP Zhong (Duke University Medical Center, Durham, NC). Except when indicated (4-5 weeks), animals were used between 6-12 months of age. No sex criteria were established for experiments. All procedures were conducted in accordance with Spanish and European directives and a protocol approved by the CNB/CSIC Ethic Committee for Animal Experimentation (RD53/2013).

BM, lymph nodes, spleen and blood analysis. BM cells were extracted in phosphate-buffered saline (PBS), counted on a Countess II (ThermoFisher), and analyzed by flow cytometry or processed for RNA analysis. Lymph nodes and spleen were removed, disaggregated, and homogenized in 40 μ m cell strainers (Becton Dickinson), then stained and analyzed by flow cytometry. Blood was collected by heart puncture in EDTA tubes and analyzed on an Abacus Junior Vet blood analyzer 24 h after extraction, or by flow cytometry.

Histology. Sternum and femur bones were decalcified in 10% EDTA for 48 h or 21 days respectively, fixed in 4% paraformaldehyde, and paraffin-embedded. Tissue sections (5 μ m) were stained with hematoxylin and eosin (H&E). BM cell density was analyzed using ImageJ software.

Immunofluorescence. BM cells were lysed with RBC lysis buffer (eBioscience) to remove erythroid cells, fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 (Sigma Aldrich), blocked with 10% bovine serum albumin (Sigma Aldrich), 0.05% Triton X-100 in PBS (30 min, RT), and incubated with antibodies to IFN γ (Abcam, ab9657) and CD3 ϵ (BD Pharmingen, 550275) (ON, 4°C) in a humidified chamber. Antigen was detected using goat anti-rabbit Cy3 (Jackson) and goat anti-Armenian hamster Alexa488 (Abcam, ab173003) (60 min, RT). Images were acquired on an Olympus FV1000 confocal laser scanning microscope, and analyzed with Fiji software.

Flow cytometry and antibodies. Antibodies used were anti-Ter119-PE, CD8-eFluor450, CD69-PeCy7, Gr1-PeCy7, Ly6G-PE (eBioscience), CD44-APC, (Coulter), CD4-PECy5, CD16/32-FITC, CD34-PE, cKit-APC, Ly6C-FITC, CD11b-PerCP5.5, Ter119-PeCy7, CD11b-PeCy7, CD4-PeCy7, CD8-PeCy7, B220-PeCy7 (BioLegend), CD69-FITC, CD62L-FITC, Scal-biotin, NK1.1-APC, CD11c-biotin (Pharmingen), annexinV-FITC (Southern), annexinV-APC (IMMUNOSTEP) and DAPI-eFluor450. For S1P1 staining, femurs were flushed with fix solution containing 0.1% paraformaldehyde, 0.5% BSA, and 2 mM EDTA (Gibco) in PBS as reported.¹³ Cell suspensions were then lysed with RBC lysis buffer (eBioscience), incubated with rat anti-mouse S1P1-APC (R&D Systems) for 1 h on ice and washed once. Samples were then incubated (30 min, 4°C) with antibody cocktail to CD4-PercP5.5 (BioLegend), CD8-eFluor450 (eBioscience), CD44-FITC (Beckman) and CD69-PE (Pharmingen). Samples were collected on a LSRII (BD Biosciences) and analyzed with FlowJo software (FlowJo LLC, Ashland, OR).

Induction of bone marrow failure (BMF). F1 progeny was generated by crossing C57BL/6 and BALB/c mice. Prior to experiments F1 mice were irradiated (3 Gy, ¹³⁷Cs source); after 5 h, BMF was induced with 5×10^7 splenocytes (intraperitoneal injection) from age- and gender-matched C57BL/6 wild type or DGK $\zeta^{-/-}$ mice, as described.¹⁴ For survival studies, mice were humanely euthanized when weight loss was less than 80% of original weight.

cDNA preparation and real time PCR. Total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (PN4368813; Applied Biosystems). Real-time PCR reactions were performed in triplicate with HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne) in an Applied Biosystems ABI PRISM 7900HT machine with SDS v2.4 software, using a standard protocol. Results were analyzed by the comparative Ct method ($\Delta\Delta Ct$). Expression was normalized using the β -actin housekeeping gene for each sample. Primers used were: IL-6: 5'-GCTACCAAACCTGGATATAATCAGGA-3' and 3'-

AAGACCTCATGTATCGATG GACC-5'; TNF- α : 5'-
CCCTCACACTCAGATCATCTTCT-3' and 3'-GACATCGGGTGCA GCATCG-5'. TGF- β :
5'-ACCATGCCAACTTCTGTCTG-3'; 3'-AGATGTTGGTTGTGTT GGGC-5'. Primers for
IFN γ and perforin,¹⁵ for granzymes A, B and C,¹⁶ for IL-10 and IL-2,¹⁷ for KLF-1, cMpl and
p57,¹⁸ and for β -actin¹⁹ as reported.

Statistical analysis. Data were analyzed using GraphPad Prism 5. Results are represented as mean $\pm$ standard error of the mean (SEM). Normality was analyzed by the Kolmogorov-Smirnov test. An unpaired two-tailed t-test with 95% confidence intervals was used for data with normal distribution and equal variances, and an unpaired t-test with Welch's correction for data sets with different variances. The Mann-Whitney U test was used for data with non-normal distribution. For more than two groups with normal distribution, ANOVA with Bonferroni correction was performed. Kruskal-Wallis test with Dunn's post hoc was applied when two groups with non-normal distribution. Survival statistics were performed with the Gehan-Breslow-Wilcoxon test. Differences were considered non-significant (ns) when $p > 0.05$, significant (*) when $p < 0.05$, very significant (**) when $p < 0.01$ and extremely significant (***) when $p < 0.001$.

Results

DGK ζ deficiency in mice causes no gross defects in T cell development and leads to a slight decrease in mature T cells in spleen, with no signs of activation in basal conditions.⁵ The BM from young (4- to 5-week old) DGK $\zeta^{-/-}$ mice showed similar total numbers of CD4⁺ and CD8⁺T cells compared to WT mice (Figure 1A). CD69 is an early activation marker that identifies recent antigen-stimulated T lymphocytes.²⁰ Both T cell pools in the BM of DGK $\zeta^{-/-}$ mice showed significantly augmented C69 populations with heightened CD69 expression on a per cell basis compared to WT animals (Figure 1B). A greater number of CD69⁺ T lymphocytes in DGK $\zeta^{-/-}$ mouse BM correlates with observations in AA patients, whose BM CD69⁺ T cell numbers are greater than those of healthy controls.¹²

Expanded numbers of IFN γ -expressing Th1 T cells is an indicator of AA,² and low IFN γ ⁺ T cell frequency correlates with responsiveness to immunosuppressive therapy.²¹ mRNA expression analysis showed a significant increase in IFN γ abundance in DGK $\zeta^{-/-}$ BM compared to WT mice (Figure 1C). Additional analysis showed higher expression of perforin and granzymes A, B and C as well as IL-2 and TNF α , with reduction in TGF β expression (Figure 1C). Greater IFN- γ and TNF α expression correlates well with the enhanced skewing toward Th1 phenotypes that results from DGK ζ deficiency,²² and resembles the increased levels of circulating IFN γ in AA patients.²³ Augmented expression of granzymes B and C and perforin in DGK $\zeta^{-/-}$ mouse BM denotes an increase in cytotoxic T cells (CTL) similar to that described in AA patients.²⁴ Activated T lymphocytes cause BM destruction in AA;²⁵ DGK $\zeta^{-/-}$ mice showed reduced BM cellularity (Figure 1D). Analysis of the TER-119⁺ erythroid lineage populations did not show differences between DGK $\zeta^{-/-}$ and WT mice (Figure 1E). Nonetheless, combined analysis of CD44 expression as a function of cell size in the TER-119⁺ population²⁶ showed lower cell numbers at stage IV in DGK $\zeta^{-/-}$ mice (Figure 1E).

The BM failure in AA is attributed in great measure to the abnormal presence of inflammatory cytokines that promote HSC differentiation, preventing their continuous self-renewal.²⁷ HSC activity in the BM is found in a small subset of the lineage marker (Lin)⁻ Sca1⁺c-Kit⁺ (LSK) population that gives rise to multipotent progenitors (MMP).²⁸ Immunophenotype analysis of HSC by flow cytometry showed no differences in the LSK pool in DGK ζ ^{-/-} mice, with a significant decrease in MMP compared to WT mice (Figure 2A). Further analysis revealed a reduction in the common myeloid progenitors (CMP) as well as megakaryocyte/erythrocyte progenitors (MEP) and granulocyte/monocyte progenitors (GMP) (Figure 2B). Analysis of some key hematopoietic factors showed severely reduced expression of Kruppel-like factor 1 (KLF-1), essential for erythroid lineage commitment and maturation in mice and humans²⁹ (Figure 2C). DGK ζ ^{-/-} mice also showed lower expression of the thrombopoietin (THPO) receptor c-Mpl (Figure 2C). THPO binding to its receptor sustains adult quiescent HSC populations by regulating cyclin-dependent kinase inhibitors including p57^{kip2}.^{30,31} Reduced p57^{kip2} expression pointed to impaired HSC quiescence in DGK ζ ^{-/-} mice (Figure 2C).

We analyzed mature myeloid populations using antibodies to target cell surface proteins, in combination with forward vs side scatter (FSC and SSC) parameters as described.³² Dendritic subsets (CD11c^h CD11b⁺) were differentiated from other CD11b⁺ myeloid subsets and were further analyzed for NK1.1⁺ natural killer (NK) and Ly6G⁺ neutrophil subsets (Figure 2D). Eosinophils (Ly6C^{Int} Ly6G⁻ SSC^{hi}) were differentiated from the monocyte/macrophage population that were further analyzed for the presence of Ly6C⁺ and more mature Ly6C^{low/neg} populations.³³ DGK ζ ^{-/-} mouse BM showed a substantial reduction in all myeloid populations except NK cells (Figure 2E).

These results demonstrate that young DGK ζ ^{-/-} mice have activated T cells in BM and a concurrent reduction in precursors of erythroid and myeloid populations. Lineage tracing

experiments show that HSC produce the majority of conventional myeloid cells and platelets within 8 months, approximately half of the mouse lifespan.³⁴ We thus explored the evolution of BM populations in adult (6-12 months) WT and DGK $\zeta^{-/-}$ mice. Total CD4⁺ T cell numbers in the BM from DGK $\zeta^{-/-}$ mice were elevated compared to those in WT mice (Figure 3A). CD69 analysis confirmed increase both in the total numbers of CD69 positive and the CD69 expression in CD4 and CD8 T cell populations (Figure 3B). In addition to acting as a T cell activation marker, CD69 functionally promotes BM retention by binding to and sequestering the sphingosine-1-phosphate receptor (S1P1), necessary for BM egress.³⁵ In accordance, the percentage of S1P⁺ CD4⁺ T cells was lower in DGK $\zeta^{-/-}$ compared to controls. A lesser, non-significant tendency was observed in CD8⁺ T cells (Figure 3C).

Similar to that observed in young mice, mRNA analysis indicated augmented expression of IFN γ and granzymes B and C (Figure 3D). No differences were observed for IL-2, TNF α , TGF β and IL-10, except for lower IL-6 expression (Figure 3E). Immunofluorescence analysis confirmed increased mean fluorescence intensity (MFI) of IFN γ in CD3⁺ cells from BM of DGK $\zeta^{-/-}$ compared to WT mice (Figure 3F).

Analysis of BM from mature WT and DGK $\zeta^{-/-}$ showed substantial loss of cellularity (Figure 4A) that correlated at this age with a marked reduction of the erythroid TER-119⁺ population³⁶ (Figure 4B). Annexin V staining suggested a higher apoptosis rate in DGK $\zeta^{-/-}$ TER-119⁺ erythrocytes compared to WT mice (Figure 4B). Based on CD44 vs. FSC gating for erythroid differentiation, DGK $\zeta^{-/-}$ showed reduced cell numbers at all differentiation stages (Figure 4C). Annexin V staining for apoptosis detection was higher at stage I, the earliest progenitors, and at stage IV, where erythroid precursors show the greatest sensitivity to apoptotic cell death³⁷ (Figure 4C). Analysis of cells from the myeloid lineage in DGK $\zeta^{-/-}$ mice showed reduced numbers of neutrophils and monocytes/macrophage populations (Figure 4D).

DGK α is another DGK family member expressed highly in T lymphocytes and has partially redundant functions in the control of Ras/ERK signals downstream of the TCR.⁴ At difference from the case for DGK ζ , DGK $\alpha^{-/-}$ mice showed normal numbers of BM CD4 and CD8 T cells (Supl Figure 1A) with no differences in CD69 expression (Supl Figure 1B). Ter119⁺ populations were not altered (Supl. Figure 1C), with similar cell numbers at all differentiation stages (Supl. Figure 1D). These results strongly suggest an isoform-specific DGK ζ contribution limiting T cell activation at the BM.

Immune-dependent destruction of BM precursors together with continuous differentiation eventually translates into peripheral cytopenia in AA patients. Blood analysis of DGK $\zeta^{-/-}$ mice showed reduction in RBC counts, as well as decreased hematocrit and hemoglobin content compared to WT mice (Figure 5A). Similar lymphocyte counts (Figure 5B) contrasted with severe reduction in circulating granulocytes and platelets, as anticipated from BM analysis (Figure 5C). These data fully agree with the impaired generation of precursors, erythrocyte and myeloid populations, and correlate with clinical symptoms observed in AA patients.

As human studies show that the most activated T cells enter the circulation,³⁸ we analyzed the activation profile of WT and DGK $\zeta^{-/-}$ peripheral blood cells (PBL). DGK $\zeta^{-/-}$ mice showed a higher percentage of circulating CD4 and CD8 T cells (Figure 5D), with large increase in CD69⁺CD4⁺ and CD8⁺ T cells (Figure 5E). Based on L-selectin (CD62L) and CD44 expression, we analyzed blood for CD62L^hCD44^{lo} naive (TN), CD62^{lo}CD44^h effector memory (TEM) and CD62^hCD44^h central memory (TCM) T cells (Figure 5F); in agreement with heightened expression of the activation marker CD69, blood analysis from DGK $\zeta^{-/-}$ mice showed reduced abundance of TN populations with an increased percentage of TEM (Figure 5F) and TCM CD4⁺ T compared to WT mice (Figure 5F). In agreement with other reports,⁵ CD69 expression was similar in DGK $\zeta^{-/-}$ and WT splenocytes and lymph nodes (Figure 5G). The analysis of DGK $\zeta^{-/-}$ PBL reflects the activation T cell phenotype observed in the BM. The

activation phenotype is reminiscent of the reduced frequency of TN $CD4^+/CD8^+$ cells³⁹ and the increase percentages of $CD4^+$ and $CD8^+$ TEM subsets⁴⁰ that are described in PBLs of AA patients.

All these experiments suggested direct correlation between DGK ζ loss and enhanced T cell-mediated destruction of the BM. To further demonstrate a causal relationship between DGK ζ deficiency in the T cell compartment and BM damage, we used a well-characterized murine model of immune-dependent BM failure.¹⁴ Severe AA was induced via sublethal irradiation of F1 hybrid mice, followed by adoptive transfer of WT or DGK $\zeta^{-/-}$ C57BL/6 splenocytes (Figure 7A). All infused animals showed symptoms rapidly and were sacrificed between 14 and 17 days (Figure 7B). In accordance with the higher IFN γ serum concentrations detected in murine lymphocyte infusion-induced BM failure models,⁴¹ the BM of mice analyzed 7 days after infusion with WT splenocytes showed enhanced IFN γ expression compared to irradiated control animals (Figure 7C). Augmented granzyme A and IL-2 expression was in agreement with the massive infiltration of $CD8^+$ (NK and T cells) described in this infusion model.⁴¹ Compared to mice infused with WT splenocytes, hosts receiving DGK $\zeta^{-/-}$ cells showed higher expression of perforin and granzymes A and B indicative of strong cytotoxic function (Figure 7C). The lack of IL-2 increase observed in hosts infused with DGK ζ -deficient cells, contrasted with enhanced expression of TNF- α and IL-6 (Fig 7C). In mouse models of graft-versus-host disease (GVHD), TNF α production correlates with the destructive phase of the disease.⁴² Enhanced IL-6 production is also an important factor in the severity of murine GVDH pathology.⁴³ In accordance with the recognized contribution of TNF α and IL-6, the extent of BM destruction was higher in F1 mice that received DGK $\zeta^{-/-}$ cells compared to those infused with WT cells (Figure 7D).

Discussion

Studies in animal models have shown that antigen recognition in the absence of DGK ζ results in stronger Ras/PKC θ activation, leading to enhanced AP-1/NF κ B-regulated transcription.⁴⁴ This promotes a CD4 Th1/ Th2 skew that provides DGK $\zeta^{-/-}$ mice with enhanced anti-viral responses,⁵ granting protection against allergic asthma.²² DGK ζ deficiency also potentiates cytotoxic programs in CD8⁺ T and NK cells, boosting antitumor functions in an antigen-dependent⁴⁵ and -independent manner.^{6,46} These and other studies prompted a growing interest in DGK ζ as a pharmacological target with immunomodulator abilities. A central issue when considering the therapeutic potential of negative regulators of T cell function is the risk of unleashing adverse autoimmune responses. Characterization of autoimmune disease symptoms in DGK $\zeta^{-/-}$ mice is critical to fully assessing the therapeutic potential of this enzyme.

Our studies identify abnormal accumulation of CD69-positive T cells in the BM of untreated DGK $\zeta^{-/-}$ mice, a situation reminiscent of the enhanced CD69 expression observed in BM T lymphocytes from AA patients. The lower cellularity, impaired erythropoiesis, and deficient progenitor population numbers found in DGK $\zeta^{-/-}$ mouse BM suggest immune-dependent BM failure similar to that described in AA. The observation of diminished DGK ζ expression in the T cells from the BM of AA patients¹² further reinforces our observations and suggests a possible role for DGK ζ in limiting T cell-dependent BM destruction.

The finding of CD69⁺ T cells in the BM of DGK $\zeta^{-/-}$ mice at a very young age, together with the presence of Th1 and cytotoxic cytokines, suggest early presence of activated T cells. CD69 is an activation marker that is induced rapidly after antigen recognition and decays very rapidly in peripheral cells. In activated CD4 T cells, CD69 expression attenuates S1P1 expression and promotes homing into the BM, where CD4 T cells evolve into memory subsets.⁴⁷ As a result, the BM is recognized as a central niche for long-term homing of

memory T cells.⁴⁸ The ability of CD69 to favor BM homing for CD4 T cells and attenuate S1P1 expression coincides with the greater CD4 T cell retention observed in the BM of DGK ζ ^{-/-} mice. DGK ζ deficiency correlates with enhanced CD69 expression in CD4 and CD8 T cells.⁴⁴ The reduction in naive populations and concomitant increase in memory T cells in PBL from DGK ζ ^{-/-} mice confirms self-activation, in accordance with the direct communication between the BM and peripheral blood.

Immune-dependent BM destruction in AA is attributed both to the direct and indirect effects of Th1 and CTL on HSC and other hematopoietic lineages.⁴⁹ In healthy conditions, elevation of inflammatory cytokines such as IFN γ induce rapid differentiation of HSC to the myeloid lineage to respond to infections. Abnormal, chronic IFN γ elevation in AA impairs HSC continuous self-renewal leading to loss of stem precursors.²⁷ The analysis of stem cell populations in DGK ζ ^{-/-} mice reveals an abnormal, high elevation of IFN γ concomitant with a significant decrease in all precursor populations and myeloid mature cells. In agreement, DGK ζ ^{-/-} mice have reduced numbers of circulating red cells, platelets, monocytes, and granulocytes. In addition to the loss of self-renewal abilities of the stem subsets, immune-dependent BM destruction is also linked to the direct induction of apoptosis. The increased apoptosis of erythroblasts observed even at a very early age, together with the lowered expression of cell cycle inhibitors probably linked to defective TP53 functions indicates that these are not opposite alternatives. These data coincides with the analysis of CD34⁺ cells from severe pediatric AA patients, which shows higher expression of apoptotic-related genes and an enhanced IFN γ signature.⁵⁰ DGK ζ ^{-/-} mice show partial BM aplasia and diminished circulating platelets and granulocytes, but not severe anemia. It is plausible that low, chronic T cell activation leads to T cell exhaustion in a manner similar to the exhausted hyporesponsive T cells found in the BM of IFN γ -induced murine models of AA.⁵¹

The phenotype of DGK $\zeta^{-/-}$ mice suggests BM destruction by autoreactive T cells. In agreement, infusion of DGK $\zeta^{-/-}$ T splenocytes in histocompatibility-mismatched hosts accelerated BM damage when compared with splenocytes from WT mice. Cytokine analysis showed enhanced TNF α and IL-6 production in hosts infused with DGK $\zeta^{-/-}$ cells. In GVHD models, transient IFN γ production associates with the initial highly proliferative phase whereas TNF α production correlates with the later more destructive phase of the disease.⁴² Our results suggest a temporality in the induction of BM failure as the result of a GVHD-like response; they suggest that DGK ζ loss accelerates the onset of the reaction from the initial proliferative phase to the final destructive phase. This hypothesis coincides with recent studies in murine models of acute AA showing that TNF α , which is generated by host macrophages in response to infused T cells, accelerates immune-mediated BM destruction.⁵³ Reduced IL-2 expression observed in mice that receive DGK ζ -deficient cells is consistent with rapid onset of activation, leading to functional decline of antigen-specific effector T cells that could result in their depletion. Exhaustion of rapidly expanding effector T cell populations is said to explain stronger antitumor effects in xenotransplanted DGK $\zeta^{-/-}$ mice.⁶ Little or no IFN γ production by BM T cells is also observed in very severe cases of AA, and is predictive of a lack of response to immunosuppressive therapy.²¹ Progressive T cell exhaustion would coincide with the lack of high IL-2 and TNF α expression observed in mature mice. It would also conform to reduced expression of IL-6, a pro-inflammatory cytokine that, as described for IFN γ and TNF α , regulates HSC differentiation.⁵²

Despite intensive studies, understanding of the autoimmune mechanism in AA remains limited. Pesticides, toxic chemicals, and viral infections are hypothesized to confer higher risk, but acquired AA is largely considered idiopathic and the autoantigen remains unknown. We found no sex differences in the phenotype of DGK $\zeta^{-/-}$ mice, whereas enhanced BM failure in older mice suggests cumulative damage. A progressive loss of BM function would agree

with recent studies that cite normal platelet counts in $DGK\zeta^{-/-}$ mice around 10 weeks of age.⁵⁴ In contrast observations in older animals, our analysis confirms normal platelet counts between 10-15 weeks (not shown). The reported abnormal platelet responses⁵⁴ could nonetheless be a result of defective differentiation. In this regard, all components of the trimeric GPIB/GPIX/GPV complex as well as GPVI, the collagen receptor on platelets, appear markedly downregulated in $CD34^{+}$ gene profiling from pediatric severe AA patients.⁵⁰

In summary, our study demonstrates that $DGK\zeta$ protects the BM from autoimmune-mediated destruction, and suggests that $DGK\zeta^{-/-}$ mice are a useful model to further explore the mechanisms that trigger this disease. Detection of $DGK\zeta$ expression could also be an important biomarker of the T cell function in AA patients. Finally, a better understanding of the mechanism by which $DGK\zeta$ protects the BM from autoimmune destruction could help to avoid the risks of aplastic anemia induced by treatment with immune checkpoint inhibitors.⁵⁵⁻⁵⁷

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Authorship contributions: IM, EA and MMS designed the research and wrote the manuscript; EA, MMS, MLS and RL performed the experiments; MMS and EA analyzed the results; MMS and EA designed the figures.

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Figure 1. Analysis of BM T cells from DGK ζ deficient mice.

BM cells from WT and DGK $\zeta^{-/-}$ mice were flushed from tibias and femurs and processed for flow cytometry analysis. **(A)** Quantification of CD4 $^{+}$ and CD8 $^{+}$ T cells counts in the BM of 4- to 5- week old mice (gated in total cells). **(B)** Left, representative histograms of CD69 expression gated on CD4 $^{+}$ or CD8 $^{+}$ T cell populations. Middle, total counts of activated T cells CD69 $^{+}$ CD4 $^{+}$ and CD69 $^{+}$ CD8 $^{+}$ cells. Right, mean fluorescence intensity (MFI) of CD69 in CD4 $^{+}$ and CD8 $^{+}$ populations. **(C)** mRNA expression was analyzed from total BM cells by RT-qPCR. Top, expression of IFN γ , perforin and granzymes (Grz.). Bottom, mRNA levels of IL-2, TNF α , TGF β and IL-10. **(D)** Left, femur sections were fixed, and H&E stained. Images show central area of each section (200x). MiddleCenter, quantification of the percentage of area covered by cells. Right, quantification of total BM cells by TrypanBlue exclusion. **(E)** Left, quantification of total erythroid cells in WT and DGK $\zeta^{-/-}$ mice by Ter119 expression. Right, analysis of erythroid differentiation. A. Mean $\pm$ SEM. n=6/genotype. CD4 $^{+}$. Unpaired t-test. CD8 $^{+}$. Mann-Whitney test. B. Mean $\pm$ SEM. n=6/genotype. Unpaired t-test. C. Mean $\pm$ SEM. WT n=4; DGK $\zeta^{-/-}$ n=5. IFN γ , perforin and Grz. C. Unpaired t-test with Welch's correction. Grz. A, Grz. B, IL-2, TNF α and TGF β . Unpaired t-test. IL-10. Mann-Whitney test. Data were acquired in two independent experiments. D. Mean $\pm$ SEM. Left and center, four sections/mouse; WT n=4, DGK $\zeta^{-/-}$ n=5. Unpaired t-test. For total cellularity, WT n=14, DGK $\zeta^{-/-}$ n=18. Unpaired t-test. E. Mean $\pm$ SEM. WT n=7, DGK $\zeta^{-/-}$ n=8. E. Mann-Whitney test. Data were acquired in two independent experiments.

Figure 2. DGK $\zeta^{-/-}$ mice show defects in HSCs and myeloid development in the BM.

(A) Left, representative flow cytometry dot plots of cKit and Sca1. Right, quantification of total of LSK cells (Lin $^{-}$ cKit $^{+}$ Sca1 $^{+}$) (gated on Lin $^{-}$ BM cells), and multipotent progenitors (MP) (Lin $^{-}$ cKit $^{+}$ Sca1 $^{-}$) (gated on Lin $^{-}$ cells). **(B)** HSCs composition was gated on Lin $^{-}$ BM

cells by flow cytometry. Left, representative dot plots of CD16/32 and CD34. Right, quantification of common myeloid progenitors (CMP) ($\text{Lin}^- \text{cKit}^+ \text{Sca1}^- \text{CD34}^+ \text{CD16/32}^{\text{int}}$), granulocyte/monocyte progenitors (GMP) ($\text{Lin}^- \text{cKit}^+ \text{Sca1}^- \text{CD34}^+ \text{CD16/32}^{\text{hi}}$), and megakaryocyte/erythrocyte progenitors (MEP) ($\text{Lin}^- \text{cKit}^+ \text{Sca1}^- \text{CD34}^- \text{CD16/32}^{\text{lo}}$) (gated on MP). (C). mRNA was purified from total BM cells and KLF1, cMpl and p57 expression was analyzed by RT-qPCR. (D) Flow cytometry dot plots of gating strategy followed for myeloid characterization. (E) Quantification of total dendritic cells ($\text{CD11b}^+ \text{CD11c}^+$), natural killer cells ($\text{NK1.1}^+ \text{Ly6G}^-$) (gated on $\text{CD11b}^{\text{low/high}} \text{CD4}^- \text{CD8}^- \text{B220}^-$), neutrophils ($\text{NK1.1}^- \text{Ly6G}^+$) (gated on $\text{CD11b}^{\text{low/high}} \text{CD4}^- \text{CD8}^- \text{B220}^-$), eosinophils (Ly6C^{dim}) (gated on $\text{CD11b}^{\text{low/high}} \text{CD4}^- \text{CD8}^- \text{B220}^- \text{NK1.1}^- \text{Ly6G}^{\text{low/dim}}$), macrophages and monocytes (Ly6C^{low}) (gated on $\text{CD11b}^{\text{low/high}} \text{CD4}^- \text{CD8}^- \text{B220}^- \text{NK1.1}^- \text{Ly6G}^{\text{low/dim}}$), and inflammatory macrophages ($\text{Ly6C}^{\text{high}}$) (gated on $\text{CD11b}^{\text{low/high}} \text{CD4}^- \text{CD8}^- \text{B220}^- \text{NK1.1}^- \text{Ly6G}^{\text{low/dim}}$). A, B. Mean $\pm$ SEM. WT n=5; $\text{DGK}\zeta^{-/-}$ n=7. Unpaired t test. Data were acquired in two independent experiments. C. Mean $\pm$ SEM. WT n=4; $\text{DGK}\zeta^{-/-}$ n=5. $2^{-\Delta\Delta\text{Ct}}$ p57. Unpaired t-test with Welch's correction. $2^{-\Delta\Delta\text{Ct}}$ KLF1 and cMpl. Unpaired t-test. D, E. Mean $\pm$ SEM. n=7/genotype. Unpaired t-test. Data were acquired in two independent experiments.

Figure 3. Analysis of BM T cells of mature $\text{DGK}\zeta^{-/-}$ mice

(A) Left, flow cytometry dot plots of CD4 and CD8 T cell populations in total BM. Right, quantification of total CD4 and CD8 T cells. (B) Left, representative histograms of CD69 expression gated either on CD4^+ or CD8^+ T cell populations. Middle, total numbers of activated T cells $\text{CD69}^+ \text{CD4}^+$ and $\text{CD69}^+ \text{CD8}^+$ T cells. Right, MFI of CD69 gated on CD4^+ or CD8^+ T cells (C) S1P1 expression was analyzed in T cells from WT and $\text{DGK}\zeta^{-/-}$ mice by flow cytometry. Left, representative flow cytometry dot plots of CD69 vs S1P1. Right, percentages of CD69^+ and S1P1^+ T cells (gated on CD4^+ or CD8^+). (D, E) mRNA expression

of IFN γ , perforin (Perf), granzymes (Grz) and interleukin (IL) 2, 6, 10 and TNF α was analyzed by RT-qPCR from total BM lysates. **(F)** CD3 ϵ and intracellular IFN γ were stained and analyzed by confocal microscopy in total BM cells. Bottom, quantification of MFI of IFN γ in CD3 ϵ cells. A, B. Data shown as mean $\pm$ SEM. WT n=13; DGK $\zeta^{-/-}$ n=14. A. Mann-Whitney test. B. Unpaired t-test with Welch's correction (for CD69 $^{+}$ CD4 $^{+}$ and CD69 $^{+}$ CD8 $^{+}$), Mann-Whitney test (for MFI CD69 in CD4) and unpaired t test (for MFI CD69 in CD8). Data were acquired in three independent experiments. C. Mean $\pm$ SEM. n=15/genotype. For % CD69. Unpaired t test with Welch's correction. For % S1P1. Unpaired t test. Data were acquired in three independent experiments. D, E. Mean $\pm$ SEM. n=10/genotype. Perf., Grz. A, B, C, IL-2 and IL-10. Mann-Whitney test. TNF α , IL-6 and TGF β . Unpaired t-test with Welch's correction. Data were acquired in three independent experiments. F. Mean $\pm$ SEM. Eleven images/mouse. n=3/genotype. Mann-Whitney test.

Figure 4. DGK $\zeta^{-/-}$ mice show signs of BM aplasia and defects in the erythroid compartment.

(A) Left, femurs from WT and DGK $\zeta^{-/-}$ mice were fixed and H&E stained. Images show central area of each section (200x). Top right, quantification of the percentage of area covered by cells in BM samples. Bottom right, BM cells obtained from tibias and femurs of WT and DGK $\zeta^{-/-}$ mice were quantified by TrypanBlue exclusion. **(B)** Total erythroid lineages and different erythroid populations within the BM were analyzed by flow cytometry. Top, total erythroid counts (Ter119 $^{+}$) (gated on total cells). Bottom, percentage of cell death Ter119 $^{+}$ population (% annexinV $^{+}$ cells + % annexinV $^{+}$ DAPI $^{+}$ cells). **(C)** Left, representative dot plots of CD44 and FSC populations in WT and DGK $\zeta^{-/-}$ mouse BM (gated on Ter119 $^{+}$ cells). Top right, quantification of erythroid populations (in Ter119 $^{+}$ cells). Bottom right, percentage of cell death (% annexinV $^{+}$ cells + % annexinV $^{+}$ DAPI $^{+}$ cells) for the different erythroid

populations. **(D)** Quantification of total dendritic cells (DC), natural killer cells (NK), neutrophils, macrophages (M ϕ)/monocytes, eosinophils and inflammatory M ϕ in the BM of WT and DGK $\zeta^{-/-}$ mice following the gating strategy depicted in Figure 2. A. Data shown as mean $\pm$ SEM. Three sections/mouse; n=3/genotype. Mann-Whitney test. For total cellularity, n=14/genotype. Unpaired t-test with Welch's correction. B. Mean $\pm$ SEM. Top, n=14/genotype. Mann-Whitney test. Bottom, n=3/genotype. Unpaired t-test. C. Mean $\pm$ SEM. Top right, n=14/genotype. Mann-Whitney test. Bottom right, n=3/genotype. Unpaired t-test. Data were acquired in three independent experiments. D. Data shown as mean $\pm$ SEM. n=3/genotype. Unpaired t-test. Data were acquired in a single experiment.

Figure 5. Analysis of peripheral blood of DGK $\zeta^{-/-}$ mice

Blood was collected by heart puncture from WT and DGK $\zeta^{-/-}$ mice and analyzed on an Abacus Junior Vet blood analyzer or processed for flow cytometry. **(A)** Left, analysis of total circulating red blood cells. Center, hematocrit analysis. Right, hemoglobin quantification. **(B)** Total circulating lymphocytes. **(C)**. Left, analysis of total granulocytes. Right, analysis of circulating platelets. **(D)** Flow cytometry analysis of blood samples for circulating T lymphocyte characterization. Percentages of CD4 $^{+}$ and CD8 $^{+}$ T cells (gated on total cells). **(E)** Percentages of CD69 $^{+}$ (gated on CD4 $^{+}$ or CD8 $^{+}$). **(F)** Left, representative flow cytometry dot plots of CD62L and CD44 cells gated on CD4 (top) or CD8 (bottom). Right, quantification of percentages of CD62L high CD44 low (naïve), CD62L high CD44 high (central memory, T $_{cm}$) and CD62L low CD44 high (effector memory, T $_{em}$) gated on CD4 $^{+}$ or CD8 $^{+}$. **(G)** CD69 expression was analyzed in spleen and lymph nodes. A-C. Mean $\pm$ SEM. n=14/genotype. A. Left, Unpaired t-test. Center, Mann-Whitney test. Right, Unpaired t-test with Welch's correction. B. Unpaired t-test with Welch's correction. C. Left, Unpaired t-test. Right, Mann-Whitney test. Data were acquired in three independent experiments. D-F. Mean $\pm$ SEM. n=13/genotype. D. Percentage

of CD4. Mann-Whitney test. Percentage of CD8. Unpaired t-test. E. Percentage of CD69 (CD4). Unpaired t-test. Percentage of CD69 (CD8). Mann-Whitney test. Percentage of CD62L^{high}CD44^{low} (naïve), CD62L^{high}CD44^{high} (central memory, T_{cm}) and CD62L^{low}CD44^{high} (effector memory, T_{em}) in CD4. Mann-Whitney test. In CD8. Mann-Whitney test. Data were acquired in four independent experiments. G. Mean ± SEM. n=3/genotype. Unpaired t-test.

Figure 6. DGKζ deficiency enhances immune-dependent BM destruction

(A) Scheme for the generation of a mouse model of severe aplastic anemia: C57BL/6 (Hb/b) and BALB/c (Hd/d) mice were crossed and F1 progeny (Hb/d) mice were sublethally irradiated (γR) and infused with 5×10^7 WT or DGKζ^{-/-} C57BL/6 splenocytes by i.p. injection. (B) Kaplan–Meier survival curve (day +17) estimates percentage of living mice after aplastic anemia induction with WT or DGKζ^{-/-} C57BL/6 splenocytes. (C) mRNA expression of Grz A, B, C, perforin, IFNγ, IL-2, TNFα, IL-10 and IL-6 was analyzed by RT-qPCR on day +7 after bone marrow failure induction. (D) Left, sternums from F1 hybrid mice were fixed and H&E stained on day +17 post disease induction. Images show central area of each section (200x). Right, quantification of the percentage of area covered by cells in sternum samples after aplastic anemia induction. B. N=10/genotype; control PBS n=2. Gehan-Breslow-Wilcoxon test. Data were acquired in two independent experiments. C. Mean ± SEM. n=3/genotype. One-way ANOVA with the Bonferroni post hoc test. D. Mean ± SEM. Three sections/mouse; WT/WT n=8; DGKζ^{-/-}/WT n=10; control PBS n=2. Kruskal-Wallis test and Dunn's post hoc. One representative image of each sample is shown. Data were acquired in two independent experiments.

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A **B** **C** **D** **E** **Figure 1**

Figure 2

Figure 4

A **B** **C** **D**

Figure 6