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Mechanisms of Acute Toxicity in NKG2D Chimeric Antigen Receptor T Cell–Treated Mice

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Targeting cancer through the use of effector T cells bearing chimeric Ag receptors (CARs) leads to elimination of tumors in animals and patients, but recognition of normal cells or excessive activation can result in significant toxicity and even death. CAR T cells based on modified NKG2D receptors are effective against many types of tumors, and their efficacy is mediated through direct cytotoxicity and cytokine production. Under certain conditions, their ligands can be expressed on nontumor cells, so a better understanding of the potential off-tumor activity of these NKG2D CAR T cells is needed. Injection of very high numbers of activated T cells expressing CARs based on murine NKG2D or DNAM1 resulted in increased serum cytokines (IFN-γ, IL-6, and MCP-1) and acute toxicity similar to cytokine release syndrome. Acute toxicity required two key effector molecules in CAR T cells—perforin and GM-CSF. Host immune cells also contributed to this toxicity, and mice with severe immune cell defects remained healthy at the highest CAR T cell dose. These data demonstrate that specific CAR T cell effector mechanisms and the host immune system are required for this cytokine release–like syndrome in murine models. ***The Journal of Immunology*****, 2016, 197: 4674–4685.**

Chimeric Ag receptor (CAR)–targeted T cells have recently shown remarkable clinical responses against B cell leukemias (1–6). CARs combine a recognition domain (e.g., scFv) with intracellular signaling domains to create an activating receptor to target a large number of T cells against a patient's tumor. Both direct tumor cell killing and cytokine production are involved in CAR T cell efficacy in immune intact tumor models (7, 8). Several different CAR strategies to target tumors are in various stages of development (9–14). However, CAR T cells also have the potential to cause harm. Tumor lysis syndrome, recognition of ligands on nontumor cells leading to tissue damage, cross-reactivity with normal proteins by modified CARs, and cytokine release syndrome (CRS) have been documented and can be severe (15–17). Activation of CAR T cells and the production of effector molecules are required for CAR T cell efficacy. A better understanding of mechanisms of toxicity, particularly the drivers of CRS, is vital to improve clinical outcomes and further develop these promising cell therapies.

NK cells recognize and kill a variety of tumor cells using evolutionarily optimized receptors, and several CARs have been developed based on these, including NKG2D and DNAM-1 CARs (18–21). This class of CARs recognizes the natural ligands of these NK cell receptors. CAR T cells not only kill ligand-expressing tumor cells, but they activate innate and adaptive host immunity through CAR T cell production of cytokines. This activation of host myeloid and lymphoid cells is involved in NKG2D CAR T cell efficacy (22–24). Human NKG2D recognizes MICA, MICB, and ULBP1–6 on human cells, whereas murine NKG2D binds to Rae1 proteins, H60 proteins, and MULT1 in mice (25). These ligands are thought to be expressed on the cell surface in low amounts and on few tissues and not at all on most healthy tissues, although they can be induced by acute infection, irradiation, and chemotherapeutic agents through mechanisms involving the ATM/ATR DNA repair pathways (19, 26–30). DNAM1 is a receptor that recognizes PVR and Nectin-2, ligands that are expressed on several normal tissues, including lung epithelium (31, 32). These molecules are often highly upregulated on many tumor types and are involved in NK cell killing of tumors (33–35). Because NKG2D ligands are highly overexpressed on many types of human tumors, they are attractive targets for cancer therapy, and a number of CAR and Ab-like approaches are under development to target them (18, 36–39).

Although attractive targets for immunotherapy, the potential of NKG2D ligands to be induced or expressed during immunotherapy resulting in off-tumor on-target toxicity remains unknown. One of the major challenges of immunotherapy is the potential to overactivate the immune system, such as occurs during CRS. To better understand how CAR T cells may activate immune-mediated toxicity, dose-escalation studies using NK cell receptor–targeting CAR T cells in immune intact animals were performed. This study demonstrates that high-dose injection of NKG2D CAR or DNAM1 CAR T cells can result in CRS-like responses in mice that are similar to those seen in many patients given CAR T cells.

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Abbreviations used in this article: AST, aspartate aminotransferase; B6, C57BL/6; CAR, chimeric Ag receptor; chNKG2D, chimeric NKG2D; CRS, cytokine release syndrome; D28Z, DNAM1-CD28-CD3z; NSG, NOD/SCID/IL-2Rγ–deficient; PMN, polymorphonuclear neutrophil; wtNKG2D, wild-type NKG2D.

Furthermore, it elucidates key effector mechanisms from NK cell receptor–based CAR T cells that lead to this immune-mediated illness and shows how host cells and molecules are involved. These murine models may provide a system to test potential therapies to prevent or limit CRS-like responses. These findings implicate both CAR T cells and host immune cells as required contributors to the development of acute immune-related toxicity.

Materials and Methods

Animals

C57BL/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD) or The Jackson Laboratory (Bar Harbor, ME). Perforin-deficient mice C57BL/6-*Prf1*^{tm1Sdz}/J (Pfp$^{-/-}$), IFN-γ–deficient mice B6.129S7-*Ifng*^{tm1Ts}/J (IFN-γ$^{-/-}$), IFN-γ receptor–deficient mice B6.129S7-*IfngR1*^{tm1Agt}/J (IFN-γ R$^{-/-}$), IL-1R–deficient mice B6.129S7-*Il1r1*^{tm1Imx}/J (IL-1R$^{-/-}$), IL-6–deficient mice B6.129S2-*Il6*^{tm1Kopf}/J (IL-6$^{-/-}$), IL-2Rγ-chain–deficient mice B6.129S4-*Il2rg*^{tm1Wjl}/J (IL-2Rγ$^{-/-}$), Cd1d1-deficient mice B6(C)-*Cd1d1*$^{tm1.2Aben}$/J (CD1$^{-/-}$), BALB/cJ, DBA/1J, DBA/2J, A/J, 129S1/SvImJ, and (B6 × 129)F$_1$ and immune-deficient mice B6.129P2(SJL)-*MyD88*$^{tm1.1Defr}$/J (MyD88$^{-/-}$), B6.129S7-*Rag1*^{tm1Mom}/J (Rag$^{-/-}$), NOD/ShiLtJ (NOD), and NOD.CB17-*Prkdc*^{scid}/J (NOD-SCID) were obtained from the The Jackson Laboratory. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wji}/SzJ (NOD/SCID/IL-2Rγ–deficient [NSG]) mice were maintained at the Geisel School of Medicine (Lebanon, NH); NKG2D-deficient mice on a B6 background (NKG2D$^{-/-}$) were provided by Dr. David Raulet (University of California, Berkeley, CA). DR5-deficient mice on a B6 background (DR5$^{-/-}$) were provided by Dr. Tak Mak (University of Toronto, Toronto, ON, Canada). GM-CSF–deficient mice were a B6/129/FVB mixed background (GM-CSF$^{-/-}$) and were provided by Dr. Jeff Whitsett (University of Cincinnati, Cincinnati, OH). Mice used in experiments were between 7 and 12 wk of age. All animal work was performed in the Geisel School of Medicine Animal Facility in accordance with institutional guidelines.

Cell lines

RMA and RMA-RG cells are B6-derived murine T cell lymphoma cells. RMA-RG cells express Rae1 and GFP, whereas parental RMA cells do not. ID8 cells are B6-derived ovarian cancer cells and express Rae1. MC38 is a murine colon cancer cell line and expresses ligands for DNAM1. All cell lines tested negative for mycoplasma by PCR.

Generation of CAR-expressing T cells

CAR T cells were generated by viral transduction of mouse splenocytes, as described previously (23, 40). Mouse splenocytes were stimulated with Con A for 18–20 h (0.75 μg/ml, lot 177K700 or 1 μg/ml, lot SLBB841V; Sigma-Aldrich), retrovirally transduced with wild-type NKG2D (wtNKG2D) (that expresses the full-length murine NKG2D protein) or chimeric NKG2D (chNKG2D) (that expresses a full-length NKG2D protein fused to the cytoplasmic portion of CD3ζ) virus as previously described, and injected into mice 8 d after activation. Cells were grown in RPMI 1640 (HyClone) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, Atlanta, GA), 100 U/ml penicillin, 100 μg/ml streptomycin (HyClone), 1 mmol/l pyruvate, 10 mmol/l HEPES, 0.1 mmol/l nonessential amino acids (Corning), and 50 μmol/l 2-ME (Sigma-Aldrich).

Animal studies

RMA-RG tumor cells (10^5 cells) in 0.4 ml of HBSS (HyClone) were injected i.v. into B6 mice on day 0. On day 5, a dose of 0, 5, 10, or 20 × 10^6 full-length wtNKG2D or NKG2D CAR (chNKG2D) T cells in HBSS was injected into tumor-bearing or non–tumor-bearing mice. In some experiments, one to three doses of 10^7 wtNKG2D, chNKG2D, DNAM1 CAR (D28z), or mock T cells in 0.4 ml of HBSS or HBSS alone were administered i.v. into tumor-bearing or non–tumor-bearing mice. For multidose experiments, a fresh cell dose was manufactured for each T cell injection. A single dose of 2 × 10^7 wtNKG2D or chNKG2D T cells derived from B6, 129, (B6 × 129)F$_1$, or gene-deficient mice in 0.4 ml of HBSS was injected i.v. into mice with different genetic backgrounds or gene deficiencies. B6 mice were injected at the same time as controls. Mice were euthanized after 18 h. A single dose of 10^7 DNAM1-based CAR T cells or mock T cells derived from B6, (B6 × 129)F$_1$, IFN-γ–, GM-CSF–, or perforin-deficient mice were injected into mice i.v. Furthermore, mice were treated with Abs to deplete specific cell populations, and the extent of cell depletion was confirmed by flow cytometry (data not shown). Mice were weighed prior to T cell injection and then two to three times per week until the time of euthanasia. Serum samples were taken and analyzed for cytokines and other analytes, as indicated in the *Results*.

Health status

The health status of mice was graded on a scale from 1 to 4 as follows: 1, normal and healthy; 1.5, some lethargy, walking a bit slowly; 2, moving slowly and (in tumor-bearing mice) a slight dragging of a limb; 2.5, hunched posture, moving slowly, and (in tumor-bearing mice) dragging limbs; 3, hunched posture with little movement; 3.5, little movement upon touch; 4, death.

Depletion of specific cell populations

For NK cell depletion studies, 50 μg of anti-NK1.1 (clone PK136; eBioscience) or control mouse γ-globulin (Jackson ImmunoResearch Laboratories, West Grove, PA) was injected i.p. 2 d before T cell injection. Depletion of NK cells was confirmed by flow cytometry by analysis of splenocytes for CD49b$^+$CD3$^-$ cells. To deplete neutrophils, 500 μg of anti–mLy-6G (clone IA8; Bio X Cell, Lebanon, NH) or control mouse γ-globulin (Jackson ImmunoResearch Laboratories, West Grove, PA) was injected i.p. 1 d prior to T cell injection. Depletion of polymorphonuclear neutrophils (PMNs) was confirmed by flow cytometric analysis of splenocytes.

Serum analysis

Mouse serum was collected via tail bleeding or cardiac puncture, and samples were allowed to coagulate for 1 h and then spun at 16,400 relative centrifugal force for 15 min and the serum was frozen at −20°C. Samples from days 3, 5, and the day of euthanasia were analyzed by multiplex cytokine analysis for G-CSF, IFN-γ, IL-1β, IL-6, IL-10, MCP1, MIG, and MIP-1β by DartLab[2], and for soluble aspartate aminotransferase (AST) or serum creatinine by ACE Alera (Alfa Wasserman, Wilmington, MA).

Histology

Dissected tissues were fixed in 10% formalin. Paraffin sections were generated and stained with H&E by the Pathology Research Resource at Dartmouth–Hitchcock Medical Center. Histological analysis and scoring was performed in an independent and blinded manner by Charles River Laboratories Animal Pathology Services (Cambridge, MA). Eight tissues were taken for analysis from each mouse: heart, lungs, liver, kidney, large intestine, small intestine, pancreas, and spleen. Tissues were rated as normal or pathology was noted, which was graded 1 (mild), 2 (moderate), or 3 (severe). When insufficient tissue was available for scoring from a particular mouse, this tissue was absent from the analysis but other tissues from the mouse were scored. A summary table of the scoring is provided in Supplemental Tables III and IV.

Flow cytometry

Cell phenotyping of transduced primary T cells was determined by staining with specific Abs: FITC-conjugated anti-CD3ε (clone 145-2C11; eBioscience), allophycocyanin-conjugated anti-NKG2D (clone CX5; eBioscience), PE-conjugated anti-CD8b (clone CT-CD8b; BioLegend), allophycocyanin-conjugated anti-CD4 (clone RM4-5; eBioscience), or allophycocyanin- or PE-conjugated isotype control Abs (eBioscience). All samples were preincubated with FcR block Ab (anti-mouse CD16/CD32, clone 93; eBioscience) to reduce nonspecific staining. Cells were analyzed using an Accuri C6 flow cytometer (BD Biosciences, Ann Arbor, MI).

Cytokine production and cytotoxicity by CAR T cells

wtNKG2D or chNKG2D T cells (10^5) were cultured with RMA, RMA-RG, and ID8 tumor cells at a 1:1 (RMA, RMA-RG) or 1:4 (ID8) ratio (10^5 T cells) or in media alone. Cell-free conditioned media were collected after 24 h and assayed for IFN-γ using mouse DuoSet ELISA kits (R&D Systems). Cytotoxicity was determined by a 4-h ^{51}Cr-release assay, as described (21). Coculture was performed at E:T ratios of 25:1, 5:1, and 1:1 in triplicate wells.

Statistical analysis

Each experiment was done at least twice with three to five animals per group in each experiment. Data are shown as combined data from the experiments, and statistical analysis was done on the complete dataset. Hence, data from 6 to 10 individual animals are shown from a given analysis. A Student *t* test was used to compare differences between groups using a two-tailed test assuming unequal variance. To compare multiple groups, ANOVA was performed using a nonparametric Kruskal–Wallis test with a Dunn test for multiple comparisons. For analysis of cell cytotoxicity in vitro, a one-way

ANOVA with a Dunnett method for multiple comparisons was used. Any individual mouse that did not receive a full injection of cells was removed from the experiment and further analysis at that time. Values of $p < 0.05$ were considered significant (Prism software; GraphPad Software, San Diego, CA).

Results

Determination of the maximum tolerated dose

To evaluate tumor-dependent, receptor-specific, and nonspecific toxicity after injection of NKG2D CAR T cells, dose escalation studies in tumor-bearing and non–tumor-bearing mice were conducted (Fig. 1A). Based on an effective dose of 5×10^6 CAR T cells from previous studies (7, 41), 5×10^6, 10^7, or 2×10^7 wtNKG2D T cells, NKG2D CAR T cells, or HBSS were injected into mice i.v. Only mice receiving NKG2D CAR T cells at the highest dose appeared ill. These mice were euthanized between 6 and 20 h after T cell injection and had an average health status of 2.6 (1–4 scale) at the time of euthanasia (Fig. 1B). This occurred in both tumor-bearing and non–tumor-bearing mice. None of the mice receiving lower doses of NKG2D CAR T cells, any dose of wtNKG2D T cells, or HBSS showed outward signs of toxicity. Compared to control mice, there was a decrease in body weight 1 d after the injection of 2×10^7 or 10^7 NKG2D CAR T cells (Fig. 1C–H). The average drop in body weight was 7.0 and 6.7%, respectively. Although weight loss was typical, it was not observed in every experiment. However, only mice given the highest CAR T cell dose exhibited signs of overt illness. Mice given 10^7 NKG2D CAR T cells showed no outward signs of illness and began to gain weight on day 2 postinjection. In tumor-bearing mice given 5×10^6 NKG2D CAR T cells, there was a small decrease in body weight (2.6%) after 1 d, which quickly recovered, but there was no change in weight for non–tumor-bearing mice given 5×10^6 CAR T cells.

Assessment of toxicity following multidoses at the maximum tolerated dose

To determine whether multiple injections of CAR T cells would result in toxicity due to accumulation of T cells, tumor-bearing and non–tumor-bearing mice were treated with up to three injections of 10^7 wtNKG2D T cells, 10^7 NKG2D CAR T cells, or HBSS. No signs of toxicity were observed due to T cell injection during 30 d. Because most of the control tumor-bearing mice had to be euthanized due to tumor growth before the third T cell injection, they only received two T cell doses. We observed a decrease in body weight (5.6%) 1 d after injection of 10^7 NKG2D CAR T cells but not in mice given wtNKG2D T cells or HBSS (Fig. 1I, 1J). Notably, this occurred after the first cell injection only. We observed no differential body weight changes after the second or third NKG2D CAR T cell injection compared with controls. Thus, multiple doses of NKG2D CAR T cells were well tolerated.

Analysis of serum analytes

To understand the physiological effects of NKG2D CAR T cell injection, specific serum analytes were measured. RMA-RG tumor cells localize in the liver and kidney, and AST values increased in tumor-bearing mice at later stages of disease. In tumor-bearing mice, the AST values from mice given wtNKG2D T cells or NKG2D CAR T cells were different 3 d after T cell infusion (day 8), but the NKG2D CAR T cell–treated and HBSS-treated groups had similar AST values. In mice without tumors, the AST values were not affected by T cell treatment. Even when acute illness was observed after the high-dose injection of NKG2D CAR T cells, there were no significant differences in AST values compared with the control groups. Creatinine values did not change, regardless of T cell injections or tumor growth (Supplemental Table I). Overall, treatment with NKG2D CAR T cells did not appear to affect kidney or liver function.

There were no significant differences in serum cytokine amounts (IL-6, IFN-γ, MCP-1, TNF-α) 3, 8, or 12 d after injection of NKG2D CAR T cells compared with controls (Supplemental Table II). Only in mice injected with 2×10^7 NKG2D CAR T cells were clinical signs of illness observed (mean health score of 3.1, Fig. 2A). An analysis of cytokines 20 h after injection of CAR T cells showed an increase in G-CSF, IL-6, IFN-γ, MCP-1, IL-10, and MIG, with the highest cell dose having significant increases compared with controls (Fig. 2B).

Analysis of histopathology

Despite their health status, mice treated with 2×10^7 NKG2D CAR T cells exhibited little pathology. The liver, pancreas, small intestine, large intestine, heart, and kidney appeared similar to wtNKG2D T cell– and HBSS-treated mice (see Supplemental Tables III, IV). There was no infiltration of leukocytes in these tissues. In the spleen, mild single-cell necrosis of lymphocytes in the white pulp of the spleen was evident in ~40% of the mice (11 of 26) treated with NKG2D CAR T cells from five independent experiments. In tumor-bearing mice, there was evidence of tumor infiltration in the liver and occasionally the kidney, as expected. Lung tissue from both wtNKG2D and NKG2D CAR T cell–treated mice had more lymphocytes, as may be anticipated after a large infusion of lymphocytes. There was no increase in cells or debris within the alveoli or airways. The epithelial cells and cells lining the alveoli were intact with no evidence of sloughing or death. In the multidose treatment experiments in tumor-bearing mice, mice from all groups showed undifferentiated round cells with atypical nuclear and cytotological features suggestive of neoplasia, and the most frequently affected tissues were liver, kidney, and lung. Splenic involvement or extramedullary hematopoietic activity was noted in a few mice treated with the CAR T cells in tumor-bearing mice. Noted observations in the exocrine pancreas of occasional mice in one multidose experiment were considered secondary to debility associated with the presence of neoplastic cells. Two of the CAR T cell–injected mice showed neutrophilic infiltration of the alveolar walls in one experiment, and one single mouse from each treatment group over these nine experiments showed alveolar hemorrhage in the lungs, each in different experiments. There was no evidence of lysis of tissues or infiltration of lymphocytes into specific tissues after injection of NKG2D CAR T cells. These findings of high amounts of serum inflammatory cytokines and no obvious tissue damage are consistent with CRS.

The role of CAR T cell effector mechanisms in acute toxicity

To determine which effector mechanisms of CAR T cells contributed to CRS, 2×10^7 NKG2D CAR T cells derived from mice that were deficient in perforin, IFN-γ, or GM-CSF were injected into mice. These molecules are needed for full efficacy of NKG2D CAR T cells (7, 8). We observed that mice injected with IFN-γ–deficient CAR T cells became acutely ill, similar to mice given B6 CAR T cells. However, when B6 mice were injected with NKG2D CAR T cells derived from GM-CSF– or perforin-deficient mice, there was no sign of illness or change in body weight (Fig. 3A). Analysis of serum cytokines 20 h after T cell injection showed that inflammatory cytokines (G-CSF, IL-6, IL-10, and MCP-1) were increased in the serum of mice treated with IFN-γ–deficient NKG2D CAR T cells compared with wtNKG2D T cell–treated mice. CAR T cells that lacked either perforin or GM-CSF resulted in much lower amounts of cytokines in the serum, which was consistent with their lack of CRS (Fig. 3B–D). MIG, which is induced by IFN-γ, was a notable exception and remained elevated.

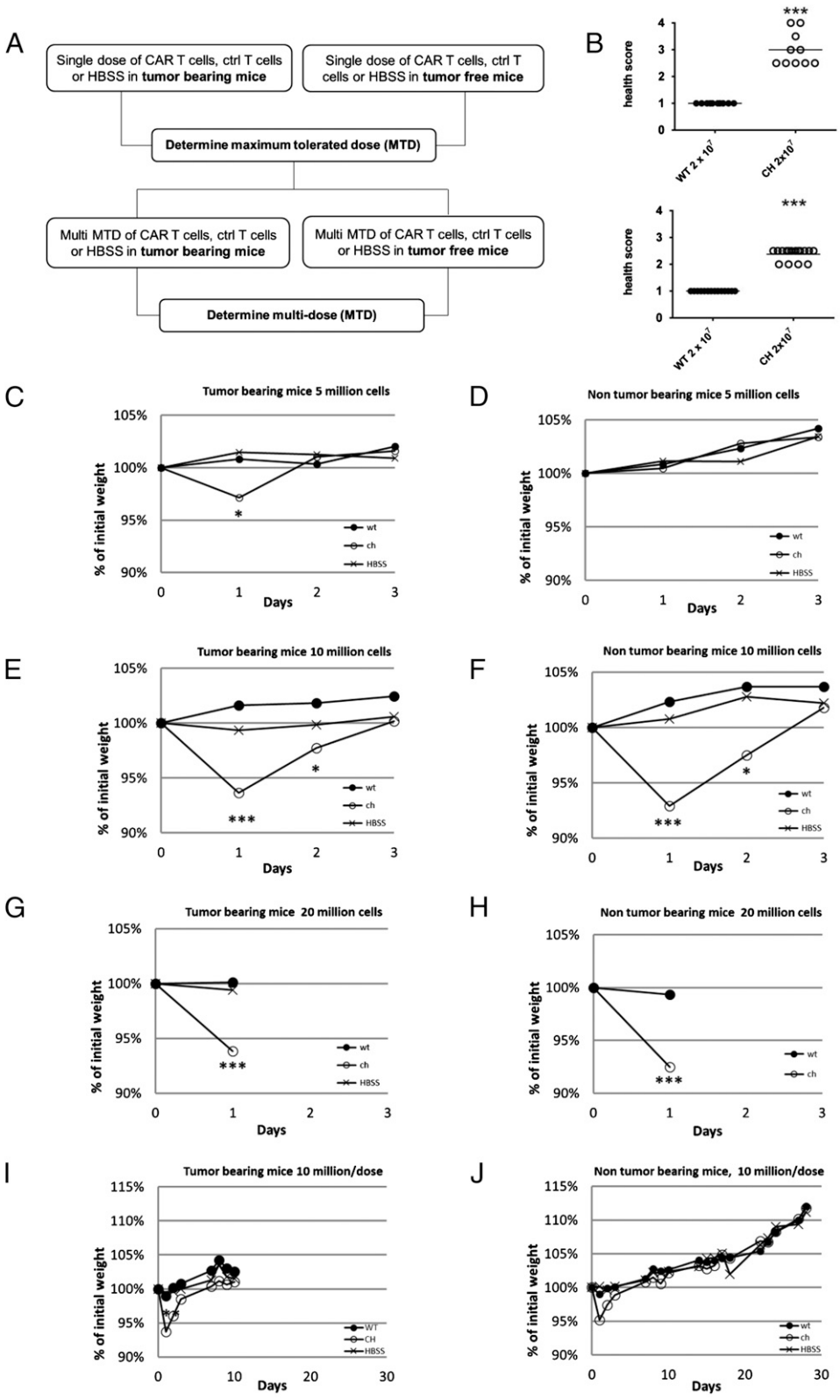

FIGURE 1. Determination of maximum tolerated dose (MTD) for NKG2D CAR T cells. (**A**) Overview of approach to determine MTD for single and multidose treatment with murine NKG2D CAR T cells. (**B**) The health score for mice injected with 2×10^7 wtNKG2D T cells (WT) or NKG2D CAR T cells (CH) in non–tumor-bearing (lower) and tumor-bearing (upper) mice is shown. Each symbol represents an individual mouse at the time of euthanasia. (**C**–**J**) The percentage change in initial weight in the days following injection of NKG2D CAR T cells, wtNKG2D T cells, or HBSS is plotted for 3–28 d after T cell injection. Average values for each group are shown ($n = 10$ per group). Mice were treated with a single dose of 5×10^6 T cells (C and D), 10^7 T cells (E and F), or 2×10^7 T cells (G and H). Mice were RMA tumor bearing (C, E, and G) or non–tumor bearing (D, F, and H). (I) RMA tumor-bearing mice (RMA cells i.v. at day −5) were injected with 10^7 T cells or HBSS on days 0 and 7. (J) Non–tumor-bearing mice were injected with 10^7 T cells or HBSS on days 0, 7, and 14. Data are from two independent experiments ($n = 10$ per group). A Student t test was used to compare differences between groups using a two-tailed test assuming unequal variance. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with wild-type. CH, NKG2D CAR T cells; MTD, maximum tolerated dose; WT, wtNKG2D T cells.

The role of host immune cells

To investigate the role of host immune cells in this process, NSG mice, NOD/SCID mice, IL-2Rγ–deficient mice, and Rag-1–deficient mice were injected with a high dose (2×10^7 cells) of either NKG2D CAR T cells or wtNKG2D T cells. The NSG and NOD/SCID mice remained healthy throughout the experiment, whereas the NOD, Rag-1–deficient, and IL-2Rγ–deficient mice became acutely ill and lost body weight after receiving the high dose of NKG2D CAR T cells, similar to B6 mice (Fig. 4A–C). In addition to their lack of T cells and B cells, the NSG and NOD/SCID mice also have significant alterations in inflammatory and myeloid cell responses. NK cells and neutrophils can be involved in rapid immune responses in vivo. Treatment of B6 mice with Abs to deplete NK cells (anti-NK1.1 mAbs) or neutrophils (anti-Ly6G mAbs), prior to injection of a high dose of NKG2D CAR

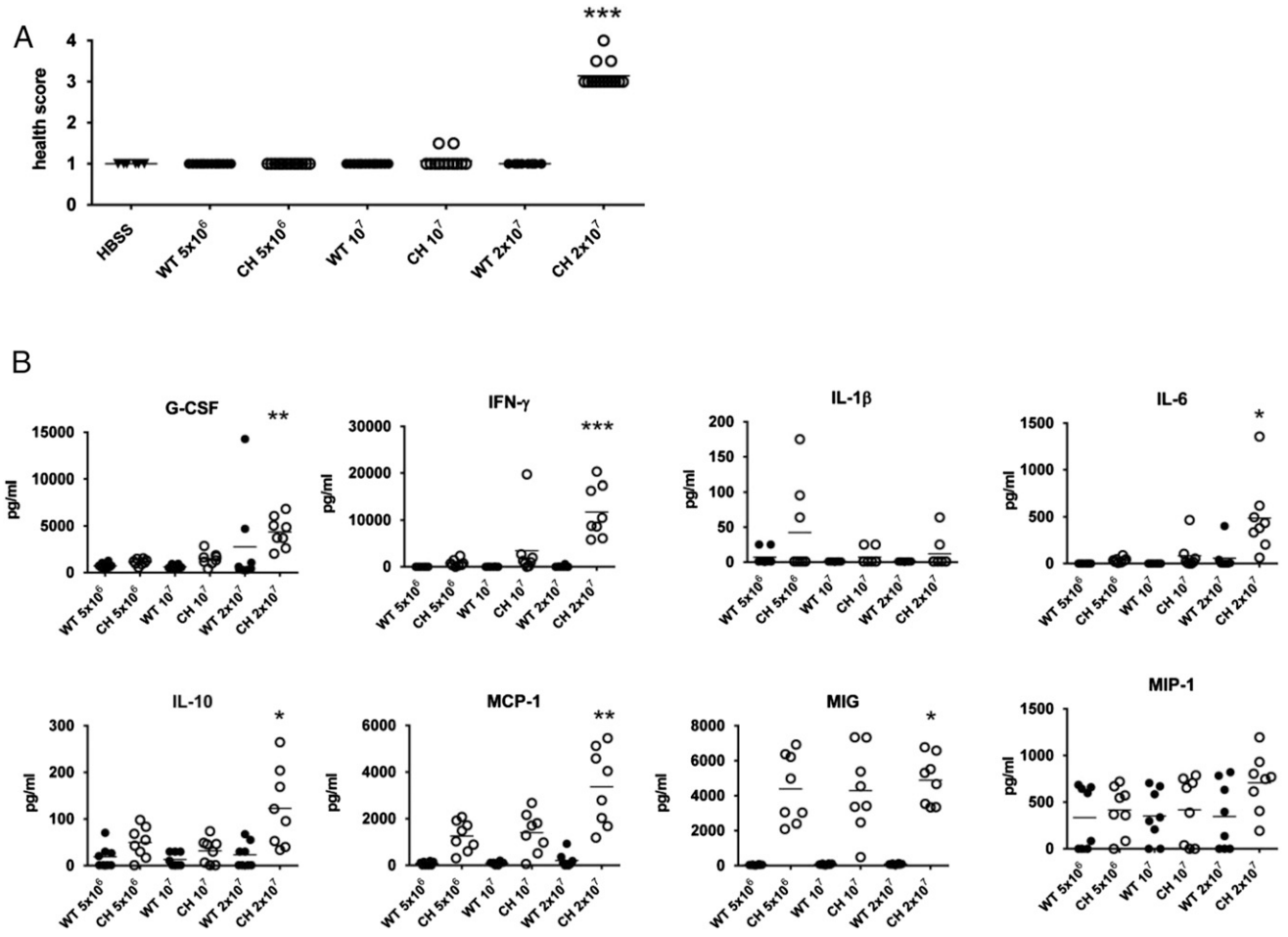

FIGURE 2. Serum cytokines of mice injected with NKG2D CAR T cells. HBSS, NKG2D CAR T cells (CH), or wtNKG2D T cells (WT) were injected i.v. into mice at 5×10^6, 10^7, or 2×10^7 cells. (**A**) Overall health score on day 1 after transfer. (**B**) Serum samples were analyzed for G-CSF, IFN-γ, IL-1β, IL-6, IL-10, MCP1, MIG, and MIP-1β by multiplex cytokine analysis. Each symbol represents an individual mouse (n = 6–14 per group). ANOVA was performed using a nonparametric Kruskal–Wallis test with a Dunn test for multiple comparisons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with wild-type. CH, NKG2D CAR T cells; WT, wtNKG2D T cells.

T cells, did not affect the development of CRS (Fig. 4D). Taken together, these data indicate that mice that had defective myeloid cells, T cells, and B cells did not develop CRS, but mice with more restricted deficiencies in immune cell populations did develop CRS.

The role of host cytokines

To determine whether specific cytokines shown to be elevated in the serum of NKG2D CAR T cell–treated mice were essential for development of CRS, 2×10^7 NKG2D CAR T cells or wtNKG2D T cells were injected into mice deficient in IL-1R, IL-6, GM-CSF, IFN-γ, or IFN-γR. All mice treated with the high dose of NKG2D CAR T cells lost weight and became acutely ill, similar to B6 mice (Fig. 5A, 5B). Whereas IL-1 and IL-6 are involved in the acute inflammatory response, the data indicate that the host's ability to respond to IL-1 or produce IL-6 was irrelevant to the outcome. GM-CSF from the CAR T cells was essential for induction of the CRS-like response, but the host did not need to produce GM-CSF for illness to occur. This suggests that GM-CSF is essential early in the induction of acute inflammation, but host production was not required. CAR T cells produce a significant IFN-γ response and this is key for antitumor immunity, yet neither the production of IFN-γ nor the response to the IFN-γ present by the host was necessary for CRS-like illness. Similarly, hosts deficient in NKG2D, DR5 (TRAIL receptor), or MyD88 also developed CRS after injection of a high dose of NKG2D CAR T cells (Fig. 5C, 5D). Although the host's endogenous NKG2D$^+$ cells might contribute to the acute inflammatory response, the absence of host NKG2D did not alter the extent of the illness. MyD88 is a key signaling molecule in responses of TLRs that leads to production of proinflammatory cytokines, such as TNF-α and IL-12 (42). However, the absence of MyD88 did not affect the development of the CRS-like illness, indicating that this signaling pathway was not essential in the acute illness observed (Table I).

The role of genetic background

Because NOD/SCID and NSG mice have a different genetic background than do B6 mice, we tested whether NKG2D CAR T cells would result in CRS in other mouse strains. Injection of 2×10^7 NKG2D CAR T cells derived from B6 mice resulted in a CRS response in BALB/c, A/J, DBA1, and DBA2 recipients (Fig. 5E). DBA/1 and A/J mice have a defect in the C5 component of the complement cascade, which when activated produces a powerful anaphylatoxin and is necessary for the generation of the membrane attack complex. However, there was no difference in the observed response in DBA/1 or A/J mice compared with the other mouse strains tested. When the NKG2D CAR was expressed in 129/Sv or (B6 × 129)F_1 T cells and injected at a high dose into B6 recipient mice, the mice all exhibited a CRS similar to when B6 T cells were used as a source of NKG2D CAR T cells (Fig. 5F). Thus, there were no differences in the CRS response when an allogeneic or F_1 combination was used.

Table I. Summary of CAR T cell injection experiments

Source of CAR T Cells	Host Strain/Treatment[a]	Immune Status/Deficiency	Outcome
B6	B6	None	Illness
IFN-γ$^{-/-}$	B6	IFN-γ from CAR T cells	Illness
GM-CSF$^{-/-}$	B6	GM-CSF from CAR T cells	Healthy
Perforin$^{-/-}$	B6	Perforin killing via CAR T cells	Healthy
B6	MyD88$^{-/-}$	Host TLR signaling pathways	Illness
B6	IL-6$^{-/-}$	Host IL-6 production	Illness
B6	IL-1R$^{-/-}$	Host responses to IL-1	Illness
B6	IFN-γ$^{-/-}$	Host IFN-γ production	Illness
B6	IFN-γ$^{-/-}$	Host responses to IFN-γ	Illness
B6	GM-CSF$^{-/-}$	Host GM-CSF production	Illness
B6	DR5	Host TRAIL receptor	Illness
B6	Anti-NK[b]	Depletion of host NK cells	Illness
B6	Anti-PMN[c]	Depletion of host PMNs	Illness
B6	RAG-1$^{-/-}$	Host T and B cells	Illness
B6	Anti-NK in CD1$^{-/-}$	Depletion of host NK and NKT cells	Illness
B6	NSG	Host T, B, and NK cells and myeloid cell defects	Healthy
B6	NOD/SCID	Host T, B, and myeloid cell defects	Mild illness
B6	NOD	Allogeneic, C5 deficiency	Illness
B6	DBA/1	Allogeneic, C5 deficiency	Illness
B6	DBA/2	Allogeneic	Illness
B6	BALB/c	Allogeneic	Illness
B6	A/J	Allogeneic, C5 deficiency	Illness
129	B6	Minor allogeneic	Illness
(129 × B6)F_1	B6	Minor allogeneic	Illness

[a]All mice were given 2 × 10^7 T cells i.v.
[b]Anti-NK1.1 mAbs.
[c]Anti-Ly6G mAbs.

DNAM1-based CARs show similar acute CRS toxicity

To test whether these observations were limited to NKG2D CARs, we also tested CARs based on the NK cell receptor DNAM1 (Fig. 6A) (21). DNAM1 CAR T cells lyse PVR- and Nectin-2–expressing tumor cells in vitro and reduce the growth of B16F10 melanoma cells in vivo (21). To test whether similar effector mechanisms were essential for the induction of CRS after injection of DNAM1 CAR T cells, perforin-, GM-CSF–, or IFN-γ–deficient DNAM1-CD28-CD3z (D28Z) T cells or intact DNAM1 CAR T cells were injected into mice. Similar to NKG2D CAR T cells, D28Z T cells derived from mice deficient in either perforin or GM-CSF did not cause CRS (Fig. 6B). However, mice injected with 10^7 D28Z T cells from B6, (B6 × 129)F_1, or IFN-γ–deficient T cells showed signs of CRS with an average health score of 2.5–2.8. Furthermore, the mice that showed signs of CRS had elevated serum inflammatory cytokines (Fig. 6C). Thus, perforin and GM-CSF were essential effector molecules of both NKG2D and DNAM1 CAR T cells for the induction of CRS. DNAM1 CARs that included CD3z, CD28, or OX40 cytoplasmic domains induced CRS in B6 mice when injected at 10^7 T cells i.v. The time to development of CRS showed a trend with the CAR T cells that had greater cytotoxicity in vitro against MC38 tumor cells, inducing a more rapid CRS-like illness in vivo (Fig. 6D, 6E).

Discussion

The present studies in an immune intact murine model using murine CAR T cells provide the opportunity to dissect critical components of CRS and particularly components of the host immune system that cannot be interrogated in xenograft models of human CAR T cells. These data show that a high dose of CAR T cells can induce CRS in immune-intact mice. CRS was dependent on cell dose, specific CAR T cell effector mechanisms, and host immune cells (Table I). Mice showed an acute inflammatory distress syndrome associated with production of proinflammatory cytokines. There was little significant histological damage in any of the eight organs examined, although some NKG2D CAR T cell–treated mice had single-cell necrosis in the splenic white pulp. There was no accumulation of T cells in any organ other than lung, and this occurred in mice given either wtNKG2D or NKG2D CAR T cells, indicating that this was not associated with toxicity and likely reflected a first pass effect of i.v. injection of a large number of T cells. At lower doses of NKG2D CAR T cells, there was a drop in body weight, but only at the first dose, and without any clinical phenotype; mice quickly recovered. Thus, toxicity was associated with a single high-dose injection of CAR cells rather than the cumulative dose of cells, and it likely involves CAR recognition of ligands. Whether these ligands are present due to endogenous expression, are induced upon an injection of a large dose of activated T cells, or are due to interaction of the CAR T cells with each other is unknown. These data indicate that NK cell receptor–based CAR T cells may lead to acute toxicities associated with proinflammatory cytokine production consistent with CRS, and also in conjunction with prior studies, they confirm that lower T cell doses and/or the use of repeated doses leads to favorable antitumor effects without toxicity.

Our previous studies have shown that NKG2D CAR T cells require IFN-γ, GM-CSF, and cytotoxicity for complete efficacy in lymphoma and ovarian cancer tumor models, although partial efficacy was observed in the absence of GM-CSF or perforin (7, 8). Even if one of these molecules is absent, the other cytokines and/or cytotoxicity pathways remain intact and active. IFN-γ from NKG2D CAR T cells has been shown to activate local macrophages and induce Ag presentation and NO production from macrophages, and there was a reduction in a large number of cytokines in the ovarian cancer tumor microenvironment when the CAR T cells were IFN-γ deficient (43). CAR T cells that lack either perforin or GM-CSF did not result in acute toxicity when injected at high cell doses, although CAR T cells derived from these mice have antitumor activity in vivo

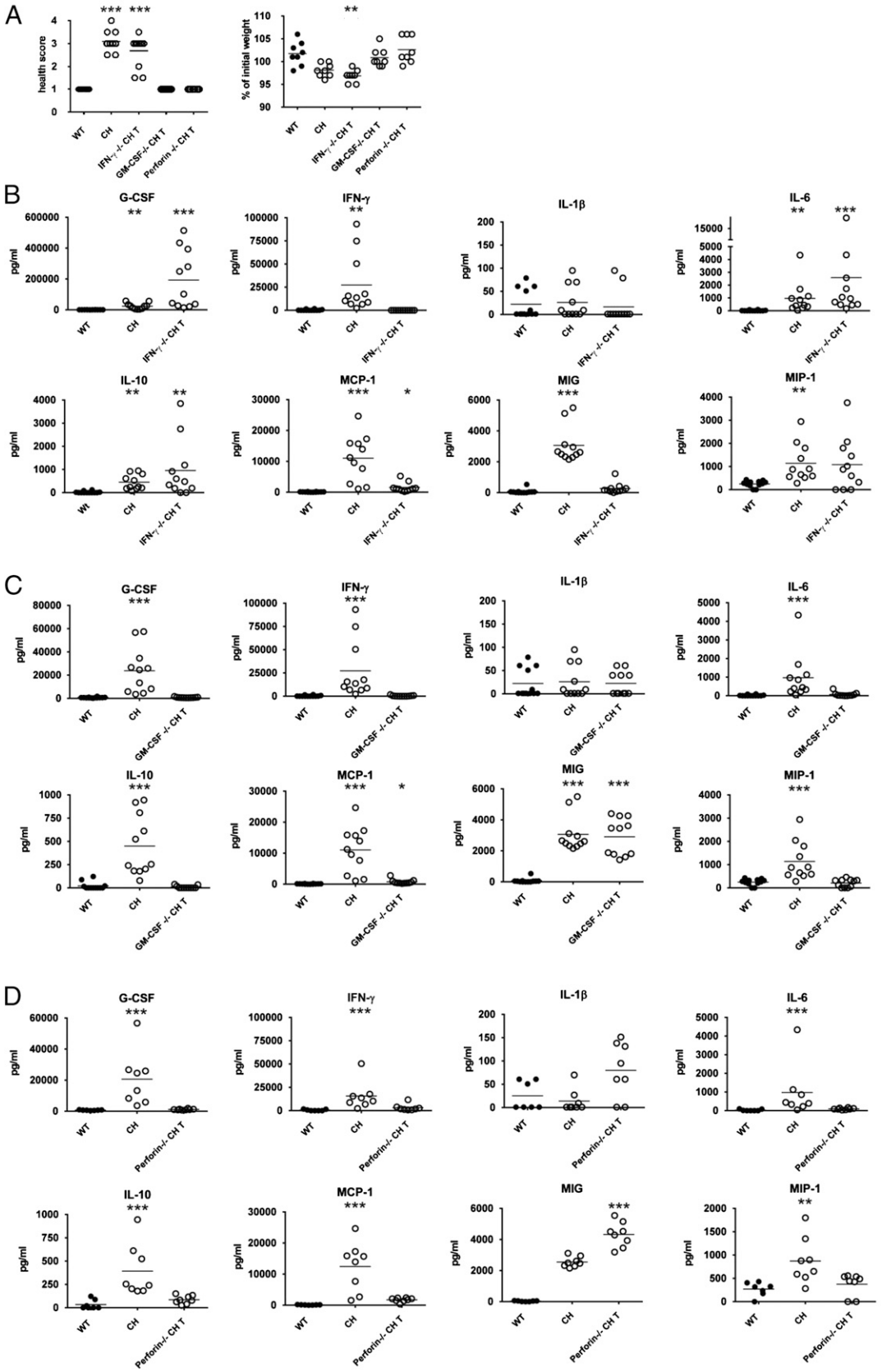

FIGURE 3. GM-CSF and perforin are essential for NKG2D CAR T cell–induced toxicity. A single dose of 2×10^7 NKG2D CAR T cells or wtNKG2D T cells were injected into mice. Changes in health status, body weight (**A**), and serum cytokines (**B**–**D**) were measured after 20 h. The amounts (picograms/milliliter) of G-CSF, IFN-γ, IL-1β, IL-6, IL-10, MCP1, MIG, and MIP-1β were measured by multiplex analysis. Serum samples from mice given B6-derived T cells that expressed wtNKG2D T cells (WT) or the NKG2D CAR T cells (CH) were compared with samples from B6 mice given NKG2D CAR T cells derived from (B) IFN-γ– (C) GM-CSF–, or (D) perforin-deficient mice. Each symbol represents an individual mouse. ANOVA was performed using a nonparametric Kruskal–Wallis test with a Dunn test for multiple comparisons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with wild-type. CH, NKG2D CAR T cells; WT, wtNKG2D T cells.

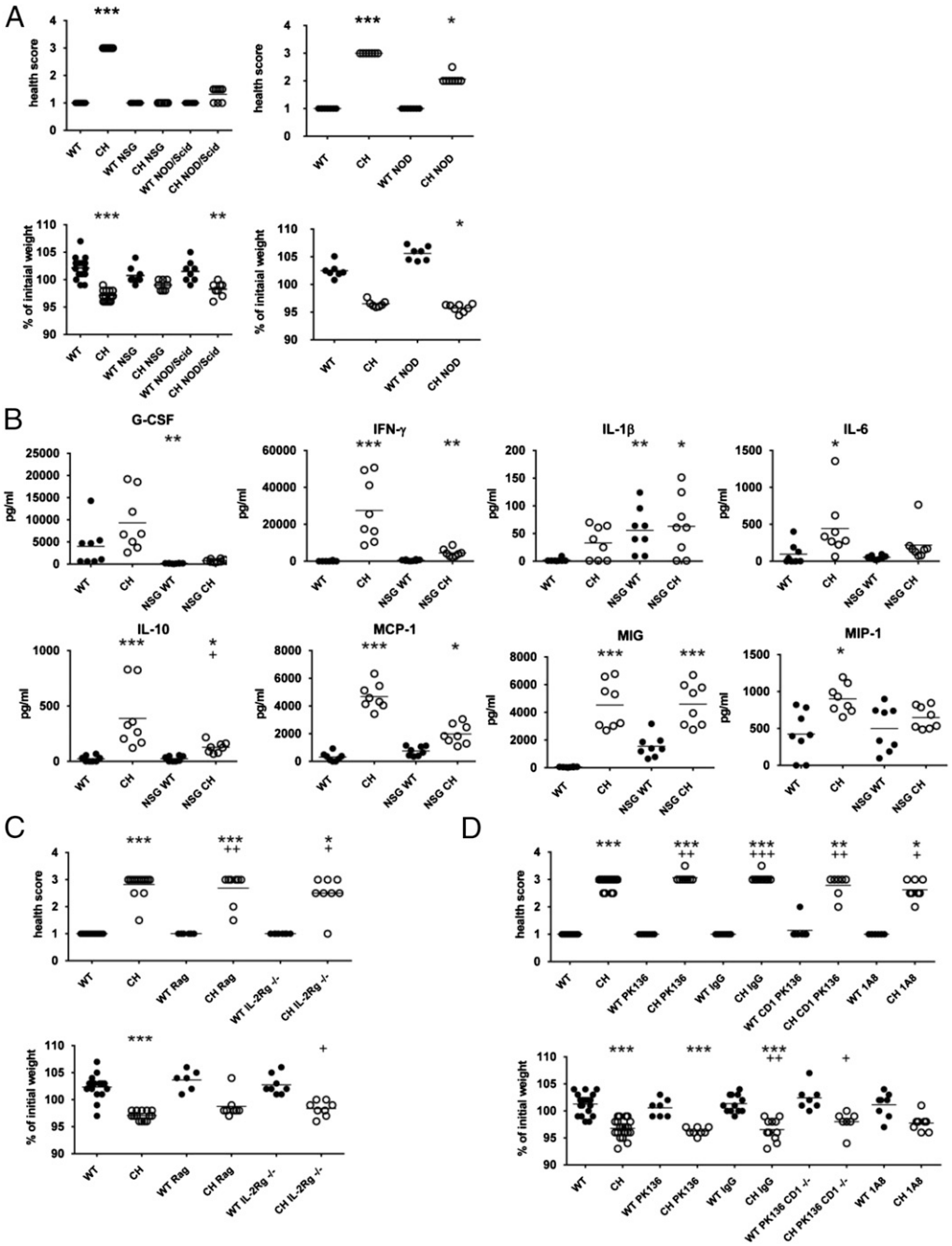

FIGURE 4. Role of host immunity in NKG2D CAR T cell toxicity. A single dose of 2×10^7 B6-derived NKG2D CAR T cells (CH) or wtNKG2D T cells (WT) was injected i.v. into mice and health status and body weight were evaluated after 20 h. (**A**) Recipients were B6, NSG, NOD/SCID, and NOD mice ($n = 8$ per group). (**B**) At the time of euthanasia, serum samples were isolated and analyzed for G-CSF, IFN-γ, IL-1β, IL-6, IL-10, MCP1, MIG, and MIP-1β by multiplex analysis. In other experiments, recipients were (**C**) Rag1-deficient or IL-2Rγ–deficient mice, (**D**) B6 mice pretreated with anti-NK1.1 mAbs (PK136), IgG controls, or CD1-deficient mice pretreated with anti-NK1.1 or anti-Ly6G (1A8) mAbs. Each symbol represents an individual mouse. ANOVA was performed using a nonparametric Kruskal–Wallis test with a Dunn test for multiple comparisons. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with B6 wild-type. $^+p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with the similar deficient or depleted wild-type. CH, NKG2D CAR T cells; WT, wtNKG2D T cells.

in lymphoma and ovarian cancer tumor models. Mice treated with DNAM1-based CAR T cells i.v. exhibited similar acute symptoms that were again dependent on perforin and GM-CSF. The manner in which these molecules drive CRS-like illness remains to be elucidated in future studies. GM-CSF from NKG2D CAR T cells drives monocyte recruitment via induction of CCR2, Ag processing, and IL-12 production (24). GM-CSF is known to activate a variety of myeloid cell activities, including the production of cytokines (44). The absence of perforin in CAR T cells does not reduce the cytokines produced or present in the tumor microenvironment to a large degree (43). The observation that the absence of the perforin cytotoxic pathway in CAR T cells prevented the CRS-like illness suggests that CAR T cell–mediated death of ligand-expressing cells and the inflammation and macrophage activation induced by the cell debris may be a key component that drives this acute clinical illness. Cell debris may drive noninfectious inflammation and wound healing responses, but the illness observed in this study was not dependent on the MyD-88/TLR–dependent signaling pathway. Moreover, these data indicate that the efficacy of CAR

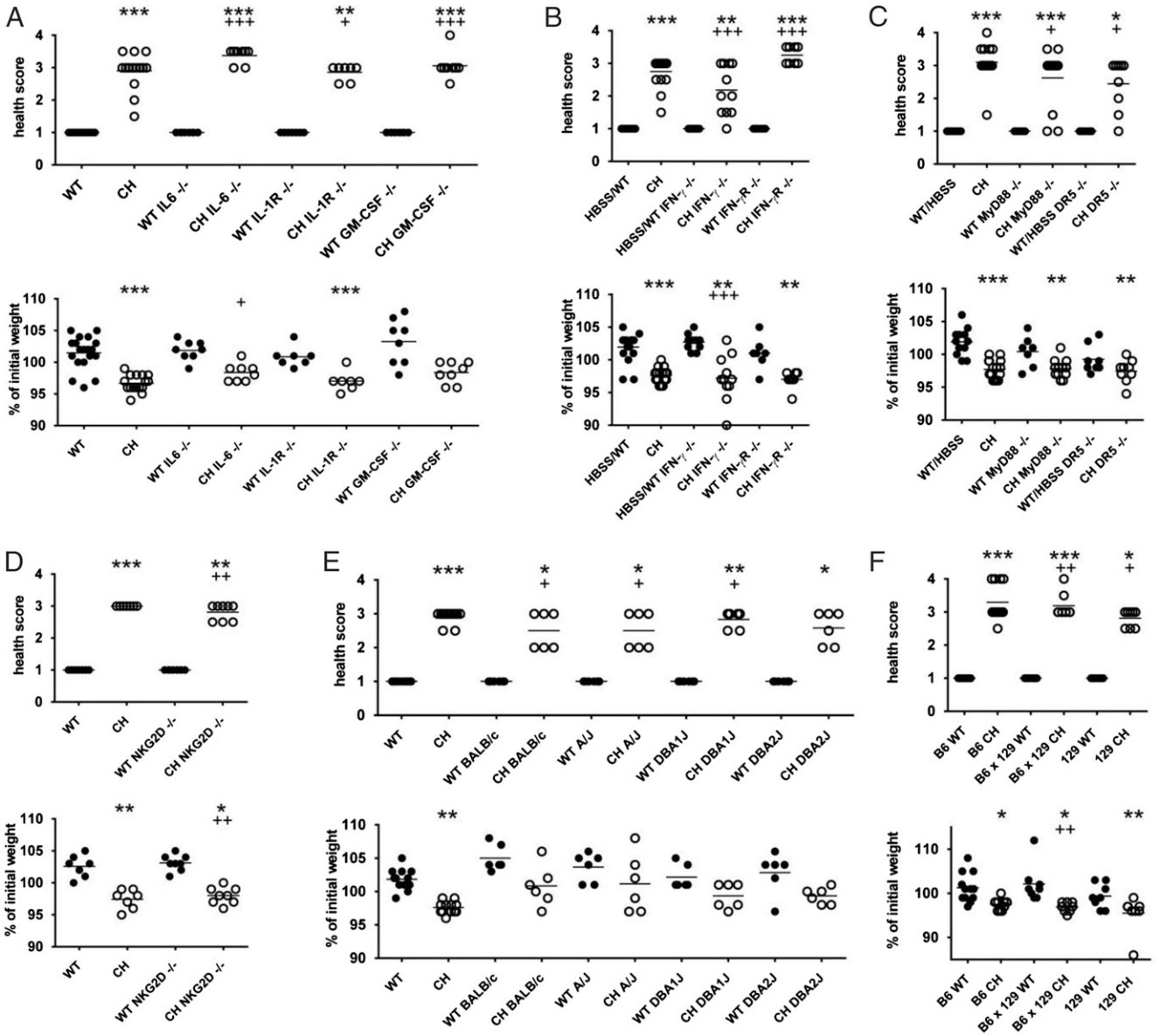

FIGURE 5. Role of host cytokines and background in NKG2D CAR T cell toxicity. A single dose of 2×10^7 B6-derived NKG2D CAR T cells (CH) or wtNKG2D T cells (WT) was injected i.v. into mice and health status and body weight were evaluated after 20 h. Recipients were (**A**) IL-6–deficient, IL-1R–deficient or GM-CSF-deficient mice, (**B**) IFN-γ-deficient or IFN-γR-deficient mice, (**C**) MyD88-deficient, or DR5-deficient mice, (**D**) NKG2D-deficient mice, (**E**) BALB/c, A/J, DBA/1, or DBA/2 mice, or (**F**) B6 mice injected with CAR or control T cells derived from B6, 129 or (B6 × 129)F_1 mice. Each symbol represents an individual mouse. ANOVA was performed using a nonparametric Kruskal–Wallis test with a Dunn test for multiple comparisons. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with B6 wild-type. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with the similar deficient or depleted wild-type. CH, NKG2D CAR T cells; WT, wtNKG2D T cells.

T cells can be detached from their ability to cause CRS-like illness. For example, IFN-γ is dispensable for the acute toxicity observed, but it is required for antitumor efficacy. Whereas partial efficacy was observed after only one dose of CAR T cells in the absence of GM-CSF or perforin, no acute toxicity was seen when either of these effector molecules was absent (7, 8). Our findings are consistent with a recent study showing that NKG2D CAR T cells did not cause significant toxicity when injected at 10^7 CAR T cells i.v. into B6 or BALB/c mice. The addition of further costimulation domains to an NKG2D CAR from CD28 resulted in increased toxicity, especially when the CAR T cells were injected following cyclophosphamide preconditioning (45). These findings suggest that methods to improve NKG2D CAR design with stronger signaling or the use of immune preconditioning should be considered carefully.

Data from patients treated with CAR T cells have shown similar patterns of elevated serum cytokines that are thought to be triggered by recognition of target Ags (2, 15, 46, 47). In these clinical studies, a set of cytokines has been elevated ≥75-fold above baseline. This set included IFN-γ, IL-6, CXCL9, CXCL10, MCP-1, IL-10, G-CSF, and GM-CSF. The development of CRS and elevation in serum cytokines was associated with the extent of expansion of anti-CD19 CAR T cells in vivo and the extent of tumor burden. However, development of severe CRS is not required for therapeutic success. Both high-dose steroids and anti–IL-6R Abs have successfully mitigated CRS in the clinic (48, 49). However, the mechanisms of CRS and required elements of immune activation have not been clearly dissected. Advances in the understanding of CRS will be critical to optimize the use of CAR T cells in patients.

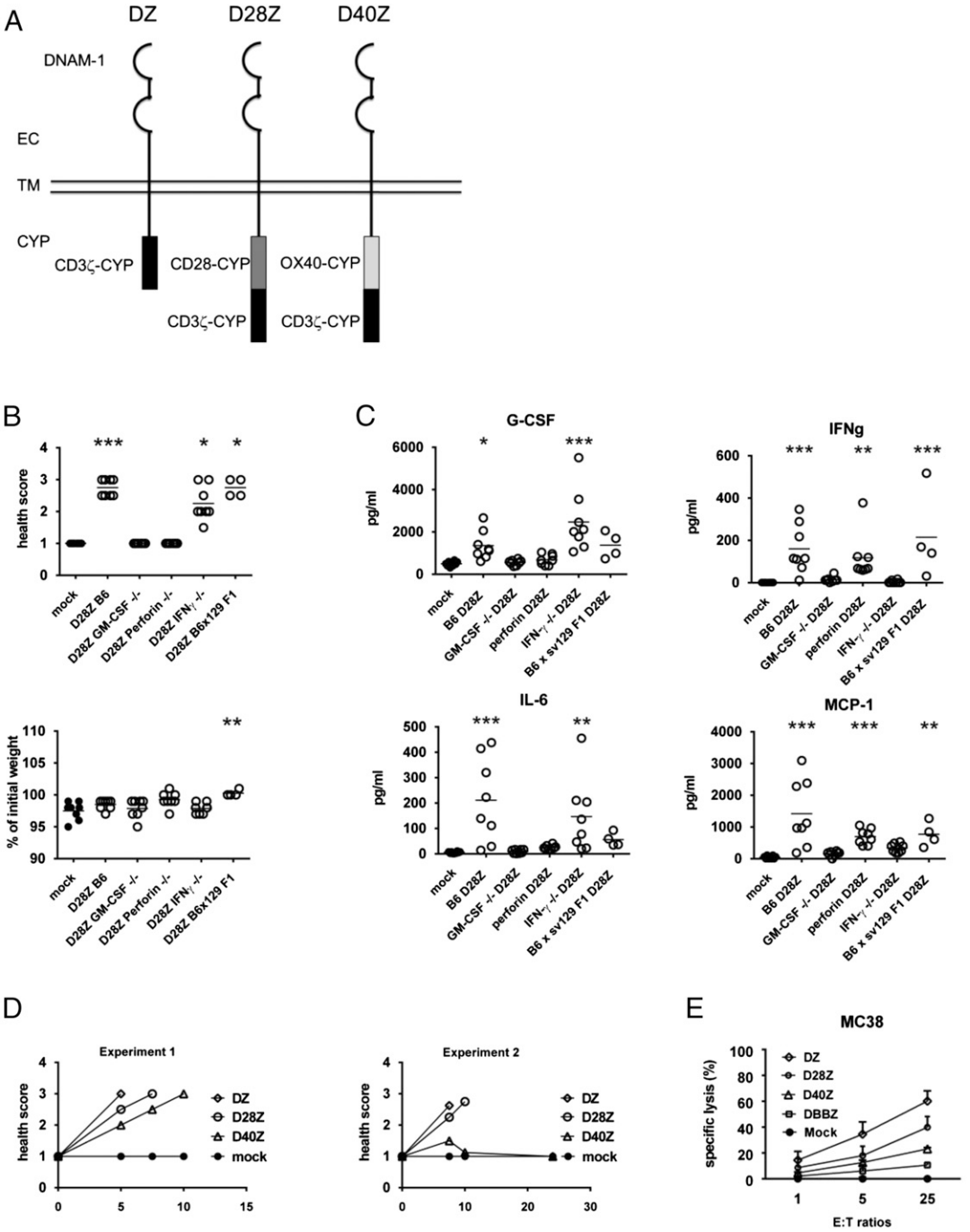

FIGURE 6. Mechanisms of DNAM1 CAR-mediated toxicity. A single dose of 10^7 DNAM1-based CAR T cells was injected into B6 mice and health status and body weight were evaluated. (**A**) The CARs used were constructed using the entire murine DNAM1 protein combined with cytoplasmic portions of CD3z (DZ), CD28 and CD3z (D28Z), or OX40 and CD3z (D40Z). (**B**) Health status and body weight of B6 mice after a single injection of HBSS or 10^7 D28Z CAR T cells derived from B6, (B6 × 129)F1, or GM-CSF–, perforin-, or IFN-γ–deficient mice. All groups had eight mice, except the $(B6 \times 129)F_1$ group, which had four mice. All mice in groups treated with HBSS or with GM-CSF– or perforin-deficient T cells had a health score of 1. (**C**) At the time of euthanasia, serum samples were isolated and analyzed for G-CSF, IFN-γ, IL-6, and MCP1 by multiplex analysis. (**D**) Health status at different times (h) after CAR T cells derived from B6 mice or HBSS injections from two independent experiments. (**E**) Specific lysis of MC38 tumor cells at various E:T ratios by T cells expressing a DNAM1 CAR–DZ, D28Z, D40Z, DBBZ (DNAM1 protein with cytoplasmic domains from 4-1BB and CD3z), or mock T cells. Error bars are SD. ANOVA was performed using a nonparametric Kruskal–Wallis test with a Dunn test for multiple comparisons. For analysis of cell cytotoxicity in vitro (E), a one-way ANOVA with a Dunnett method for multiple comparisons was used. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with wild-type.

The use of genetic mouse models (which lack one or several key molecules or cell types) and the use of cell-depleting Abs allowed the evaluation of the host immune cells and effector molecules essential for CRS-like illness observed after injection of NKG2D CAR T cells. The data demonstrated that many cells and molecules known to contribute to inflammation under different conditions, including B cells, T cells, NK cells, IL-6, and IFN-γ, were not required for toxicity. That a particular gene knockout or cell depletion did not lead to an altered phenotype does not indicate that the gene or cell plays no role in the acute CRS-like illness, only that the gene or cell

is not a critical component. In contrast, mice of the NOD/SCID background, including NSG mice, developed none or at most a mild illness due to the severe immune deficiency associated with NOD/SCID mice. Not only do NOD/SCID and NSG mice lack T cells and B cells, they also have a number of other immune deficiencies, such as decreased NK cells, altered PMNs, more immature macrophages, reduced macrophage activation, and altered lymphoid tissue architecture. This severe immune deficiency is in contrast to Rag1-deficient mice that only lack T and B cells, or IL-2Rγ–deficient mice that have no NK cells and defects in T and B cell numbers and responses. Thus, a defect in multiple immune cell populations in the host, including the monocyte/macrophage compartment, protected against the development of the CRS-like illness, and this is consistent with a clinical phenotype that resembles macrophage activation syndrome. This is an important observation because severely immunodeficient NSG mice are often used to evaluate and optimize CAR T cells in vivo. However, toxicity associated with CAR T cell treatment may not be observed in these mice. There are notable human cytokines that do not function in mice. One of these cytokines is GM-CSF, so GM-CSF from human CAR T cells will not affect mouse immune responses. The data from this study showed that acute illness was independent of genetic background in the strains tested. Collectively, the data suggest that host myeloid cells and innate cells are key players in this CRS-like illness that we observe at high doses of CAR T cells. Overall, the data show that murine NKG2D CAR T cells can result in an acute CRS-like phenotype, the bolus dose is more important than the cumulative cell dose for CRS development, and that specific effector mechanisms from the CAR T cells and host are involved.

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Disclosures

C.L.S. has patents and financial interests in NK receptor–based CAR therapies. C.L.S. is a scientific founder for Celdara Medical, a consultant, and receives research support from Celdara Medical. These conflicts are managed under the policies of Dartmouth College. M.-L.S. has an immediate family member with financial interests in NK receptor–based CAR therapies. J.M.M., J.R., and M.W.F. are employed by Celdara Medical, which has a material financial interest in NK receptor–based CAR intellectual property assigned to the Trustees of Dartmouth College. S.H.B. received Small Business Innovation Research grant support through Celdara Medical. G.D. is currently an employee of Novartis, which has material financial interests in other CAR T cell therapies. The other authors have no financial conflicts of interest.

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Supplemental Table 1. *AST and creatinine are not altered by CAR T cell injection*

Tumor	Treatment	Day‡	10^7 T cells				5×10^6 T cells			
			AST§	±SD	CREA‖	±SD	AST§	±SD	CREA‖	±SD
RMA-RG	**wt***	8	54.0	5.3	0.3	0.1	58.0	11.6	0.3	0.1
	wt*	12	97.1	35.1	0.4	0.1	111.6	55.1	0.3	0.1
	ch†	8	71.7*	22.9	0.3	0.1	77.0*	32.5	0.3	0.1
	ch†	12	88.6	37.7	0.4	0.1	85.9	27.2	0.4	0.1
	HBSS	8	67.0*	12.0	0.3	0.1	66.2	7.9	0.3	0.1
	HBSS	12	70.0	18.0	0.3	0.1	87.9	33.3	0.3	0.1
No Tumor	**wt***	3	74.8	14.2	0.3	0.1	95.1	45.3	0.4	0.1
	wt*	8	62.8	8.2	0.3	0.1	83.0	34.8	0.3	0.1
	ch†	3	68.0	10.3	0.4	0.1	95.2	42.0	0.3	0.1
	ch†	8	64.8	11.0	0.4	0.1	68.8	9.9	0.2	0.1
	HBSS	3	79.6	19.6	0.4	0.1	67.5	6.9	0.3	0.1
	HBSS	8	67.2	19.6	0.4	0.1	83.1	31.2	0.3	0.1

* wt= wild type NKG2D T cells, † ch= NKG2D CAR T cells

‡ Day= Day sample was collected after RMA-RG tumor injection (T cells given on day 5), and day sample was collected after T cell injection in mice without tumors.

§ Values reported as U/L

‖ Values reported as mg/dL

ANOVA compared to wt at the same day and dose, * $p<0.05$

Supplemental Table 2. *Serum cytokines are not changed 3 to 12 days after CAR T cell injection.*

			10^7 T cells								5×10^6 T cells							
Tumor	**Treatment**	**Day**‡	**IL-6**§	±SD	**IFN-γ**§	±SD	**MCP-1**§	±SD	**TNF-α**§	±SD	**IL-6**§	±SD	**IFN-γ**§	±SD	**MCP-1**§	±SD	**TNF-α**§	±SD
RMA-RG	**wt***	8	2.6	1.5	4.2	2.9	167.5	41.8	15.2	7.3	2.0	1.1	3.0	3.2	162.8	49.0	27.0	11.6
	wt*	12	2.7	0.9	2.4	3.1	451.5*	219.8	9.7	11.3	2.9	1.4	0.9	1.3	239.7	101.3	10.8	11.3
	ch†	8	8.4	9.1	3.2	3.4	177.8	86.0	19.3	10.7	4.2	7.0	3.1	3.2	200.3	121.8	26.2	14.4
	ch†	12	5.5	7.4	0.4	0.8	277.2	145.0	9.5	9.3	2.8	2.1	1.9	1.7	169.7	89.2	14.4	11.4
	HBSS	8									5.4	6.7	3.0	3.9	197.4	119.1	19.1	11.1
	HBSS	12									4.9	5.4	1.0	1.8	201.4	112.3	10.6	12.6
No Tumor	**wt***	3	3.5	5.2	1.6	2.5	143.2	100.6	15.3	11.7	2.7	1.4	1.0	2.1	95.5	50.4	56.1	87.7
	wt*	8	2.8	1.7	0.9	2.0	161.5	80.8	34.2	42.6	2.8	1.3	0.7	1.9	154.8	41.4	49.4	54.3
	ch†	3	2.1	1.4	2.8	4.3	149.0	39.4	11.8	20.7	3.3	3.7	1.7	3.3	104.1	41.2	36.5	72.4
	ch†	8	2.8	2.0	1.3	2.6	143.9	40.1	17.5	16.2	5.3	6.3	0.9	0.0	268.2	200.0	25.3	15.8
	HBSS	3									2.5	1.7	2.5	4.4	168.3	43.7	12.5	15.4
	HBSS	8									4.4	7.1	2.1	2.9	182.9	160.7	17.6	14.6

* wt= wild type NKG2D T cells, † ch= NKG2D CAR T cells

‡ Day= Day sample was collected after T cell injection

§ Values reported as pg/mL

ANOVA compared to HBSS at the same day and dose, * $p<0.05$

Table 3 *Histological analysis of tissues harvested after a dose of 2 x 10^7 CAR T cells.*

		RMA-RG tumor bearing mice						Non tumor bearing mice							
		WT NKG2D T cells		NKG2D CAR T cells		HBSS		WT NKG2D T cells			NKG2D CAR T cells			HBSS	
Tissue/diagnosis		**expt 1**	**expt 2**	**expt 1**	**expt 2**	**expt 1**	**expt 2**	**expt 1**	**expt 2**	**expt 3**	**expt 1**	**expt 2**	**expt 3**	**expt 1**	**expt 2**
Heart	normal	4/4	5/5	5/5	5/5	5/5	4/4	5/5	5/5	6/6	5/5	5/5	6/6	5/5	4/5
Kidney	normal	4/4	5/5	5/5	5/5	5/5	4/4	5/5	5/5	6/6	5/5	5/5	6/6	5/5	4/5
	tumor, undifferentiated round cell														
Liver	normal	3/5		4/5	4/4	2/5	3/5	4/5	3/5	5/6	4/5	5/5	6/6	2/5	2/5
	infiltration, mixed cell	2/5	5/5	1/5			2/5		2/5	1/6	1/5			3/5	2/5
	depletion, glycogen					3/5									
Lung	Normal	5/5	5/5	5/5	3/5	5/5	5/5	5/5	5/5	6/6	5/5	5/5	6/6	5/5	3/5
	infiltration, neutrophilic, alveolar wall				2/5										
	Hemorrhage, alveolar														1/5
Large intestine	normal	5/5	5/5	5/5	3/3	5/5	5/5	5/5	5/5	6/6	5/5	5/5	6/6	5/5	4/5
Small intestine	normal	4/4	5/5	4/4	5/5	5/5	4/4	5/5	5/5	6/6	5/5	5/5	6/6	5/5	4/5
Pancreas	normal	4/5	5/5	4/5	4/5	5/5	4/5	5/5	5/5	6/6	4/5	5/5	6/6	5/5	4/5
	Few/no islets in section	1/5		1/5	1/5		1/5				1/5				
Spleen	normal	5/5	5/5	4/5		5/5	5/5	5/5	5/5			5/5	6/6	5/5	4/5
	necrosis, single cell; white pulp			1/5	5/5					6/6	5/5				
	hematopoietic activity, increased														
	tumor, undifferentiated round cell														

note: pathology was scored mild (1), but 2 single cell necrosis, spleen were scored moderate in expt 2.

Table 4 *Histological analysis of tissues harvested after three doses 10^7 CAR T cells.*

		RMA-RG tumor bearing mice						Non tumor bearing mice					
		WT NKG2D T cells		NKG2D CAR T cells		HBSS		WT NKG2D T cells		NKG2D CAR T cells		HBSS	
Tissue/diagnosis		**expt 1**	**expt 2**	**expt 1**	**expt 2**	**expt 1**	**expt 2**	**expt 1**	**expt 2**	**expt 1**	**expt 2**	**expt 1**	**expt 2**
Heart	normal	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Kidney	normal	3/5	5/5	3/5	3/5	5/5	2/5	5/5	5/5	5/5	5/5	5/5	5/5
	tumor, undifferentiated round cell						3/5						
Liver	normal			2/5	1/5	1/5		4/5	4/5	4/5	2/5	3/5	5/5
	infiltration, mixed cell	2/5	2/5			2/5	1/5	1/5	1/5	1/5	3/5	2/5	
	tumor, undifferentiated round cell	3/5	5/5	3/5	4/5	3/5	4/5						
Lung	Normal	5/5	3/5	2/5		4/5	5/5	4/5	5/5	5/5	4/5	5/5	4/4
	Hemorrhage, alveolar							1/5			1/5		
	tumor, undifferentiated round cell		2/5	3/5	5/5	1/5							
Large intestine	normal	5/5	5/5	5/5	5/5	5/5	5/5	4/4	5/5	5/5	5/5	5/5	5/5
Small intestine	normal	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Pancreas	normal	5/5	5/5	5/5	3/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5
	Depletion, Zymogen granules				2/5		1/5						
Spleen	normal	5/5	5/5		2/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
	necrosis, single cell; white pulp			1/5	2/5								
	hematopoietic activity, increased			3/5									
	tumor, undifferentiated round cell			2/5	1/5								