NOD2 regulates hematopoietic cell function during graft-versus-host disease

Olaf Penack,1,3 Odette M. Smith,1 Amy Cunningham-Bussel,4 Xin Liu,4 Uttam Rao,1 Nury Yim,1 Il-Kang Na,1,3 Amanda M. Holland,1,4 Arnab Ghosh,1 Sydney X. Lu,1 Robert R. Jenq,1,2 Chen Liu,5 George F. Murphy,6 Katharina Brandl,1 and Marcel R.M. van den Brink1,2

Abbreviations used: allo-BMT, allogeneic BM transplantation; allogeneic HCT, allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; GVHD, graft-versus-host disease; MDP, muramyl dipeptide; MLN, mesenteric LN; MLR, mixed leukocyte reaction; NOD2, nucleotide-binding oligomerization domain 2; SNP, single nucleotide polymorphism; TCD-BM, T cell–depleted BM; TLR, toll-like receptor.

Nucleotide-binding oligomerization domain 2 (NOD2) polymorphisms are independent risk factors for Crohn’s disease and graft-versus-host disease (GVHD). In Crohn’s disease, the proinflammatory state resulting from NOD2 mutations have been associated with a loss of antibacterial function of enterocytes such as paneth cells. NOD2 has not been studied in experimental allogeneic bone marrow transplantation (allo-BMT). Using chimeric recipients with NOD2+/− hematopoietic cells, we demonstrate that NOD2 deficiency in host hematopoietic cells exacerbates GVHD. We found that proliferation and activation of donor T cells was enhanced in NOD-deficient allo-BMT recipients, suggesting that NOD2 plays a role in the regulation of host antigen-presenting cells (APCs). Next, we used bone marrow chimeras in an experimental colitis model and observed again that NOD2 deficiency in the hematopoietic cells results in increased intestinal inflammation. We conclude that NOD2 regulates the development of GVHD through its inhibitory effect on host APC function.

NOD2 has been studied in human Crohn’s disease and in mouse models for inflammatory bowel disease. Single nucleotide polymorphisms (SNPs) near, or within, the NOD2 leucine-rich repeat region (G908R, L1007insC, and R702W) constitute genetic risk factors for the development of Crohn’s disease (Hugot et al., 2001; Ogura et al., 2001). Mutations in the NACHT region of NOD2 are linked to other inflammatory diseases such as Blau syndrome (Miceli-Richard et al., 2001) and early onset sarcoidosis (Kanazawa et al., 2005). Several groups have investigated the role of NOD2 in experimental models for intestinal inflammation, which has led to the development of three hypotheses regarding the function of NOD2. Kobayashi et al. (2005) found greater susceptibility to enteral infection with Listeria monocytogenes and reduced expression of α-defensins, antibacterial peptides produced by intestinal Paneth cells, in NOD2−/− mice. Watanabe et al.

© 2009 Penack et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/). After six months it is available under a Creative Commons License Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.jem.org/misc/terms.shtml). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike–No Mirror Sites).
combination with the MLRs (unpublished data). To deter-
gunds (MDP, LPS, Pam3Cys, Flagellin, and PGN) alone or in
we added different toll-like receptor (TLR) and NOD2 li-
gation rate between WT T cells and NOD2
addition, we found no significant differences in the prolifera-
tcell proliferation (Fig. 1 A), T cell activation (Fig. 1 B),
lar T cell proliferation (Fig. 1 C). Next, we performed mixed
and expression of the gut-homing molecule LPAM (Fig. 1 D).
We transferred CFSE-labeled B6 WT or B6 NOD2
whether NOD2 deficiency affects allogeneic donor T cells.
To address contradictory clinical studies showing that NOD2
does not affect the development of GVHD
NOD2 deficiency in donor BM cells and/or donor T cells
does not affect the development of GVHD
To address contradictory clinical studies showing that NOD2
SNPs of the allo-HSCT donor can either exacerbate GVHD
(Holler et al., 2004, 2006) or ameliorate GVHD (Elmaagachi et al., 2006), we investigated the effect of NOD2 deficiency of allo-BMT donors in our GVHD models. We first assessed whether NOD2 deficiency affects allogeneic donor T cells. We transferred CFSE-labeled B6 WT or B6 NOD2−/− T cells to lethally irradiated BALB/c recipients and found similar T cell proliferation (Fig. 1 A), T cell activation (Fig. 1 B), and expression of the gut-homing molecule LPAM (Fig. 1 C). Next, we performed mixed leukocyte reactions (MLRs) using CD3+–selected B6 WT or B6 NOD2−/− splenocytes as effectors and irradiated BALB/c splenocytes as stimulators. We found no significant differences in the proliferation rate between WT T cells and NOD2−/− T cells (Fig. 1 D). In addition, we found no significant differences in the proliferation rate between WT T cells and NOD2−/− T cells when we added different toll-like receptor (TLR) and NOD2 ligands (MDP, LPS, Pam3Cys, Flagellin, and PGN) alone or in combination with the MLRs (unpublished data). To deter-
the role of NOD2 in Crohn’s disease (Hugot et al., 2001; Watanabe et al., 2004, 2006, 2008; Kobayashi et al., 2005). NOD2 polymorphisms resulting in impaired NOD2 function were associated with increased susceptibility to Crohn’s disease. Studies in NOD2-deficient mice demonstrated an increased susceptibility to experimental colitis, whereas mice with a NOD2 transgene resulting in increased NOD2 function were resistant to experimental colitis.

The activation and proliferation of alloreactive donor T cells is increased in NOD2−/− allo-BMT recipients

To study the mechanisms underlying increased GVHD in NOD2−/− allo-BMT recipients, we first transferred CFSE-labeled allogeneic B10BR T cells into B6 WT or B6 NOD2−/− allo-BMT recipients. We found that the in vivo proliferation of alloreactive donor T cells was significantly increased in NOD2−/− allo-BMT recipients (Fig. 2 F). Next, we performed allo-BMTs in the B10BR→B6 model and harvested organs at day 14 after allo-BMT. We found that the number of donor T cells was significantly increased in spleen and mesenteric LNs (MLNs) in NOD2−/− allo-BMT recipients (Fig. 2 G, left). However, the percentages of CD4+ T cells, CD8+ T cells, and CD44+/CD62L− effector T cells were not significantly different (Fig. S1). We next determined the alloactivation status of donor T cells by their CD25 expression, which is a reliable marker for alloactivation in our

Figure 1. NOD2 deficiency in donor BM cells and/or donor T cells does not affect the development of GVHD. (A–C) Lethally irradiated (8.5 Gy) BALB/c allo-BMT recipients received 5 × 10⁶ CFSE-labeled B6 WT or B6 NOD2−/−CD5+ T cells. Recipient spleens were harvested 96 h after transfer of T cells; n = 8/group; combined data from two independent experiments are shown. (A) Identical CFSE histogram overlays of WT and NOD2−/− donor T cells (left), as well as similar total number of CFSEhigh and CFSELow donor T cells. (B) Similar up-regulation of the alloactivation marker CD25 on WT B6 donor T cells versus NOD2−/− donor T cells. (C) Similar expression of the gut-homing marker LPAM on WT B6 donor T cells versus NOD2−/− donor T cells. (D) MLR with WT versus NOD2−/−CD5+ T cells and irradiated BALB/c stimulators. Combined data of three independent experiments are shown; n = 12/group. (E) Lethally irradiated BALB/c (8.5 Gy) or LP (11Gy) allo-BMT recipients were transplanted with 5 × 10⁶ B6 WT TCD-BM versus B6 NOD2−/− TCD-BM and 10⁶ B6 WT T cells versus B6 NOD2−/− T cells. Combined data from four independent experiments are shown; n = 15–45/group. Error bars indicate SEM.
Figure 2. NOD2−/− allo-BMT recipients have increased systemic and target organ GVHD because of enhanced activation and proliferation of alloreactive donor T cells. (A) Lethally irradiated (11 Gy) B6 WT versus B6 NOD2−/− allo-BMT recipients received 5 × 10^6 B10BR TCD-BM or 5 × 10^6 LP TCD-BM with or without 10^6 B10BR T cells or 2 × 10^6 LP T cells. Combined data from four independent experiments are shown; n = 45/group. (B-E) Lethally irradiated (11 Gy) B6 WT versus B6 NOD2−/− allo-BMT recipients received 5 × 10^6 B10BR TCD-BM + 10^6 B10BR T cells. Organs were harvested at day 21 after allo-BMT. Combined data from two independent experiments are shown; n = 10/group. (B) Histopathologic GVHD scores in target organs showing increased GVHD in NOD2−/− allo-BMT recipients. (C) CD3+ histochemistry images showing increased infiltration of T cells (brown) in liver tissue of NOD2−/− allo-BMT recipients. Bars, 50 µm. (D) Quantification of T cell infiltration in GVHD target organs by CD3+ histochemistry. (E) Increased thymic GVHD in NOD2−/− allo-BMT recipients demonstrated by reduced numbers of CD4+/CD8− double-positive thymocytes. Shown are flow cytometric plots and the absolute numbers of DP (CD4+/CD8−), DN (CD4−/CD8−), CD4+, and CD8+ thymocytes. (F) Lethally irradiated (11 Gy) B6 WT versus B6 NOD2−/− allo-BMT recipients received 5 × 10^6 CFSE-labeled B10BR CD5+ T cells. Recipient spleens were harvested 96 h after transfer of T cells; n = 8/group; combined data from two independent experiments are shown. CFSE histogram overlays of donor T cells in WT allo-BMT recipients versus NOD2−/− allo-BMT recipients (left); ratio of proliferating donor T cells (right, top) and absolute numbers of donor T cells (right, bottom) showing increased donor T cell proliferation in NOD2−/− allo-BMT recipients. (G and H) Lethally irradiated (11 Gy) B6 WT versus B6 NOD2−/− allo-BMT recipients were transplanted with...
GVHD models (Alpdogan et al., 2003), and found that donor T cells were significantly more activated in NOD2+/− allo-BMT recipients as compared with WT allo-BMT recipients (Fig. 2 G, middle). When we analyzed FoxP3+/CD4+ regulatory T cells in the spleen, we found a significantly reduced percentage of FoxP3+/CD4+ cells (of total CD4+ splenocytes) in NOD2+/− allo-BMT recipients (Fig. 2 G, top right). The percentages of IL-17+CD4+, IFN-γ+CD4+, and Tbet+CD4+ T cells were not different (Fig. S1). There was no difference between the expression of the gut-homing molecule LPAM (α4β7 integrin) on donor T cells in MLNs in NOD2+/− and WT allo-BMT recipients (Fig. 2 G, bottom right). We detected no differences in donor cell types, such as B cells, NK cells, granulocytes, monocytes, and DCs in NOD2+/− allo-BMT recipients (Fig. 2 H). We conclude that increased GVHD in NOD2+/− allo-BMT recipients is the result of enhanced alloactivation and proliferation of donor T cells.

NOD2 deficiency in the hematopoietic system of the allo-BMT recipient exacerbates GVHD

Given that NOD2 is expressed in both hematopoietic and nonhematopoietic cell types, including antigen-presenting cells and intestinal epithelial cells, respectively, it is not clear what relative contributions of these cell types is to the development of GVHD. To address this, we created BM chimeras in which the NOD2 deficiency was only manifest in one of these systems. BM chimeras were created by performing syngeneic BMTs after lethal irradiation, using B6 WT and B6 NOD2−/− as donors or as recipients (WT→NOD2−/− or NOD2+/−→WT). Using B6 Ly5.1+ mice as donors, we found the donor chimerism of hematopoietic cell subsets to be >95% in GVHD target organs, as well as in secondary lymphoid organs at day 90 after syngeneic BMT (Fig. S2). We then used the BM chimera, WT mice, and NOD2−/− mice as allo-BMT recipients in the B10BR→B6 GVHD model. We found that NOD2−/− and NOD2+/−→WT chimeric allo-BMT recipients had a greater GVHD-induced mortality rate as compared with WT and WT→NOD2−/− chimeric allo-BMT recipients (Fig. 3 A, left). We confirmed our findings in the minor mismatched LP→B6 model (Fig. 3 A, right). To analyze target organ GVHD, we performed histopathological analyses and found significantly more GVHD in the terminal ileum, colon, liver, and skin in NOD2−/−→WT chimeric allo-BMT recipients versus WT→NOD2−/− chimeric allo-BMT recipients (Fig. 3 B). The numbers of tissue-infiltrating CD3+ T cells in GVHD target organs were increased in NOD2−/−→WT chimeric allo-BMT recipients (Fig. 3 C). We found that the number of donor T cells was significantly increased in the spleen and MLNs in NOD2−/−→WT chimeric allo-BMT recipients (Fig. 3 D, left). We next determined the alloactivation status of donor T cells by their CD25 expression and found that donor T cells were significantly more activated in NOD2−/−→WT chimeric allo-BMT recipients as compared with WT→NOD2−/− chimeric allo-BMT recipients (Fig. 3 D, middle). Additionally, we found a significantly reduced percentage of FoxP3+ CD4+ regulatory T cells in the spleen (percentage of FoxP3+ in total CD4+ splenocytes) in NOD2+/−→WT chimeric allo-BMT recipients (Fig. 3 D, top right). The expression of LPAM on donor T cells in MLNs was similar in both chimeric allo-BMT recipients (Fig. 3 D, bottom right). We conclude that NOD2 deficiency in the hematopoietic system exacerbates GVHD, whereas NOD2 deficiency in the nonhematopoietic system has no significant impact on the development of GVHD in NOD2−/− allo-BMT recipients.

NOD2−/− DCs have a higher activation status and increased ability to induce T cell proliferation during GVHD

Based upon our finding that donor T cell proliferation is increased in allo-BMT recipients with NOD2 deficiency in the hematopoietic system, we hypothesized that NOD2−/− DCs might have a higher activation status and increased ability to induce T cell proliferation during GVHD. We therefore performed allo-BMTs in the B10BR→B6 model and harvested spleens at day 7 after allo-BMT. We found that the absolute numbers of host DC subsets in the spleen were not significantly different before and after allo-BMT in WT versus NOD2−/− allo-BMT recipients (Fig. 4 A). We then quantified the expression of the activation markers and costimulatory molecules CD40, CD80, and CD86 on host (CD11c+H2kb+) DCs before allo-BMT and at day 7 after allo-BMT. We found a significantly increased up-regulation of activation markers on host NOD2−/− DCs in contrast to WT DCs during GVHD (Fig. 4, B and C). The increase in the expression of MHC class II molecules, which was already high at baseline, did not reach statistical significance (unpublished data). To study DC function, we selected splenic DCs from WT and NOD2−/− allo-BMT recipients with GVHD (day 7) and used them as stimulators in MLRs. We found that B6 NOD2−/− DCs had a significantly increased ability to induce proliferation of allogeneic B10BR T cells as compared with B6 WT DCs (Fig. 4 D). These data supports the hypothesis that NOD2 can negatively regulate the activity and function of host DCs, resulting in increased alloactivation and proliferation of donor T cells in NOD2−/− allo-BMT recipients. Our results provide a possible cellular mechanism for recent clinical studies suggesting a regulatory role of NOD2 on the incidence of GVHD and transplant-related mortality in allo-HSCT recipients with SNPs in the gene locus encoding NOD2 (Holler et al., 2004, 2006; Elmaagacli et al., 2006).
In previous studies using NOD2−/− mice in inflammatory bowel disease models, it was suggested that increased TLR signaling led to a massively increased production of cytokines such as IL-12. (Watanabe et al., 2004, 2006, 2008; Strober et al., 2006; Yang et al., 2007) To determine whether increased cytokine/chemokine production is the underlying mechanism for the enhanced ability of NOD2−/− DCs to induce T cell proliferation, we performed allo-BMTs in the B10BR→B6 model and measured cytokines/chemokines in serum with a multiplex system at day 7 after allo-BMT. We did not detect significant differences in serum levels of IFN-γ, IL-1α, IL-1β, IL-2, IL-6, IL-7, IL-9, IL-10, IL-12 p40,

Figure 3. NOD2 deficiency of the hematopoietic system of the allo-BMT recipient aggravates GVHD. (A–D) Chimeric mice with NOD2 deficiency either in the hematopoietic system or in the nonhematopoietic system were created by syngeneic BMT (B6 WT→B6 NOD2−/− or B6 NOD2−/−→B6 WT). After 90 d, lethally irradiated (11 Gy) B6 WT versus B6 NOD2−/− versus chimeric allo-BMT recipients were transplanted with 5 × 10^6 B10BR TCD-BM or 5 × 10^5 LP TCD-BM + 2 × 10^6 B10BR T cells or 3 × 10^6 LP T cells. (A) Survival curves. Combined data from two independent experiments are shown; n = 16/group. (B–E) Lethally irradiated (11 Gy) B6 WT→B6 NOD2−/− or B6 NOD2−/−→B6 WT chimeric allo-BMT recipients were transplanted with 5 × 10^6 B10BR TCD-BM + 10^5 B10BR T cells. Organs were harvested at day 14 after allo-BMT. Combined data from two independent experiments are shown; n = 8/group. (B) Histopathologic GVHD scores in GVHD target organs. (C) Quantification of T cell infiltration in GVHD target organs by CD3+ histochemistry. (D) Absolute number and activation status (CD25+) of donor CD3+ T cells in spleen and MLNs, percentage of FoxP3+ regulatory T cells (top right), and expression of the gut-homing marker LPAM in MLNs (bottom right). Error bars indicate SEM.
IL-12 p70, IL-15, IL-17, IP-10, LIX, MCP-1, MIG, MIP-1α, MIP-1β, Rantes, and TNF in NOD2−/− allo-BMT recipients versus WT allo-BMT recipients (Fig. S3). Although serum levels were not different we cannot exclude differences in cytokine production in specified cell populations. However, our results are in agreement with recent data, which showed that APCs from humans with NOD2 SNPs do not have increased cytokine production (van Heel et al., 2005). Consequently, there might be alternative molecular mechanisms that can enhance APC function and activity in NOD2−/− allo-BMT recipients during inflammatory diseases such as colitis and GVHD.

**NOD2 deficiency of the hematopoietic system exacerbates experimental colitis**

Next, we analyzed whether results from our GVHD experiments also apply to other inflammatory diseases. Because the pathophysiology of GVHD and inflammatory bowel disease has many similarities, we performed experiments using the TNBS (2,4,6-trinitrobenzenesulfonic acid) colitis model. TNBS colitis is a transmural inflammation often used as a model for inflammatory bowel disease (Scheiffele and Fuss, 2002). Administration of TNBS leads to modification of intestinal proteins that function as haptens. We rectally challenged B6 WT or B6 NOD2−/− mice with 5 mg TNBS in 50% ethanol. We found a significantly higher weight loss in NOD2−/− mice than in WT mice during colitis (Fig. 5 A). We next compared the NOD2−/−→WT chimeric to the WT→NOD2−/− chimeric mice (90 d after syngeneic BMT) in the TNBS colitis model. We found significantly more weight loss in NOD2−/−→WT chimeric mice versus the WT→NOD2−/− chimeric mice (Fig. 5 B). We harvested colons at day 3 after induction of colitis, performed histopathological analysis, and found significantly higher colitis scores in NOD2−/−→WT chimeric mice as compared with the WT→NOD2−/− chimeric mice (Fig. 5, C and D). Our data demonstrate that NOD2 deficiency in the hematopoietic system, but not in the nonhematopoietic system, exacerbates both experimental GVHD and experimental TNBS colitis. These results suggest that loss of intestinal epithelial cell function causing impaired antibacterial resistance, which has been proposed as a mechanism for increased inflammation in NOD2−/− mice (Kobayashi et al., 2005), is not responsible for the increased GVHD and colitis. Our results are in agreement with previous findings (Watanabe et al., 2004, 2006, 2008; Strober et al., 2006; Yang et al., 2007) that found increased susceptibility to different types of experimental colitis in NOD2−/− mice, which was the result of the enhanced ability of NOD2−/− APCs to trigger inflammatory T cell responses.

**NOD2−/− mice have intact resistance against experimental tumors**

In response to mixed clinical results on a possible role of NOD2 in antitumor immunity (Mayor et al., 2007; Holler et al., 2008), we performed tumor challenges with hematologic and solid tumors. We studied the role of NOD2 in antitumor responses in two tumor models: acute myeloid leukemia (AML; C1498) and melanoma (B16). When we challenged B6 WT or B6 NOD2−/− mice intravenously with C1498 or B16, we found no significant differences in survival (unpublished
Flow cytometry and MLR. Most antibodies were obtained from BD. DCs were purified by positive selection with CD11c (N418) MicroBeads (Miltenyi Biotec) from digested spleens. Purity was between 90 and 95%. Splenic T cells were enriched by CD5+ selection and cocultured. After 48 h, thymidine (1 µCi [0.037 MBq] [3H]thymidine/well) was added and, after 72 h, thymidine uptake was measured (TopCount; Packard).

Proliferation of CFSE-labeled T cells in vivo. T cells were selected using CD5 MicroBeads (Miltenyi Biotec), stained with 5 µM CFSE (Invitrogen), and transplanted into lethally irradiated allogeneic recipients.

Measurement of cytokines and chemokines. Serum levels were determined using the Milliplex Mouse Cytokine/Chemokine Immunoassay (Millipore).

Figure 5. NOD2 deficiency in the hematopoietic system regulates experimental colitis. (A) TNBS colitis (5 mg TNBS in 50% ethanol) was induced in B6 WT or B6 NOD2−/− mice. Higher weight loss of NOD2−/− mice during colitis. Combined data from three independent experiments are shown; \( n = 12 \) /group. (B–D) First, chimeric mice with NOD2 deficiency either in the hematopoietic system or in the nonhematopoietic system were created by syngeneic BMT (B6 WT→B6 NOD2−/− or B6 NOD2−/−→B6 WT). After 90 d, TNBS colitis (5 mg TNBS in 50% ethanol) was induced. Colons were harvested at day 3 after induction of colitis. Combined data from two independent experiments are shown; \( n = 8 \) /group. (B) Higher weight loss of NOD2−/−→WT chimera versus WT→NOD2−/− chimera during colitis. (C) Hematoxylin and eosin–stained images of the colon at day 3 after induction of colitis. Bar, 200 µm. (D) Histological scoring showing increased colitis in the hematopoietic NOD2-deficient chimera. Error bars indicate SEM.

MATERIALS AND METHODS

Mice and allo-BMT. Animal protocols were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee. NOD2−/− mice (Kobayashi et al., 2005) were backcrossed to a B6 background (\( n = 11 \)). BMT experiments and bioluminescent imaging was performed as described previously (Terwey et al., 2005; Zakrzewski et al., 2006, 2008).

Figure 5. NOD2 deficiency in the hematopoietic system regulates experimental colitis. (A) TNBS colitis (5 mg TNBS in 50% ethanol) was induced in B6 WT or B6 NOD2−/− mice. Higher weight loss of NOD2−/− mice during colitis. Combined data from three independent experiments are shown; \( n = 12 \) /group. (B–D) First, chimeric mice with NOD2 deficiency either in the hematopoietic system or in the nonhematopoietic system were created by syngeneic BMT (B6 WT→B6 NOD2−/− or B6 NOD2−/−→B6 WT). After 90 d, TNBS colitis (5 mg TNBS in 50% ethanol) was induced. Colons were harvested at day 3 after induction of colitis. Combined data from two independent experiments are shown; \( n = 8 \) /group. (B) Higher weight loss of NOD2−/−→WT chimera versus WT→NOD2−/− chimera during colitis. (C) Hematoxylin and eosin–stained images of the colon at day 3 after induction of colitis. Bar, 200 µm. (D) Histological scoring showing increased colitis in the hematopoietic NOD2-deficient chimera. Error bars indicate SEM.
Induction and assessment of TNBS colitis. Induction of TNBS colitis was performed as published previously (Scheiffele and Fuss, 2002). Severity of colitis was assessed using a semiquantitative scoring system (Scheiffele and Fuss, 2002).

Statistics. Group-wise comparisons were done with the Mann-Whitney test, one-way ANOVA test, or the Wilcoxon matched pairs test where appropriate. Survival data were analyzed with the Mantel-Cox log-rank test.

Online supplemental material. Fig. S1 shows that donor T cell subsets are not different in NOD2−/− versus WT albo-BM recipients. In Fig. S2, the donor chimerism at day 90 is given. Fig. S3 shows serum levels of cytokines and chemokines during GVHD. Fig. S4 demonstrates that NOD2−/− mice have intact resistance against experimental tumors. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090623/DC1.

We would like to thank Renier Brentjens, Richard Flavell, Ivan J. Fuss, Atsushi Kitani, Kaija Manova, Van Nhiknam, Gabriel Nunez, Eric Pamer, Carles Ubeda-Morant, Joan Zhiqiong Yang, and James Young.

REFERENCES


Supplemental Material

Penack et al., http://www.jem.org/cgi/content/full/jem.20090623/DC1

Figure S1. The percentages of CD4+, CD8+, CD44+/CD62L, IL-17a+/CD4+, IFN-γ+/CD4+, and Tbet+/CD4+ donor T cells are not significantly different in B6 NOD2−/− allo-BMT recipients as compared with B6 WT allo-BMT recipients. Lethally irradiated (11 Gy) B6 WT versus B6 NOD2−/− allo-BMT recipients were transplanted with 5 x 106 B10BR TCD-BM + 106 B10BR T cells. Organs were harvested at day 14 after allo-BMT. Combined data from two independent experiments are shown; n = 10/group. Error bars indicate SEM.
Figure S2. The donor chimerism of hematopoietic cell subsets is >95% in multiple organs at day 90 after syngeneic BMT after lethal irradiation. Lethally irradiated (11 Gy) B6 mice were transplanted with $5 \times 10^6$ B6 Ly5.1 BM. Organs were harvested at day 90 after BMT, digested, and analyzed by flow cytometry. Cells of donor origin were defined by their Ly5.1 positivity. The percentage of B6 Ly5.1$^+$ cells in hematopoietic cell subsets in multiple organs is shown; $n = 5$/group. PLN, pelvic LN. Error bars indicate SEM.

Figure S3. The serum levels of multiple cytokines and chemokines during GVHD are not significantly different in B6 NOD2$^{-/-}$ allo-BMT recipients as compared with B6 WT allo-BMT recipients. Lethally irradiated (11 Gy) B6 WT versus B6 NOD2$^{-/-}$ allo-BMT recipients were transplanted with $5 \times 10^6$ B10BR TCD-BM + $2 \times 10^6$ B10BR T cells. Serum was obtained by eye bleeding at day 7 after allo-BMT. Combined data from two experiments are shown; $n = 10$/group. Error bars indicate SEM.
Figure S4. **NOD2<sup>−/−</sup> mice have intact resistance against experimental tumors.** B6 WT and B6 NOD2<sup>−/−</sup> mice were challenged intravenously with $2 \times 10^5$ C1498 AML cells or $10^5$ B16 melanoma cells. $n = 10$ per group; one out of two independent experiments for each tumor is shown. Bioluminescence imaging shows that early tumor growth is slightly reduced in B6 NOD2<sup>−/−</sup> mice as compared with B6 WT mice.