T cell inhibitory mechanisms in a model of aggressive Non-Hodgkin’s Lymphoma

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**ABSTRACT**

A reduced immune surveillance due to immune deficiency or treatment with immunosuppressive drugs is associated with a higher risk to develop aggressive Non-Hodgkin’s lymphoma (NHL). Nevertheless, NHL also develops in immunocompetent patients indicating an escape from the immune system. T cell function in advanced aggressive lymphoma is not well characterized and the molecular mechanisms how malignant B cells influence T cell function are ill-defined. We therefore studied T cell function in Eμ-myc transgenic mice that develop an aggressive B cell lymphoma with some similarities to human Burkitt-lymphoma (BL). In advanced lymphoma, the number of T cells was severely reduced and the remaining CD4+ and CD8+ T cells lost the capacity to produce effector cytokines and expand upon re-stimulation. T cells in lymphoma-bearing mice were characterized by the expression of the immune inhibitory molecules programmed death (PD)-1, 2B4 and lymphocyte activation protein (LAG)-3. The proto-oncogene c-Myc not only drives cell proliferation and disease progression but also induces apoptosis of the malignant cells. We found that apoptotic lymphoma cells release purine metabolites that inhibit T cell function. Taken together, our data document that the characteristic high cell turnover and apoptotic rate in aggressive NHL induce a severe T cell dysfunction mediated by several immune-inhibitory mechanisms including ligation of inhibitory ligands and purine metabolites. Blocking a single mechanism only partially restored T cell function and did not increase survival of lymphoma mice.

**Introduction**

Malignant transformation of mature B cells and the development of Non-Hodgkin’s lymphoma (NHL) require genetic alterations of oncogenic drivers. During an immune response, germinal center (GC) B cells are at risk of acquiring mutations in important oncoenes during the process of hyper-mutation and affinity maturation. Nevertheless, NHL is a rare disease. This may be due to an efficient immune control of malignant B cells at early stages. Indeed, the frequency of high-grade NHL is substantially increased in immunodeficient patients. Close to 100% of post-transplant NHL are associated with Epstein-Barr virus (EBV) infection. In contrast, only 30–60% of NHL in patients with congenital immunodeficiency syndromes are EBV-associated. EBV-derived antigen latent membrane protein 1 (LMP1) expressed in B cells is a strong lymphoma-associated antigen that activates specific cytotoxic T cells. However, immune surveillance of NHL is not limited to EBV antigens. Recently, Afshar-Sterle et al. demonstrated that T cells prevent spontaneous lymphoma development by Fas ligand-mediated cytotoxicity. Additional effector mechanisms such as perforin-, granzyme- and cytokine-mediated cytotoxicity contribute to the immune control of lymphoma cells. Besides T cells, NK cells are important for the immune control of B cell lymphomas. The fact that high-grade lymphomas develop in immunocompetent patients implies that malignant B cells escape immune surveillance. Documented immune escape mechanisms in NHL include downregulation of major histocompatibility class I and II (MHC I and II) and costimulatory molecules that render lymphoma cells unrecognizable for effector T cells. Alternatively, the malignant B cells escape immune control by impairing T cell function, a process that is well established in solid tumors. In contrast, the mechanisms that lead to T cell dysfunction in advanced lymphoma are poorly defined.

In this study, we used Eμ-myc transgenic mice that constitutively express the Myc oncogene under the control of the potent Igh enhancer (Eμ). These mice develop aggressive B cell lymphoma with some similarities to human Burkitt-lymphoma (BL), i.e. lymphoblastic morphology and rapidly progressive lymphadenopathies. However, human BL typically arise from germinal center (GC) B cells with somatically mutated Ig variable regions whereas MYC-driven lymphomas in Eμ-myc transgenic mice typically arise from pre-B or naïve B cells.
Mice with advanced lymphoma had severely reduced T cell numbers and an impaired T cell function. T cells expressed the co-inhibitory molecules programmed death 1 (PD-1), 2B4 and lymphocyte-activation gene 3 (LAG-3). Blocking the programmed death ligand 1 (PD-L1)/PD-1 interaction in vitro partially restored T cell expansion but did not improve immune control of the lymphoma in vivo. Furthermore, malignant B cells undergoing apoptosis released metabolites that additionally induced T cell dysfunction. An unbiased screen of molecules secreted by malignant B cells by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy revealed lactic acid and purine metabolites as additional components inducing T cell dysfunction. Blocking PD-1 signaling and blocking the effect of ATP and its degradation improved T cell function in vitro and partially in vivo. However, neither blocking PD-1 signaling nor ATP-degradation prolonged survival of lymphoma-bearing Eμ-myc mice. Our experiments indicate that in aggressive lymphoma, purge degradation products released by apoptotic lymphoma cells induce a severe T cell dysfunction. Thus, blocking one single immune-inhibitory pathway may not be sufficient to improve the outcome of aggressive lymphomas which are characterized by high cell turnover and apoptosis.

**Results**

**T cells in lymphoma-bearing mice are reduced in numbers and express T cell inhibitory molecules**

First, we characterized the phenotype and function of T cells in the Eμ-myc lymphoma model. Eμ-myc mice developed a c-myc-oncogene-driven aggressive B cell lymphoma with enlarged lymph nodes (LN) and splenomegaly between 6 weeks and 12 months after birth (Fig. 1A, B). Malignant B cells were highly abundant in lymphoid organs whereas CD4+ and CD8+ T cells were significantly reduced in numbers in lymphoma-bearing Eμ-Myc (Eμ-myc+L) mice when compared with naïve BL/6 or non-lymphoma-bearing Eμ-Myc mice (Eμ-myc-L; Fig. 1A, B). Phenotypically, CD4+ and CD8+ T cells were characterized by increased expression of the T cell inhibitory molecules PD-1, 2B4 and LAG-3 in blood, spleen and LN (Fig. 1C, D and data not shown). In Eμ-myc+L mice approximately 40% of all CD8+ T cells and 29% of all CD4+ T cells were significantly reduced in numbers in lymphoma-bearing Eμ-Myc (Eμ-myc+L) mice when compared with naïve BL/6 or non-lymphoma-bearing Eμ-Myc mice (Eμ-myc-L; Fig. 1A, B). To this end, we crossed Eμ-myc mice to lymphocytic choriomeningitis virus (LCMV) gp33 TCR transgenic p14 mice (Eμ-myc x p14). In Eμ-myc x p14 mice, T cells are of known non-lymphoma antigen specificity. Nevertheless, up to 16% of p14 (Vα2+Vβ8.1+CD45.1+) CTLs from lymphoma-bearing Eμ-myc x p14 (Eμ-myc x p14+L) mice expressed one or more inhibitory receptors (Fig. 1E). However, the expression of inhibitory receptors on p14 T cells (Fig. 1E) was substantially lower than on total CD8+ T cells in Eμ-myc mice (Fig. 1D), indicating that the expression of inhibitory receptors is driven by T cell receptor-dependent and independent mechanisms. We next analyzed whether the expression of T cell inhibitory receptors correlates with lymphoma load. Indeed, the expression of PD-1, 2B4 and LAG-3 on CD8+ and on CD4+ T cells significantly correlated with the number of B cells in spleen (Fig. 1F). The expression of T cell inhibitory markers has been associated with T cell dysfunction, exhaustion and cell death. In accordance with these studies, [3H]-TdR uptake of CD8+ (Fig. 1G) and CD4+ T cells (Fig. 1H) isolated from spleen of Eμ-myc+L mice was significantly reduced. In addition, a higher fraction of CD8+ and CD4+ T cells in spleens of Eμ-myc+L mice underwent apoptosis (Fig. 1, J). Therefore, CD4+ and CD8+ T cells in BL-like disease undergo apoptosis and are reduced in numbers whereas the remaining T cells are dysfunctional.

**CTLs are functionally impaired in lymphoma-bearing mice in vivo**

To investigate whether CTLs in Eμ-myc+L mice can be activated by a physiologic stimulus in vivo, we immunized Eμ-myc+L mice with mature dendritic cells (DCs) derived from H8 mice ubiquitously expressing the MHC-class I restricted peptide gp33 from LCMV (H8 DCs). Eight days after primary immunisation, the frequencies and the absolute numbers of interferon gamma (IFN-γ) and tumor necrosis factor α (TNF-α)-producing CD8+ T cells were substantially lower in lymphoma-bearing mice than in controls (Fig. 2A-G, S2A). Activated CD8+ T cells produced lower levels of IFN-γ and TNF-α per cell when compared with controls as indicated by a lower MFI Index (Fig. 2D, G). The lytic function of gp33-specific CD8+ T cells in vivo was analyzed by transferring gp33-loaded CFSElo and unpulsed CFSEhi labeled B cells into Eμ-myc+L mice and BL/6 controls 8 d after immunisation with H8 DCs. Antigen-specific killing was completely absent in Eμ-myc+L mice whereas BL/6 mice eliminated 35% and 50% of antigen-pulsed B cells 1 and 4 h after transfer, respectively (Fig. 2H). LCMV infection in mice leads to a very strong inflammatory stimulus and to a massive expansion of antigen-specific CTLs. To therefore asked whether LCMV infection could overcome the T cell dysfunction observed in Eμ-myc+L mice. Upon LCMV infection the number of IFN-γ and TNF-α single positive and IFN-γ/TNF-α double positive antigen specific CD8+ T cells was significantly lower in Eμ-myc+L mice than in controls (Fig. 2I-L, S2B). In addition, activated CD8+ T cells from Eμ-myc+L mice produced less IFN-γ and TNF-α per cell (Fig. 2L). This resulted in approximately 1000–10000 fold higher virus titers in livers and spleens of Eμ-myc+L mice 8 d post infection (Fig. 2M). Similarly, the LCMV gp13-specific CD4+ T cell function was impaired in Eμ-myc+L mice with reduced numbers of IFN-γ and TNF-α single and double positive CD4+ T cells (Fig. 2N, O, S2C). Again, each activated CD4+ T cell produced less cytokine in Eμ-myc+L mice than in controls (Fig. 2P). These experiments document a severe T cell dysfunction in lymphoma-bearing mice.
CTL dysfunction in B cell lymphoma-bearing mice is reversible after transfer into naive BL/6 mice

Lymphoma cells also infiltrated primary lymphoid organs such as bone marrow and thymus (data not shown) and thereby may affect T cell development. To analyze the effect of the B cell lymphoma on naïve CD8+ T cells that developed in the absence of lymphoma, we transferred naïve p14 CTLs to Em-myc-L+ mice and BL/6 controls. p14 CTLs were activated with either H8 DCs (Fig. 3A-D; S3A) or with 200 pfu LCMV-

Figure 1. (For figure legend, see page 4).
WE i.v. (Fig. 3E-H; S3B). Significantly fewer p14 CTLs produced IFN-γ and TNF-α in lymphoma-bearing mice (Fig. 3A-C, E-G, S3A, B). Similarly, the IFN-γ and TNF-α production per cell was reduced in Eμ-myc-L mice as indicated by MFI index (Fig. 3D, H).

Next we investigated whether the dysfunction of T cells from Eμ-myc-L was restored in a healthy microenvironment. CTLs from Eμ-myc x p14-L mice incorporated significantly less [3H]-TdR than CTLs isolated from Eμ-myc x p14-L mice (Fig. 3I). Isolated p14 T cells were injected into naïve BL/6 mice and activated with LCMV-WE. p14 CTLs originating from Eμ-myc x p14-L and -L mice expanded similarly (Fig. 3J). In addition, the frequency and absolute numbers of IFN-γ and TNF-α producing p14 CTLs in the spleen were comparable (Fig. 3K-M).

These results indicate that BL-like lymphoma induces a severe CD8+ T cell dysfunction that is reversible in a healthy host environment.

**Blocking PD-1 signaling partially restores T cell function**

Interfering with the PD-L1/PD-1 interaction restores T cell function in different cancer entities and prolongs survival of patients with melanoma, non-small-cell lung cancer and others. PD-L1 was expressed on CD19+ B cells and on CD11b+ and CD11c+ cells in the spleen of BL/6 and Eμ-myc+L mice (Fig. 4A). Co-culture of naïve CD8+ T cells with B cells from Eμ-myc+L mice inhibited [3H]-TdR uptake in a dose-dependent manner (Fig. 4B). A similar trend was observed in co-culture experiments with B cells from naïve BL/6 mice, but to a much lesser extent (Fig. 4B). Blocking PD-1 by mAb did not reduce the inhibitory effect of malignant CD19+ B cells on PD-1 expressing CD8+ T cells isolated from lymphoma-bearing mice (Fig. 4C). In contrast, blocking PD-1 signaling by mAb increased [3H]-TdR uptake of CD8+ T cells in co-culture experiments with antigen-presenting CD11b+ / CD11c+ APCs from Eμ-myc+L mice (Fig. 4D). To analyze the effect of PD-1 signaling on the immune control of BL-like disease in vivo, we transferred MACS-sorted CD19+ B cells from Eμ-myc+L into naïve BL/6 mice followed by treatment with an αPD-1 mAb or control IgG. Blocking PD-1/PD-L1 axis resulted in significantly higher frequencies of CD4+ and CD8+ T cells and a similar trend in absolute T cell numbers in spleen (Fig. 4E, F). In addition, the function of T cells was improved after treatment with αPD-1 mAb as documented by an enhanced expansion of CD8+ T cells ex vivo and higher cytokine production especially of CD4+ T cells after re-stimulation in vitro (Fig. 4G-K, S4A).

Interestingly, αPD-1 treatment increased the expression of all analyzed inhibitory receptors on CD8+ T cells and of 2B4 on CD4+ T cells (Fig. S4B-D), indicating partial redundancy and compensation of T cell inhibitory mechanisms. Despite the fact that αPD-1 treatment partially restored T cell function and numbers, this did not result in a reduction of the lymphoma load as indicated by similar B cell numbers in spleen (Fig. 4L). In addition, PD-1 blockade by mAb treatment (Fig. 4M) had only a minor, non-significant, effect on the survival of lymphoma mice (p = 0.1094). Furthermore, we transferred MACS-sorted CD19+ B cells from Eμ-myc+L into naïve PD-1-/- and BL/6 mice and monitored lymphoma development and survival. Genetic ablation of PD-1 signaling on immune cells did not improve survival of lymphoma mice (Fig. 4N).

Therefore, although T cells in lymphoma bearing animals express high levels of PD-1, blocking PD-1 signaling did only partially restore T cell function, mainly of CD4+ T cells, but did not significantly improve the immune control of BL in our model.

**CD8+ T cell dysfunction in B cell lymphoma is induced by soluble factors < 1kD**

To characterize additional immunosuppressive mechanisms we first analyzed whether cell-cell interactions or soluble factors are responsible. Therefore, we performed a trans-well assay using CD8+ T cell responders and B cells from BL/6 or Eμ-myc+L mice. The results revealed a comparable dose-dependent inhibition of T cell proliferation in a trans-well assay (Fig. 5A) as observed during direct cell-cell interaction (Fig. 4B). In line with this, the addition of sera from Eμ-myc+L mice to CD8+ T cells reduced their proliferation in a dose-dependent manner (Fig. 5B). Similarly, B cell-conditioned medium (BCM) from Eμ-myc+L mice inhibited the expansion of naïve CD8+ T cells (Fig. 5C, D). BCM from Eμ-myc+L mice even suppressed the proliferation of human CD8+ T cells from healthy donors, indicating that a conserved molecule mediates T cell inhibition (Fig. 5C). Proteins as possible mediators of T cell dysfunction could be excluded as neither heat inactivation (Fig. 5E) nor Proteinase K treatment (Fig. S5A, B) of Eμ-myc+L BCM prevented inhibition of CD8+ T cell expansion. Filter size exclusion according to molecular size revealed that only the fraction containing molecules <3kDa impaired CD8+ T cell proliferation (Fig. 5F). Similarly, the
Figure 2. Impaired CD8\(^+\) T cell function in lymphoma-bearing mice after immunization with dendritic cells (DCs) or LCMV. (A-H) BL/6, Eµ-myc-L and Eµ-myc+L mice were immunized day 0 and day 2 i.v. with \(2 \times 10^5\) H8 DCs and CD8\(^+\) T cells were analyzed in spleen 8 d after primary immunization. (A, B, E) Frequencies, (C, F) absolute numbers and (D, G) MFI index of cytokine expressing CD8\(^+\) T cells from BL/6, Eµ-myc-L and Eµ-myc+L mice after 5 h of in vitro re-stimulation with LCMV gp-33 are shown (n = 5 mice/group). (H) \(3 \times 10^7\) gp33-pulsed CFSE\(^{lo}\) labeled B cells and unpulsed CFSE\(^{hi}\) labeled B cells were injected into H8 DC-immunized BL/6 and Eµ-myc+L mice (n = 3–6 mice/group). Naive BL/6 and Eµ-myc-L mice served as controls (n = 2 mouse/group). In vivo cytolytic activity of CD8\(^+\) T cells was determined by measuring antigen-specific elimination of gp33-pulsed B cells 1 and 4 h after transfer by flow-cytometry. (I-P) BL/6 and Eµ-myc+L mice were infected with 200 pfu LCMV-WE i.v. CD8\(^+\) and CD4\(^+\) T cells were analyzed in the spleen 8 d after infection. (I, J) Frequencies, (K) absolute numbers and (L) MFI indexes of IFN-\(\gamma\) and TNF-\(\alpha\) positive CD8\(^+\) T cells after re-stimulation with LCMV gp13 in vitro (n = 4 mice/group). LCMV titres in livers and spleens (n = 4 mice/group). Dotted line indicated detection limit of the assay. (N) Frequencies, (O) absolute numbers and (P) MFI indexes of IFN-\(\gamma\) and TNF-\(\alpha\) positive CD4\(^+\) T cells after re-stimulation with LCMV gp33 in vitro (n = 4 mice/group). For (A) and (I), one representative dot plot out of 5 is depicted and frequencies are displayed as Mean ± SEM (n = 5 mice/group). Expression values for D, G, L, and (P) are displayed as MFI Index (= intensity of expression in cytokine positive / cytokine negative cells). Data are displayed as mean ± SEM. Statistics: (B-G) One-way ANOVA, (H, J-P) Student’s t test. \(p < 0.05\), \(** p < 0.01\), *** \(p < 0.0001\).
Figure 3. Dysfunctional p14 CTLs from lymphoma-bearing mice regain effector function after adoptive transfer into healthy recipient mice. (A-D) $1 \times 10^6$ congenic CD45.1$^+$ p14 T cells were adoptively transferred into lymphoma-bearing Eμ-myc or BL/6 mice (n = 5–8 mice/group). Mice were immunized twice with $2 \times 10^5$ HB-DCs (day 0 and day 2). (A, B) Frequency, (C) total numbers and (D) MFI index of IFN-γ- and TNF-α-producing p14 CTLs in the spleen 6 d after immunisation. (E-H) $1 \times 10^6$ p14 CTLs were adoptively transferred into Eμ-myc x L or BL/6 mice (n = 9 mice/group). 18 h later p14 CTLs were activated with $1 \times 10^4$ pfu LCMV. (E, F) Frequency, (G) total number and (H) MFI index of IFN-γ and TNF-α-producing p14 CTLs in the spleen after re-stimulation in vitro with gp33. One representative dot plot out of 4–5 is depicted. (I) MACS-purified p14 CTLs from Eμ-myc x p14-L and +/L mice were isolated and re-stimulated in vitro with gp33-pulsed irradiated splenocytes for 72 h and $[^{3}H]$-TdR incorporation of p14 CTLs was measured. (J-M) $1 \times 10^6$ MACS-purified p14 CTLs from Eμ-myc x p14-L and +/L mice were adoptively transferred i.v. in naive BL/6 recipients and activated 18 h later with $1 \times 10^3$ pfu LCMV i.v. (I) Total number, (J) frequencies (L) absolute numbers and (M) MFI index of IFN-γ and TNF-α producing p14 CTLs after re-stimulation with gp33 in vitro were assessed in the spleen 6 d after LCMV infection (n = 9 mice/group). For A, E and K one representative dot plot out of 5 or 9 is depicted and frequencies are displayed as mean ± SEM (n = 5; A); n = 9 (E, K) mice/group). Expression values for D, (H) and, M are displayed as MFI index (intensity of expression in cytokine positive / cytokine negative cells). Data are displayed as mean ± SEM. Statistics: Student’s t test. "p < 0.05, ""p < 0.01, """"p < 0.0001.
fraction < 3kDa from BL/6 BCM inhibited CD8⁺ T cell expansion, however, to a significantly lesser extent (Fig. 5F). A further filtration step revealed that the inhibitory molecule was of a molecular size <1kDa (Fig. 5G).

The Eμ-myc+L BCM fraction <3kDa increased apoptosis of naive CD8⁺ T cells in vitro as analyzed by Annexin-V staining (Fig. 5H). In contrast, BCM fractions >3kDa did not induce apoptosis. In addition, the Eμ-myc+L fractions <3kDa,
Purine degradation products induce CD8$^+$ T cell dysfunction in B cell lymphoma

To characterize the molecule(s) inducing CD8$^+$ T cell dysfunction in lymphoma-bearing mice, the BCM fraction <1kDa from Eμ–myc-L and BL/6 mice was analyzed by Mass Spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Fig. 6A). The MS analysis identified 32 different masses that were present at significantly higher concentration in Eμ–myc-L BCM compared with BL/6 BCM including 9 metabolites of documented inhibitory effects on T cells (Fig. 6 A, Table 1). NMR analysis identified higher concentrations of hypoxanthine (Fig. 6B, C), a member of the purine metabolism, and lactic acid in Eμ–myc-L BCM (Fig. S6A). Lipids including sphingosine-1-phosphate (SIP), lactic acid and metabolites of the purine pathway have been shown to inhibit T cell function.26-30 We therefore analyzed if one or several of these molecules are responsible for the T cell dysfunction induced by malignant B cells.

Lactic acid levels were elevated in Eμ–myc-L BCM by NMR (Fig. S6A) as well as by a multiple biochemical analyzer (Fig. S6B). Indeed, lactic acid inhibited T cell proliferation in a concentration-dependent manner (Fig. S6C). Importantly, the concentration of lactic acid necessary to reduce T cell expansion was substantially higher (6.7–20 mM) than the concentrations found in BCM form Eμ–myc-L mice (0.87 mM). Inhibition of lactic acid by the lactic acid dehydrogenase (LDH) inhibitor Sodium Oxamate (S.O.) in Eμ–myc-L BCM did not restore T cell proliferation (Fig. S6D). This suggests that this single pathway does not substantially contribute to the observed T cell dysfunction in our model.

Titrated amounts of SIP did not inhibit CD8$^+$ T cell proliferation in vitro (Fig. S7A). In addition, depletion of SIP in Eμ–myc-L BCM did not reduce the inhibitory effect on CD8$^+$ T cell proliferation (Fig. S7B). Similarly, the polar lipid containing fractions of Eμ–myc-L BCM did not inhibit T cell proliferation (Fig. S7C). These experiments excluded polar lipids such as SIP, psychosine and D-glucosyl-sphingosine as mediators of CD8$^+$ T cell inhibition in our model.

Increased production of purine metabolites and elevated serum levels of uric acid are a hallmark of rapidly proliferating lymphoma cells and frequently detected in patients with aggressive NHL.31,32 We confirmed increased levels of ATP and uric acid in the sera of Eμ–myc-L mice (Fig. 6D, E). Moreover, we found substantially higher ATP concentrations in lyses and in supernatants of Eμ–myc-L B cells than of normal BL/6 B cells (Fig. 6F and data not shown). All degradation products of ATP analyzed such as adenosine, adenine, hypoxanthine, xanthine and uric acid were present at higher concentrations in Eμ–myc-L BCM than in BL/6 BCM (Fig. 6F). Next, we analyzed which of these molecules potentially inhibit CD8$^+$ T cell proliferation in vitro. We found that ATP, ADP, AMP, adenosine, adenine and uric acid had an inhibitory effect on T cell expansion (Fig. 6F). The role of adenosine was also tested with its stable functional analog 2-Chloro-Adenosine (2-CADO) (Fig. 6F). No effect on T cell proliferation was found for hypoxanthine, xanthine and uridine (Fig. 6F). Moreover, the combination of ATP, 5′ADP, and AMP suppressed T cell function significantly stronger than each of these purine metabolites alone (Fig. 6G).

These data indicate that adenosine containing purines are elevated in sera and BCM of Eμ–myc-L mice and that ATP degradation products are potent suppressors of CD8$^+$ T cell proliferation in vitro.

Blocking apoptosis or purine receptors partially restores T cell function in B cell lymphoma

It is well documented that purines are released by apoptotic cells.33,34 In addition, an increased cell proliferation accompanied by an increased apoptosis rate is a hallmark of BL.35 We therefore asked if blocking apoptosis of Eμ–myc-L B cells reduced the inhibitory effect on T cells. Treatment of Eμ–myc-L B cells with the pan-caspase inhibitor QVD significantly reduced the concentration of ATP and of its degradation products (hypoxanthine, xanthine and thymidine) in the BCM (Fig 7A). Indeed, incubation of Eμ–myc-L B cells with QVD restored $^{3}$H–TdR uptake of naive CD8$^+$ T cells (Fig. 7B). Similarly, degradation of ATP by the enzyme apyrase partially reduced the T cell suppressive effect of Eμ–myc-L BCM (Fig. 7C). The ectonucleotidases CD39 and CD73 act as extracellular degradation enzymes of purines and have increased

Figure 4. (see previous page) Blockade of PD-1 signaling partially restores T cell function. (A) PD-L1 expression was analyzed on CD19$^+$, CD11b$^+$ or CD11c$^+$ cells on splenocytes from BL/6 or Eμ–myc-L mice by flow-cytometry. One representative histogram out of 3 is shown. (Black: staining, gray: isotype; n = 3 mice/group) (B) MACS-purified CD8$^+$ T cells from naive BL/6 mice (respondors) were activated with plate-bound αCD3 mAb (2 μg/ml) in the presence of titrated numbers of MACS-purified CD19$^+$ B cells from naive BL/6 or Eμ–myc-L mice for 72 h in triplicate cultures. $^{3}$H–TdR was added to the culture for the last 18 h of incubation. 1 representative out of 12 experiments is shown. (C) CD8$^+$ T cells were cultured with titrated amounts of MACS-purified CD19$^+$ B cells from Eμ–myc-L in the absence or presence of a blocking αPD-1 mAb (10 μg/ml) or control IgG and proliferation was measured as described in (B). Naive BL/6 CD8$^+$ T cells were cultured in the presence or absence of a αPD-1 or isotype control mAb served as controls. (D) Titrated numbers of MACS purified, irradiated (1000 rad) CD11b$^+$ and CD11c$^+$ APCs were cultured with CD8$^+$ T cell responders (as described in (B) in the presence or absence (hatched bars) of an αPD-1 mAb (10 μg/ml) or control IgG and $^{3}$H–Tdr incorporation was measured. (E-M) 3 × 10$^6$ MACS-purified CD19$^+$ B cells from Eμ–myc-L mice were transplanted into BL/6 recipients and mice were treated with 200 μg αPD-1 mAb (blue bars) or control IgG (yellow bars) i.p. every third day. Day 17 after treatment, splenocytes were isolated and (E) frequencies and (F) absolute numbers of CD4$^+$ and CD8$^+$ T cells were analyzed using flow-cytometry. (G) MACS purified CD8$^+$ T cells were activated with plate-bound αCD3 mAb (2 μg/ml) and $^{3}$H–Tdr incorporation was measured. (H-K) MACS purified CD4$^+$ and CD8$^+$ T cells were stimulated with PMA/ionomycin for 5 h in vitro. Then, IFN-γ and TNF-α production was measured by flow-cytometry. (H, I) Total numbers and (J, K) MFI index of IFN-γ and TNF-α-producing T cells is shown. (L) Absolute CD19$^+$ B cell numbers (n = 3–5 mice/group). (M) Kaplan-Meier curve (n = 5 mice/group). (N) 3 × 10$^6$ MACS-purified CD19$^+$ B cells from Eμ–myc-L mice were transplanted into BL/6 or PD-1$^+$ recipients and survival was monitored. Kaplan-Meier curve (n = 5 mice/group). Expression values for L, K are displayed as MFI index ( = intensity of expression in cytokine positive/cytokine negative cells). Data are displayed as mean ± SEM. Statistics: (B-L) Student’s t-test; (M, N) log-rank test. ”p < 0.05, “p < 0.01, “”p < 0.001. See also Supplementary Figure 5.
Figure 5. Lymphoma B cells induce mouse and human CD8\(^+\) T cell dysfunction by secreting a soluble compound smaller than 1kDa. (A) MACS-purified CD8\(^+\) T cells from naive BL/6 mice (responders) were activated with plate-bound αCD3 mAb (2 \(\mu g/ml\)) in the presence of titrated numbers of MACS-purified CD19\(^+\) B cells from naive BL/6 or Eμ-myc\(-\)BL cells for 72 h in transwell tissue culture plates in triplicate cultures and \[^{3}H\]-TdR incorporation was determined after 72 h of culture. One representative out of 3 experiments is shown. (B) BL/6 CD8\(^+\) T cells were stimulated with αCD3 mAb as described in (A) in the presence and absence of titrated amounts of serum from Eμ-myc\(-\)BL mice (n = 2 different sera) and \[^{3}H\]-TdR incorporation was determined after 72 h of culture. (C) Mouse and human naive CD8\(^+\) T cells were stimulated with αCD3 mAb as described in (A) or OKT-3 (2 \(\mu g/ml\)) respectively, in the presence and absence of titrated amounts of B cell conditioned medium (BCM), derived from BL/6 or Eμ-myc\(-\)BL B cell cultures. \[^{3}H\]-TdR incorporation was determined after 72 h of culture. One representative out of 3 experiments is shown. (D) CD8\(^+\) T cells were labeled with CFSE and CFSE dilution was measured after stimulation of T cells with αCD3 mAb in the presence and absence of indicated BCMs at a dilution of 1:3 for 72 h. One representative out of 2 experiments is shown. (E-G) CD8\(^+\) T cells were cultured as described in (A) in the presence and absence of titrated amounts of (E) untouched or heat-inactivated BCM and (F, G) size fractionated BCM from BL/6 or Eμ-myc\(-\)BL B cell cultures. \[^{3}H\]-TdR incorporation was determined after 72 h of culture. One representative out of 6 experiments is shown. (H) Naive BL/6 cells were cultured in the presence or absence of size fractionated BCM together with αCD3 mAb and viability was determined by Annexin-V staining after 24 h of culture. Apoptosis rate of CD8\(^+\) T cells in medium was 41% (data not shown). 1 representative out of 2 experiments is shown. (I) Naive BL/6 cells were cultured in the presence or absence of size fractionated BCM and αCD3 mAb for 72 h. Then, IFN-\(\gamma\) and TNF-\(\alpha\) production was measured by flow-cytometry after additional 5 h of in vitro stimulation with PMA/ ionomycin. In medium, TNF-\(\alpha\) production of CD8\(^+\) T cells was 29%, IFN-\(\gamma\) production was 18% (data not shown). Data are displayed as mean ± SEM. Statistics: Student’s t test: *p < 0.05, **p < 0.01, ***p < 0.0001. See also Supplementary Figure S5.
Figure 6. Lymphoma B cells produce and secrete purines that inhibit T cell function. (A) <1kDa fractions of BCM from BL/6 or Eμ-myc+L mice (n = 4 mice/group) were analyzed for metabolites using mass spectrometry. Results are indicated as S-Plot correlation of equal masses, detected in BL/6 (−1) or Eμ-myc+L (+1) mice. Masses with a very high (Eμ-myc+L (+1)) or very low (BL/6 (−1)) correlation reflect the highest significance. Masses selected for further screening are displayed in red and depicted in Table 1. (B) NMR analysis. (C) NMR signal after spike-in of Hypoxanthine standard or Eμ-myc+L BCM (n = 4 mice/group). (D, E) Serum concentration of (D) ATP and (E) uric acid (n = 3 mice/group). (F) ATP concentrations in MACS-purified B cell lysates (n = 3 lysates/group). Concentrations of indicated purines in BCM (n = 3–5 BCM/group). Proliferation of purified, naïve CD8+ T cells from BL/6 mice was determined after addition of titrated amounts of different purines. (G) Titrated amounts of ATP, 5’ADP or AMP were added separately or combined (ATP + 5’ADP and ATP + 5’ADP + AMP) to purified, naïve CD8+ T cells from BL/6 mice and [3H]-TdR incorporation was determined. Data are presented as mean value of triplicate measurements and one representative experiment out of 2 independent experiments is shown. Data are displayed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.0001 (Student’s t test); (G) One-way ANOVA. See also Supplementary Figure S5-S7.
activity in cancer.\textsuperscript{26,36,37} In line with this, we found an approximately 100-fold higher expression of CD39 on CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells of E\textsubscript{mu-myc}+L mice when compared with controls, but only a slight difference in CD73 expression (Fig. 7D). To further confirm the role of ATP degradation products in T cell dysfunction, we used naive CD8\textsuperscript{+} T cells from CD39\textsuperscript{-/-} (Fig. 7E) or A\textsubscript{2a}/-/- mice (Fig. 7F) in a \textsuperscript{3}H-TdR incorporation assay in the presence of BL/6 or E\textsubscript{mu-myc}+L BCM. BL/6 BCM and E\textsubscript{mu-myc}+L BCM inhibited the expansion of CD39\textsuperscript{-/-} or A\textsubscript{2a}/-/- T cells to a lesser extent than the expansion of BL/6 T cells (Fig. 7E, F).

In CD39\textsuperscript{-/-} mice, extracellular ATP cannot be converted into ADP or AMP leading to approximately 1000-fold higher concentrations of ATP and reduced levels of hypoxanthine, xanthine and inosine in serum when compared with BL/6 mice (Fig. S8A, B). To investigate T cell function and lymphoma development in the absence of ATP degradation products, we crossed E\textsubscript{mu-myc} mice with CD39\textsuperscript{-/-} mice. Spleen weights and absolute numbers of splenocytes of E\textsubscript{mu-myc}+L mice were significantly lower when compared with E\textsubscript{mu-myc}+L controls (Fig. 7G, H). CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell to B cell ratios were significantly increased in E\textsubscript{mu-myc}+L mice compared to E\textsubscript{mu-myc} x CD39\textsuperscript{-/-}+L mice (Fig. 7I). Moreover, \textsuperscript{3}H-TdR incorporation of ex vivo isolated T cells from E\textsubscript{mu-myc} x CD39\textsuperscript{-/-}+L mice was significantly higher than from E\textsubscript{mu-myc} x CD39\textsuperscript{-/-}+L mice (Fig. 7J, K).

However, absence of ATP degradation products in E\textsubscript{mu-myc} x CD39\textsuperscript{-/-}+L mice did not improve survival of lymphoma mice when compared E\textsubscript{mu-myc} x CD39\textsuperscript{-/-}+L controls (Fig. 7L). Of note, T cells from E\textsubscript{mu-myc} x CD39\textsuperscript{-/-}+L mice expressed higher levels of PD-1 (Fig. S9A, B), most likely due to pro-inflammatory environment induced by high ATP concentrations.\textsuperscript{38,39}

These results suggest that the T cell dysfunction in NHL is induced by several redundant pathways and that blocking one major immune inhibiting pathway is not sufficient to treat aggressive NHL.

### Discussion

As lymphomas grow in lymphoid organs, it would be expected that they should be efficiently recognized and eliminated by the immune system. Indeed, experimental studies and clinical data clearly document an important role of T, NK and probably NKT cells in the immune control of lymphoma.\textsuperscript{2,3,5-7} Clinically manifest lymphoma in immune-competent hosts must have evaded immune surveillance. One well-documented mechanism of immune escape of lymphoma cells is the loss of antigen-presentation. Approximately 60% of DLBCL have an aberrant expression of β2-microglobulin preventing the presentation of antigenic peptides on MHC class I (HLA class I) to specific CD8\textsuperscript{+} T cells.\textsuperscript{10} Similarly, loss of MHC II protein expression and thereby escape of recognition by CD4\textsuperscript{+} T cells has been documented in B-cell neoplasms and is associated with an aggressive clinical course.\textsuperscript{40-42}

In addition, experiments with lymphoma cells expressing a model antigen indicate that in advanced lymphoma specific T cells are dysfunctional.\textsuperscript{43,44} How malignant B cells induce T cell dysfunction \textit{in vivo} has not been documented so far. A siRNA library screen revealed that inhibitory molecules of the B7 family such as CD200, CD274 (PD-L1) and CD276 (B7-H3) and the TNF-receptor superfamily member CD270 (HVEM) impair the formation of the immunological synapse between T cells and chronic lymphatic leukemia cells \textit{in vitro}.\textsuperscript{45} Similarly, PD-L1 is expressed in DLBCL.\textsuperscript{45} In the present study, we document a high expression of PD-L1 on normal and on malignant B cells and on non-malignant CD11c\textsuperscript{-} and CD11b\textsuperscript{-} myeloid cells. This is comparable to data from solid tumors where the expression of PD-L1 has been documented on cancer cells and on tumor-infiltrating immune cells.\textsuperscript{46} In addition, a large fraction of CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells expressed PD-1. Blocking PD-1 signaling increased the proliferation of CD8\textsuperscript{+} T cells \textit{in vitro} when incubated with CD11c\textsuperscript{-}CD11b\textsuperscript{-} cells isolated from lymphoma-bearing mice. This is in line with and extends a study by Rehm et al.,\textsuperscript{51,52} indicating that a C/EBPβ driven upregulation of immunomodulatory cytokines and growth factors in dendritic cells contributes to lymphoma progression in E\textsubscript{mu-myc} mice.\textsuperscript{47}

Blocking PD-1 signaling by treatment with mAbs is efficacious in solid tumors and in refractory Hodgkin’s lymphoma with alterations in the PD-L1- and PD-L2-encoding chromosome 9p24.1.\textsuperscript{48-50} Furthermore, there is early evidence of efficacy in follicular lymphoma and in high-grade lymphoma after autologous haematopoietic stem cell transplantation.\textsuperscript{51,52} Surprisingly, although blocking PD-1 did increase the frequencies and partially also the function of T cells, it did not significantly prolong survival in our model. This is in agreement with a recent study documenting that PD-1 blockade does not improve the control of Myc-driven lymphoma whereas a α-GaCer vaccine together with a stimulating 4-1BB mAb prolonged survival.\textsuperscript{53} Importantly, 20–25% of total T cells in E\textsubscript{mu-myc}+L mice expressed 2 or 3 immune inhibitory receptors including PD-1, 2B4 and LAG-3 and PD-1 blockade increased the expression of other immune inhibitory receptors.

An unbiased screen of BCM from lymphoma cells using MS and NMR revealed some candidate molecules that we functionally tested \textit{in vitro}. Interestingly, molecules that are associated with an increased cell turn-over and cell death such as lactate and the purine hypoxanthine were detected in high concentrations. Similarly, Johnston H.E. et al. recently documented in a proteomic analysis of plasma from lymphoma-bearing mice a tumor lysis signature.\textsuperscript{54}

**Table 1.** Identification of lipids and members of the purine pathway in BCM from E\textsubscript{mu-myc}+L mice. <1kDa fractions of BCM from E\textsubscript{mu-myc}+L mice or BL/6 mice were analyzed for metabolites using Mass Spectrometry (as described in material and methods). Masses that appeared with highest intensity and significance in E\textsubscript{mu-myc}-L BCM were further verified by fragmentation analysis. Identified masses contained bioactive lipids and members of the purine pathway, listed in Table 1. (n = 4 mice/group).

| Search Mass | Compound Name             |
|------------|---------------------------|
| 179,076    | D-mannosamine             |
| 179,076    | galactosamine             |
| 280,086    | adenosine 5'-carboxamide  |
| 280,086    | linoleate                 |
| 281,037    | 1-methyladenosine         |
| 323,313    | linoleoyl ethanolamide    |
| 379,121    | sphingosine-1-phosphate   |
| 461,092    | psychosine                |
| 461,092    | D-glucosyl-sphingosine    |

**Identiﬁed masses E\textsubscript{mu-myc} : BL/6 BCM (n = 4)**

**Identified masses E\textsubscript{mu-myc} : BL/6 BCM (n = 4)**
**Figure 7.** Metabolites of the purine degradation pathway are released by apoptotic lymphoma B cells and induce CD8+ T cell dysfunction. (A, B) MACS-purified B cells from Eμ-myc-L mice were cultured in the presence or absence of the caspase inhibitor QVD (50 μM) and BCM was harvested after indicated time points. (A) The concentrations of ATP, hypoxanthine, xanthine and thymidine in BCM from Eμ-myc-L mice were determined 1 h (ATP) and 24 h (hypoxanthine, xanthine and thymidine) after QVD or control (DMSO) treatment. (B) BCM was harvested 6 h after incubation and added to CD8+ T cell responders at a 1:1 ratio and 3[H]-Tdr incorporation was measured. 1 representative out of 2 experiments is shown. (C) MACS-purified B cells from Eμ-myc-L mice were cultured in the presence or absence of the ATP degrading enzyme apyrase at different concentrations. BCMs were harvested 24 h after in vitro culture. BCMs were added to BL/6 CD8+ T cell responders at a 1:3 ratio and 3[H]-Tdr incorporation was measured. 1 representative out of 3 experiments is shown. (D) CD4+ and CD8+ T cells from spleens of BL/6 or Eμ-myc-L mice were analyzed for the expression of ATP ectonucleotidases CD39 and CD73 using flow cytometry ex vivo. (n = 2; black: staining, gray: isotype, MFI: MFI staining – MFI isotype). One representative FACS plot out of 4 independent experiments is shown. (E, F) CD8+ T cells responders from naïve BL/6 or (E) CD39−/− (red bars) or (F) A2A−/− (green bars) mice were cultured in the presence or absence of Eμ-myc-L BCM at a 1:3 ratio and 3[H]-Tdr incorporation was measured. Data are presented as mean value of triplicate measurements and one representative experiment out of 2 independent experiments is shown. (G) Spleen weights, (H) total splenocyte numbers and (I) T cell/B cell ratios of Eμ-myc x CD39−/−L (n = 30) and Eμ-myc x CD39−/−L and 3[H]-Tdr incorporation was measured (n = 2 mice/group). (L) Kaplan-Meier survival curve of Eμ-myc x CD39−/−+L (n = 30) and Eμ-myc x CD39−/−+L (n = 4). Survival is depicted as Kaplan-Meier curve (n = 5 mice/group). Data are displayed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.0001 (Student’s t test). See also Supplementary Figure S8 and S9.
In addition, MS and NMR analysis indicated the presence of purines in BCM from lymphoma cells. ATP and its degradation products ADP, AMP, adenosine, adenine, hypoxanthine, xanthine, uric acid and uridine were present at elevated concentrations in lymphoma BCM or serum when compared with BL/6 controls. ATP, ADP, AMP, adenosine, adenine inhibited T cell proliferation when added to the culture in a concentration-dependent manner, whereas hypoxanthine, xanthine, uric acid and uridine had no effect, suggesting that adenosine/adenine-containing purines were the main molecules that inhibit T cell proliferation in our model. In accordance, P2X7 stimulation by high ATP concentrations has been shown to trigger cell death and inhibit T cell functions in different models.38,55,56 However, depending on the concentrations, ATP has opposite effects on immune cells.38,39,56,57 It has been shown that low ATP concentrations have immune-stimulating properties due to activation of the NALP3-inflammasome and caspase-1, resulting in the release of pro-inflammatory cytokines such as IL-1β, IL-18 and IL-33, important inducers of innate and adaptive immune responses.58

Experiments with apyrase that leads to the degradation of ATP to ADP and AMP, with CD39+/− T cells that prevent the degradation of ATP and with A2A+/− T cells indicated that ATP itself and its degradation products reduce T cell proliferation. Moreover, T cells isolated from Eμ−myc x CD39+/− L mice proliferated more efficiently ex vivo than T cells isolated from control lymphoma-bearing mice. These data indicate that although CD39 deficiency results in substantially higher concentrations of ATP, the lack of its degradation products leads to an improved T cell response in lymphoma bearing CD39+/− x Eμ−myc mice. However, although T cell function was partially improved, there was no significant survival difference between CD39-competent or -deficient lymphoma mice. Of note, absence of CD39 resulted in substantially higher concentrations of ATP that by itself mediates T cell suppression.65

Recent studies suggest that only combinatorial blockade of different T cell inhibitory mechanisms enhance anti-tumoral T cell immune responses.59,60 For example, blocking PD-1 leads to increased A2A expression.61 In line with this, Kobayashi et al. identified a successful vaccine approach using the lipid antigen α-GalCer in combination with stimulating α4–1BB against a transplantable B cell clone derived from the Eμ−myc model.62 These successful vaccination approaches,55,62 and the reduced survival of RAG-deficient mice challenged with malignant B cells,63 indicate that T cell immunosurveillance at least partially controls Eμ−myc-driven lymphoma. Nevertheless, the antigenicity of Myc-driven lymphoma might be substantially lower than that of EBV associated lymphoma in humans.64

In summary, our study revealed that in advanced aggressive lymphoma different and partially redundant mechanisms contribute to a severe dysfunction of CD4+ and CD8+ T cells. This includes the expression of inhibitory cell-surface receptors such as PD-1, LAG-3 and 2B4. However, purine degradation products released by cells undergoing apoptosis crucially contribute to the suppression of the T cell response. It is well documented that c-myc activates pathways that induce cell proliferation but also sensitize cells to apoptotic stimuli.65 Blocking apoptosis of lymphoma cells in vitro reduced the T cell inhibitory potential. Our findings are in accordance with a recent study that indicated that the "immunological visibility" and the immunosurveillance of Eμ−myc mice critically depend on the expression of the anti-apoptotic protein Bcl−2.56 Therefore, successful immunotherapy of advanced aggressive NHL may depend on the combined blockade of several immune inhibitory pathways.

Material and methods

Mice

C57 BL/6 J (BL/6) mice were from RCC Ltd. (Füllinsdorf, Switzerland) and Charles River Laboratories (Sulzfeld, Germany). CD45.1+ mice, RAG-1+/− mice, p14 TCR transgenic mice specific for the LCMV glycoprotein epitope 33–41 (gp33) (CD45.1+ x RAG−−/− x Vex2Vβ8.1+) and H8 transgenic mice,23 ubiquitously expressing LCMV-gp33 under the control of a MHC class I promoter were from the Institute for Laboratory Animals (Zürich, Switzerland). Eμ−myc mice were obtained from Prof. Dr. med. Frank Heppner and Dr. Roland Kälin Institute for Neuropathology at Charité University of medicine (Berlin, Germany).20 CD39+/− mice were provided by Prof. Simon C. Robson, Division of Gastroenterology and Hepatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA.66 A2A−/− mice were provided by Karen Dwyer and were bred at St Vincent’s hospital, Melbourne, Victoria, Australia.69 All mice were on a BL/6 background and were sex and age matched before entering the experiment. Animal experiments were approved by the experimental animal committee of the Canton of Bern, Switzerland, and performed according to Swiss laws for animal protection.

Virus

LCMV strain WE was provided by R.M. Zinkernagel (University of Zürich), propagated and titrated as described previously.70 Virus was injected intravenously (i.v.) at a dosage of 2 × 106 or 1 × 106 pfu per mouse. T cell function was analyzed 6–8 d after immunisation.

Reagents

Brefeldin-A (Cat.#B5936), Phorbol-12-myristate-13-acetate (PMA; Cat.#P8139), ATP (Cat.#34369–7–8), 5’ADP (Cat. #20398–34–9), AMP (Cat.# 4578–31–8), adenosine (Cat.# 58–61–7), cAMP, 2-chloro-adenosine (2-CADO; Cat.#sc-300835; Santa Cruz Biotech); LCMV peptides gp33 (amino acid sequence: KAVYNFATM) and gp13 (amino acid sequence: GLKGPDIYKGVYQFKSVEFD) (Cat.#SC1347; NeoMPS SA.); anti-α4 (BioXCell); Q-VD-OPH (QVD) (Cat.#U2875), apyrase (Cat.# A6237), lactic acid (Cat.#O2751), adenosine (Cat. #74–24–2), cAMP, 2-chloro-adenosine (2-CADO; Cat.#sc-300835; ONCOIMMUNOLOGY e1365997-13)
**Antibodies and flow cytometry**

αIFN-γ-PE (Clone: XM1G1.2; Cat.#127311), αTNFα-FITC (Clone: MP6-XT22; Cat.#117321), αCD80-PE (Clone: 16–10A1; Cat.#120801) were purchased from eBioscience. αCD8-FITC, -PE, -PerCP-Cy5.5, -PE-Cy7, -APC-Cy7 and -PacificBlue (Clone: 53–6.7; Cat.#1007XX), αCD4-FITC, -PE, -PerCP-Cy5.5, -PE-Cy7, -APC (Clone: GK1.5; Cat.#1004XX), αCD19-APC-Cy7 (Clone: 6D5; Cat.#115530), αCD45.1-PE, -PerCP-Cy5.5, -PE-Cy7, -APC (Clone: A20; Cat.#1107XX), αPD-1-PE-Cy7 (Clone: RMP1–30; Cat.#109110), αCD223 (LAG3)-PE (Clone: C9B7 W; Cat.#125208), αCD244a-FITC (2B4) (Clone: M2B4; Cat.#133504), αCD39-PE (Clone: Duha59; Cat.#143804), αCD73-AlexaFluor647 (Clone: TY/11.8; Cat.# 127208), αCD11 PE-Cy7 (Clone: N418; Cat.#117318), αMHC-II-PE-Cy7 (Clone: M5/114.15.2; Cat.# 107630), αCD86-APC (Clone: GL1; Cat. #105012), αVo2-PerCP-Cy5.5 (Clone: B20.1; Cat.#127814), αCD62 L-PacificBlue (Clone: MEL-14; Cat.#104423); αCD44-APC-Cy7 (Clone: IM7; Cat.#103028) were from BioLegend. Annexin-V-PE was from Immunotools and αV/β1,8,2-PE (Clone: MR5–2; Cat.#140104) was from BD Pharmingen. All samples were acquired on a BD LSRII (BD Bioscience). Data were analyzed using FlowJo™ software (TreeStar). Mouse αPD-1 mAb (Clone: 2A3; Cat.#BE0146) was from BioXCell and rat IgG2 a (Cat.#I4131) from Sigma.

**Transplantation of lymphoma B cells**

Isolation of CD19+ lymphoma B cells from the spleen of lymphoma-bearing Eμ-Myc mice was performed using MACS™ αCD19 MicroBeads and LS columns (Milteny Biotec). 3 × 10^6 purified lymphoma B cells were injected into BL/6 recipient mice, which developed lymphoma 3 to 4 weeks after transplantation. In some experiments, mice were treated with anti-mouse PD-1 mAb (RMP1–14) or rat IgG2 a (2A3) as isotype control (200 μg; i.p.; every third day).

**Intracellular IFN-γ and TNF-α assay**

1–2 × 10^6 splenocytes were re-stimulated in vitro for 5 h with 1 μM gp33, 1 μM gp13 or with 1 μg/ml Ionomycin and 100 ng/ml PMA, in the presence of 5 μg/ml Brefeldin A and 25 U/ml of recombinant mouse interleukin-2 (rmIL-2). Cells were stained for surface molecules and fixed with 4% paraformaldehyde. Cell membranes were permeabilized with Perm-Buffer (PBS, 2% FCS, 5 mM EDTA, 0.1% saponin, 0.2% NaN3) and stained with αIFN-γ and αTNF-α antibodies. The fluorescence intensity index was calculated by dividing the intensity of expression in cytokine positive cells by that of negative cells.

**H8 DC immunisation protocol**

BM cells from H8 mice (7–10 × 10^7 cells) were cultured in 20 ml of DC medium (RPMI 10%, GM-CSF 10%) in non-tissue-culture-treated petri dishes (VWR) for 10 days, performing half medium changes every 3 d. At day 10 of culture, the non-adherent cells were washed away and resuspended. 1–3 × 10^7 cells were re-plated per tissue-culture-treated Petri dish (Falcon) and 9 ml of RPMI 10%, GM-CSF 5% and 10 μg/ml LPS was added for DC maturation. At day 12 of culture, the maturation status of DCs was determined by flow cytometry. Mice were immunized twice i.v. with 2 × 10^5 H8 DCs at an interval of 2 d.

**Generation of effector CTLs**

1 × 10^6 splenocytes from p14 mice were adoptively transferred intravenously (i.v.) into BL/6 or Eμ-myc recipient mice. 12–18 h after injection cells were activated by l.v. injection of 2 × 10^5 H8 DCs or 1 × 10^6 pfu LCMV-WE. Six days after immunization, spleens were isolated and T cell function of adoptively transferred cells was analyzed. Alternatively, 1 × 10^6 MACS-purified Eμ-myc x p14 T cells were adoptively transferred into BL/6 mice. 18 h after transfer, p14 cells were activated with 1 × 10^5 pfu LCMV-WE. Six days after infection, spleens were isolated and T cell function was analyzed.

**In vivo cytotoxicity assay**

MACS-purified splenic CD19+ lymphoma B cells from malignant Eμ-myc mice were either labeled with 0.1 μM Carboxyfluorescein succinimidyl ester (CFSEhi) or with 5 μM CFSE (CFSElo) for 10 min at 37°C. Cells were then washed and only the CFSElo B cell population was loaded with gp33 peptide (1 μM) for 90 min at 37°C. 15 to 30 × 10^6 peptide loaded CFSElo and non-peptide loaded CFSEhi B cells were adoptively transferred at a 1:1 ratio into BL/6 or Eμ-myc mice, that were immunised 8 d earlier with or without H8 DCs. Spleens were isolated 1 h and 4 h after i.v. injection of CFSE+ B cells and specific killing was analyzed by flow cytometry. The percent killing was calculated as: 100 – (((% peptide pulsed in infected / % un-pulsed in infected)/(% peptide pulsed in uninfected / % un-pulsed in uninfected) × 100).

**LCMV immunoplaque assay**

Virus titers were determined after homogenization of total organs from Eμ-myc mice or BL/6 mice 8 d after infection with 200 pfu LCMV-WE or 6 d after adoptive transfer of 1 × 10^6 p14 TCR transgenic T cells followed by infection with 1 × 10^4 pfu LCMV-WE. Dilutions of supernatants from organs were used to infect a monolayer of MC57 cells after adding an methycellulose (Sigma) overlay in MEM 10% FCS. 2 d later, cells were fixed and stained with VL-4 rat αLCMV mAb and a color reaction of ortho-phenylenediamine (Sigma) served for determining the focus-forming units. Virus titers were calculated by counting the focus-forming units and were divided by the weight of the respective organs from individual mice.

**Proliferation assay**

To measure the proliferation of T cells 2 × 10^5 MACS-purified naïve human PBMC-derived or mouse splenic CD8+ T cells were stimulated in vitro. Briefly, 96-U-bottom-well-plates were coated with plate bound anti-human OKT-3 or anti-mouse CD3ε-antibody at a concentration of 2 μg/ml overnight in PBS at 4°C. T cells were plated for in vitro
expansion of 48 h. 0.5 μCi / well well tritiated thymidine ([3H]-
Tdr) was added to each well for the last 17 h of culture. Cells were harvested with an Omnifilter-96-Harvester (PerkinElmer) on UniFilter® GF/C plates (PerkinElmer). Incorporation of the radioactively labeled nucleoside was measured after pre-wetting filters with Microsynth Scintillation liquid (PerkinElmer) on a top count microplate scintillation counter (PerkinElmer). In vitro proliferation of naïve Eμ-myc x p14 TCR transgenic T cells was analyzed by co-culturing 2 × 10^5 MACS-purified CD8^+ T cells with 2 × 10^5 gp33-pulsed and irradiated splenocytes (1000 rad, γ-irra-
diator; Gammacell 1000, Nordion). After 3 d of co-culture proliferation was measured as described above.

**CFSE-dilution assay**

MACS-purified splenic CD8^+ T cells were labeled with 0.1 μM CFSE for 10 min at 37°C. Cells were then washed and transferred into αCD3e-coated 96-well plates. BCM from lymphoma-bearing Eμ-myc or BL/6 mice was added and CFSE dilution was assessed 72 h later by flow cytometry.

**Co-culture of T cells with B cells or APCs**

2 × 10^5 MACS-purified splenic T cells were co-cultured with MACS-purified and irradiated (1000 rad) splenic CD19^+ B cells or with irradiated (1000 rad) CD11b/CD11c double positive APCs at different dilutions (1:1; 1:3; 1:10). APCs were isolated from spleens using biotinylated CD11b and CD11c primary antibodies and subsequent MACs purification with streptavi-
dine Microbeads. In some experiments a blocking mouse αPD-1 mAb or control IgG was added (10 μg/ml). Trans-well plates (3.0 μm pore size and 6.5 mm diameter, Corning Costar) were used to separate B and T cell populations, whereas B cells were cultured in the upper chamber and T cells in the αCD3-coated lower chamber.

**Filtration of B cell-conditioned media (BCM)**

Splenic B cells were cultured at a concentration of 5 × 10^6 cells/ ml in tissue culture-treated 6-well plates. 24 h later, BCM was removed and filtered through a 0.22 μm filter (VWR). The supernatants were then either directly added at different dilu-
tions (1:1; 1:3; 1:10) to naïve T cell responders or additional fil-
ter steps were performed: 100.000 NMWL (Nominal molecular weight limit); 10.000 NMWL; 3.000 NMWL; Amicon ultra cen-
trifugal filter devices (Merck, Millipore, Billerica, Massachu-
setts); high recovery ultracel ultrafiltration membrane (1.000
NMWL, 25 mm diameter, Merck, Millipore, Billerica, Massa-
chusetts) suited in a Amicon filter chamber (model 8003, stirred cell, Merck, Millipore, Billerica, Massachus-
tts). Different fractions of BCM (>100 kDa, 100–10 kDa, 10–3 kDa,
<3 kDa, <1 kDa) were then separately added to T cells and proliferation was assessed.

**Determination of ATP concentration**

ATP concentration in mouse-sera, in whole B cell lysates and in BCM was determined using the ATP Bioluminescence Assay Kit HSII from Roche. Bioluminescence was detected using a Tecan Infinite 200 Plate Reader.

**Determination of purine metabolites**

Mouse serum levels of Uric Acid and BCM levels of Lactic Acid were determined at the Center of Laboratory Medicine, University Institute of Clinical Chemistry, Inselspital, Bern University Hospital and University of Bern, Switzerland using a multiple biochemical analyzer (cobas® 8000 modular ana-
lyzer, Roche). Hypoxanthine, xanthine, uridine, adenine and inosine concentrations were determined by high performance liquid chromatography (HPLC) at the University Institute of Clinical Chemistry, Inselspital, Bern University Hospital and University of Bern, Switzerland.

**Protein digestion and measurement**

Proteins in BCM were digested by using immobilized Proteinase K containing reaction columns, on 200 μl of matrix G3 m (MoBiTec). After enzymatic treatment, the amount of residual protein in the supernatants was assessed using NanoDrop™
2000.

**MS analysis using a UHPLC-TOF mass spectrometer**

BCM and conditioned cell media were injected into a reverse-
phase Acquity UPLC HSS T3 C18 Column, 100 A, 1.8 μm,
1 mm x 100 mm (Waters Corp.) with a gradient mobile phase comprising 0.1% formic acid and acetonitrile containing 0.1% formic acid. Each sample was resolved for 15 min at a flow rate of 0.28 mL/min with the linear gradient consisted of 2% B for 1 min, 1–9.5 min from 2 to 98% B; 9.5–12 min 98% B, and 2% B for 3 min. The column temperature was 60°C. The eluent was introduced by electrospray ionization into the MS (Waters Synapt G2 QTOF), operating in either positive ion (ESI+) or negative ion (ESI-) electrospray ionization modes. In both modes the capillary voltage was set to 700 V and the sampling cone to 30 V. The dissolving gas flow was set to 950 L/h and the temperature to 550°C. The cone gas flow was 50 L/h, and the source temperature was set to 140°C. To maintain mass accuracy, Leucin-Enkephalin ([M-H])^+ = m/z 556.2771 or (M-H)^- = m/z 554.2615) at a concentration of 2 μg/ml in 50% acetonitrile was used as a lock mass and injected at a rate of 20 μl/min. Data were acquired in continuum mode from 50 to
1200 m/z at a scan time of 0.3 sec. The MS collision energy was scanned from 15 to 40 V.

The continuum data was converted to centroid with the integrated program in MassLynx. Centroided and integrated chromatographic mass data from 50 to 1200 m/z were pro-
cessed by MarkerLynx (Waters) to generate a multivariate data matrix. Pareto-scaled MarkerLynx matrices including information on sample identity were analyzed by principal components analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) using Markerlynx XS (Waters).To determine which ions contribute to the difference between healthy (y = −1) and lymphoma-bearing (y = +1) mice orthogonal partial least squares (OPLS) was used. The loadings scatter S-plots and the contribution lists were used to describe the candidate markers.
that were significantly different between healthy and lymphoma-bearing mice. To identify the structure of high-contribution score metabolites, elemental compositions were generated with MassLynx (Waters) based on the exact masses of the top ions. The exact masses and the generated formula were used to screen for biomarkers in different databases like Metacyc and Metlin. Results were compared with the literature to define bioactive compounds with known immune modulatory effects. Other masses with unknown effects were excluded. The remaining possible biomarkers were analyzed using a MS/MS fragmentation spectrum with collision energy ramping from 15 to 40 V.

**Nuclear magnetic resonance (NMR) spectroscopy**

550 μl of filtered supernatants of Eμ1-myc or BL/6 B cells or the non-conditioned medium control were mixed with 50 μl of 0.2% 3-(4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate) (DSA) (w/v) in an eppendorf tube. After being thoroughly mixed, eppendorf contents were transferred to a labeled NMR tube (WILMAD Labglass). Prepped samples were stored at −40°C. Prior to NMR runs, samples were thawed and inverted several times to mix. Samples were run on a Bruker DRX600 spectrometer fitted with a 5 mm BBI Probe (Bruker Biospin) with a BACS 60 automated sample changer. 1D Carr-Purcell-Meiboom-Gill (CPMG) 1H spectra were acquired for each media sample with a T2 relaxation time of 32 ms to suppress any broad resonances. Final obtained spectra for each sample were the sum of 128 scans. Sample spectra were imported into Matlab (release 2010, The MathWorks, USA), phased, baseline corrected and calibrated to the DSA resonance (0 ppm). Metabolite assignments were made using Chenomx NMR Suite 7.0 software, online metabolome databases and previously published.71-73 Using Matlab, resonances from metabolites of interest were integrated and normalized to the integral of DSA to give relative metabolite concentrations.

**Statistical analysis**

Statistical analysis was performed by GraphPad Prism 5.0 (GraphPad Software). Results are represented as mean ± SEM. Data were analyzed using Two-tailed student’s t-test, one-way ANOVA or log-rank test. P ≤ 0.05 was considered significant.

**Disclosure of potential conflicts of interest**

The authors declare no conflict of interest.

**Authorship contribution**

T. Hilmenyuk designed and performed experiments, interpreted the data, prepared the figures and wrote the paper; C.A. Ruckstuhl designed and performed experiments, interpreted the data, prepared the figures and wrote the paper; M. Hayoz and C. Berchtold performed MS measurements; J. M. Nuofter performed HPLC experiments, S. Solanki and H. C. Keun performed NMR measurements; P. A. Beavis performed experiments and provided us with materials, facilities and A2A−/− mice; C. Riether prepared figures, designed experiments and wrote the paper; A.F. Ochsenbein designed and supervised experiments and wrote the paper.

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