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Reversal of tumor acidosis by systemic buffering reactivates NK cells to express IFN-γ and induces NK cell-dependent lymphoma control without other immunotherapies

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Like other immune cells, natural killer (NK) cells show impaired effector functions in the microenvironment of tumors, but little is known on the underlying mechanisms. Since lactate acidosis, a hallmark of malignant tissue, was shown to contribute to suppression of effective antitumor immune responses, we investigated the impact of tissue pH and lactate concentration on NK-cell functions in an aggressive model of endogenously arising B-cell lymphoma. The progressive loss of IFN-γ production by NK cells observed during development of this disease could be ascribed to decreased pH values and lactate accumulation in the microenvironment of growing tumors. Interestingly, IFN-γ expression by lymphoma-derived NK cells could be restored by transfer of these cells into a normal micromilieu. Likewise, systemic alkalization by oral delivery of bicarbonate to lymphoma-developing mice was capable of enhancing IFN-γ expression in NK cells and increasing the NK-cell numbers in the lymphoid organs where tumors were growing. By contrast, NK-cell cytotoxicity was dampened *in vivo* by tumor-dependent mechanisms that seemed to be different from lactate acidosis and could not be restored in a normal milieu. Most importantly, alkalization and the concomitant IFN-γ upregulation in NK cells were sufficient to significantly delay tumor growth without any other immunotherapy. This effect was strictly dependent on NK cells.

Natural killer (NK) cells are innate effector lymphocytes, which are able to combat virally infected and malignant cells in an MHC-nonrestricted way.[1] NK effector functions include direct cytotoxicity against target cells and production of IFN-γ.[2] The latter is pivotal for initiating specific T-helper 1 (Th1) and cytotoxic CTL responses,[3,4] which are necessary for effective antitumor protection exerted by the adaptive immune system.[4–7] Yet, direct evidences for a role of NK cells in tumor control are sparse. In solid carcinoma, a correlation between NK-cell infiltration and clinical prognosis was reported,[8–15] and in mouse models, impairment of NK cells or NK-cell receptors was shown to entail accelerated tumor growth.[16–18]

Key words: endogenous B-cell lymphoma, λ-myc mouse, tumor escape, NK-cell activation, bicarbonate
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Generally, however, NK cells are unable to effectively impede tumor growth, which is ascribed to numerous strategies that malignancies evolve to escape from immune surveillance[19] and from NK-cell attack, in particular. For example, NK cell-mediated tumor suppression can be obviated by downregulation of activating NK-cell receptors occurring in the presence of cancer cells[20–23] or by loss of NK cell-activating ligands expressed by the tumor cells.[16,17]

While most information on NK cell-tumor interaction was gathered in solid carcinoma, little is known on the role of NK cells in lymphoma. Since transplantation of lymphoma cells in mice creates a milieu where high IFN-γ production by NK cells promotes a strong Th1 bias eventually inducing a specific CTL response,[4,24] we are using a model of endogenously arising B-cell lymphoma, which more closely reflects the immunosuppressive situation as found in patients.[25] In this model, we showed that NK cells are effectively recruited to the tumor sites[24] and become strongly activated in early disease stages,[23] but progressively lose effector functions like IFN-γ expression and cytotoxicity during further tumor growth.[17,23]

Among a plethora of factors that might compromise NK-cell functions in the tumor microenvironment,[26,27] lactate acidosis may play a crucial role. Lactate accumulation and extracellular acidosis were described as a common phenomenon

What's new?

Natural killer cells show impaired effector functions in the microenvironment of tumors, but the underlying mechanisms remain unclear. Since lactate acidosis contributes to suppression of antitumor immune responses, here the authors investigate the impact of tissue pH and lactate concentration on NK-cell functions. Tissue acidosis compromises IFN-γ expression of NK cells in the microenvironment of endogenously arising B-cell lymphoma, thereby contributing to tumor immune escape. Systemic alkalization augments IFN-γ expression and significantly delays tumor growth. The study demonstrates the role of NK cells and IFN-γ for lymphoma control as well as the relevance of the tumor microenvironment for NK-cell function.

in solid tumors,[28] which is either due to anaerobic tumor metabolism under hypoxic conditions or to glycolysis despite the presence of oxygen, a phenomenon referred to as the "Warburg effect."[29] Studies indicate that tumor acidosis has adverse effects on the clinical course of several carcinoma entities[30,31] and that increasing the intratumoral pH by systemic buffers can inhibit malignant growth.[32–34] It was further shown that lactic acid negatively affects the functions of immune cells like T lymphocytes[34–36] or antigen-presenting cells.[37,38]

In this article, we asked the question whether, in endogenous mouse lymphoma, an acidotic milieu is relevant for the progressive functional impairment of NK cells, which was shown to develop despite strong initial NK-cell activation.[23] Lactic acid heavily compromised IFN-γ expression and cytotoxicity, but the former could rapidly recover in a nonacidotic milieu. Neutralizing the lymphoma pH by systemic delivery of bicarbonate alone conveyed a significant retardation of tumor growth in mice, which was critically dependent on NK cells.

Material and Methods

Animal experiments

C57BL/6 mice were purchased from Bommice (Ry, Denmark), and λ-myc mice[25] were bred in our animal facility. Breeding of mice and all animal experiments were approved by the responsible authority. For cell transfer experiments, NK cells were isolated from wildtype (wt) or tumor-bearing λ-myc spleens by immunomagnetic negative selection using the NK Cell Isolation Kit (Miltenyi, Bergisch-Gladbach, Germany). 1.5×10^6 CFSE-labeled NK cells were then i.v. injected into C57BL/6 wt mice or in λ-myc animals bearing visible tumor burdens. Mice were sacrificed 18 to 72 hrs later and spleens and lymph nodes (LNs) were isolated for further analyses.

For increasing the tissue pH *in vivo*, λ-myc mice received drinking water supplemented with 200 mM sodium bicarbonate as of Day 40 after birth. At this early time point, NK-cell functions already became compromised due to the presence of tumor cells although clinical symptoms were not yet visible. When tumor growth became clinically apparent, spleens were removed and pH values in the spleen tissues from mice that had or had not received bicarbonate water were compared by using a micro fiber pH transmitter (Presens, Regensburg, Germany) according to the manufacturer's instructions. NK-cell depletion *in vivo* was performed by injecting anti-Asialo-GM1 antibody (Ab; eBioscience, San Diego) i.p. in weekly to biweekly intervals starting at Day 50.

Tumor supernatants

Tumor-conditioned supernatants were generated by incubating 10^6 spleen or LN cells from tumor-bearing λ-myc mice or 10^6 291 cells (a cell line derived from a λ-myc mouse) in 200 μl RPMI medium in 96-well plates. As a control, normal splenocytes or LN cells were used. After 48 hrs, supernatants were harvested and pH values were determined. Lactate concentrations were measured using the OSR6193 reagent and the AU680 analyzer (Beckman Coulter, Krefeld, Germany). The identity of the 291 cells was regularly confirmed on the basis of cell morphology and the expression of the *myc* transgene.

NK-cell culture

Highly enriched 0.5×10^6 CFSE-labeled NK cells from normal wt or tumor-bearing λ-myc mice were cocultivated with 4×10^6 wt or λ-myc non-NK cells, which were obtained from the same immunomagnetic separation. In another setting, wt NK cells were cultivated in tumor-conditioned medium or in RPMI medium whose lactate concentration and/or pH value were adjusted to the levels as found in tumors. To this end, lactic acid or sodium lactate was added, and the pH was titrated by adding HCl or NaOH. After 18 hrs, IFN-γ production was quantitated by flow cytometry. All culture experiments were done in the presence of 50 ng/ml IL-15 and 30 U/ml IL-2.

Quantitation of IFN-γ expression in NK cells

IFN-γ expression was measured intracellularly by flow cytometry. To this end, total splenocytes from mice were stimulated with PMA/ionomycin for 4 hrs in the presence of Brefeldin A, fixed and permeabilized (Fixation and Permeabilization Kit, eBioscience) and stained with APC-conjugated XMG-1.2 mAb (Biolegend, San Diego). NK cells were defined as $NK1.1^+$ $CD3^-$ by counterstaining for NK1.1 and CD3.

Alternatively, IFN-γ expression was determined by qRT-PCR in a LightCycler 2.0 real-time PCR system (Roche Diagnostics, Mannheim, Germany). Total RNA was prepared

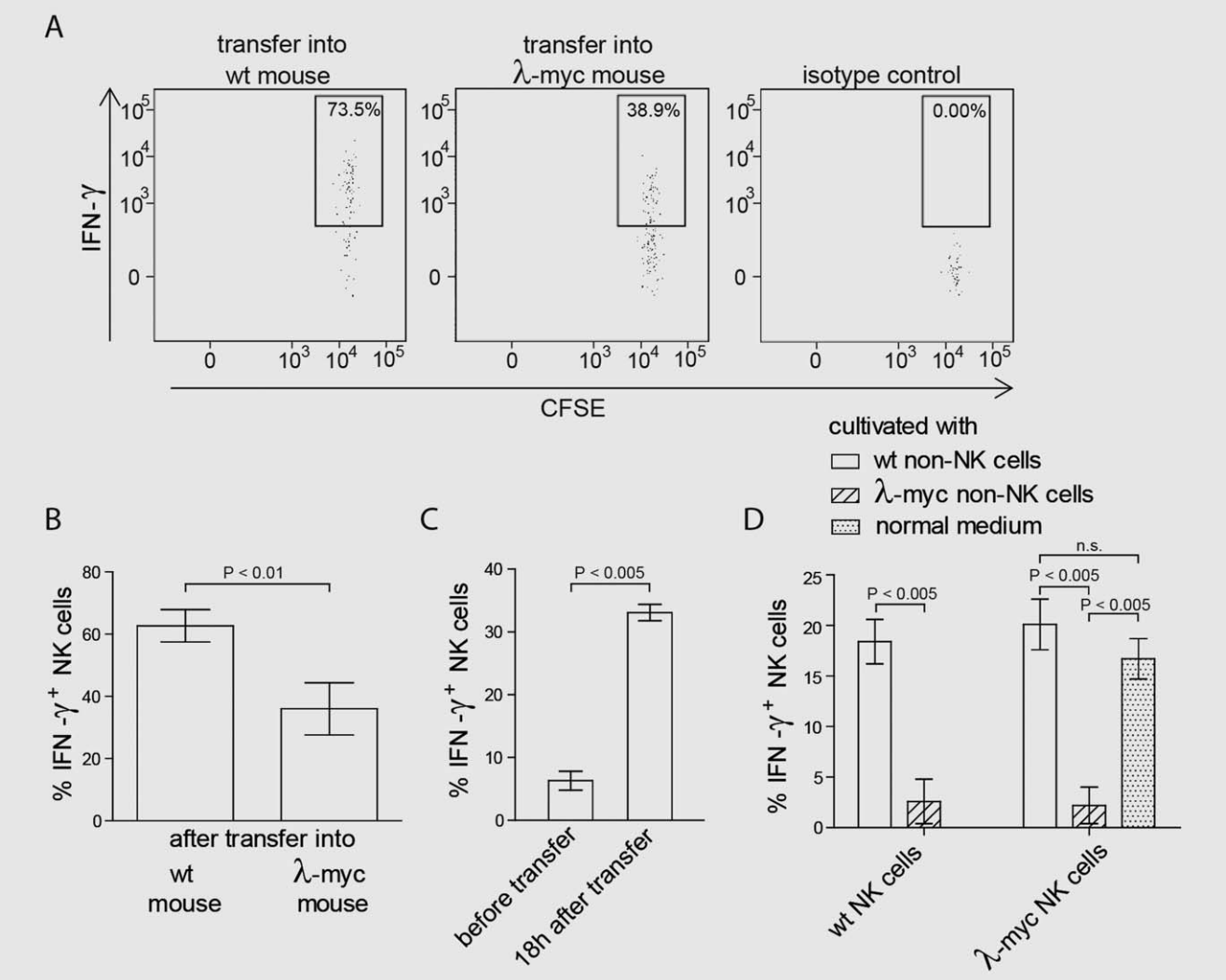


Figure 1. Dependence of IFN-γ expression by NK cells *in vivo* on the microenvironment. (*a*) Downregulation of IFN-γ in normal NK cells after transfer into a tumor micromilieu. Highly enriched NK cells derived from spleens of wt animals were labeled with CFSE and i.v. injected into wt mice or tumor-bearing λ-myc mice. IFN-γ expression was determined by PMA/I stimulation and intracellular FACS staining of NK1.1$^+$ CD3$^-$ cells recovered from lymphoid organs 3 days after transfer. (*b*) Compilation of six *in vivo* transfer experiments as described in (*a*). The difference is significant with $p < 0.01$ (Mann–Whitney). For endogenous NK cells, differences between λ-myc and wt mice were similarly detected irrespective of whether IFN-γ was quantitated after PMA/I stimulation of total splenocytes (which contained tumor cells in the case of λ-myc mice) or by determining transcript levels in highly purified NK cells (Fig. 4*e* and Ref. 23). An impact of the PMA/I stimulation on the different outcome seen for transferred NK cells is therefore unlikely. (*c*) Recovery of IFN-γ production in tumor spleen-derived NK cells after their transfer into a normal tissue milieu. NK cells were highly enriched from tumor spleens of λ-myc animals, labeled with CFSE and i.v. injected into wt mice or analyzed without transfer. Eighteen hours after *in vivo* transfer, spleens were removed and IFN-γ expression of transferred cells was analyzed ($n = 3$). The difference is significant with $p < 0.005$ (Mann–Whitney). (*d*) Suppression and restoration of IFN-γ expression *in vitro*. Enriched NK cells from wt or tumor-bearing λ-myc mice were labeled with CFSE and incubated with the indicated non-NK cell fractions for 18 hrs. Then, IFN-γ expression was quantitated as described above ($n = 3$; $p < 0.005$). All columns show mean values and standard deviations.

from immunomagnetically enriched NK cells using 200 µl TRIzol reagent (Invitrogen, Karlsruhe, Germany) per 1×10^6 cells, extracting with 1-Bromo-3-chloro-propane (Sigma, Deisenhofen, Germany) and precipitating with isopropanol. RNA was reverse-transcribed with the First Strand cDNA Synthesis Kit (Roche Diagnostics) and quantified with primer pairs (Search-LC, Heidelberg, Germany) specific for IFN-γ or the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (*hprt*). The specific signals were normalized to that obtained for *hprt*.

Chromium release assay

Highly enriched NK effector cells were incubated with 2×10^3 ^{51}Cr-labeled YAC-1 target cells at the indicated ratios for 4 hrs in standard chromium release assays. After transfer of supernatants to Luma-Plates (Perkin-Elmer, Boston), radioactivity was

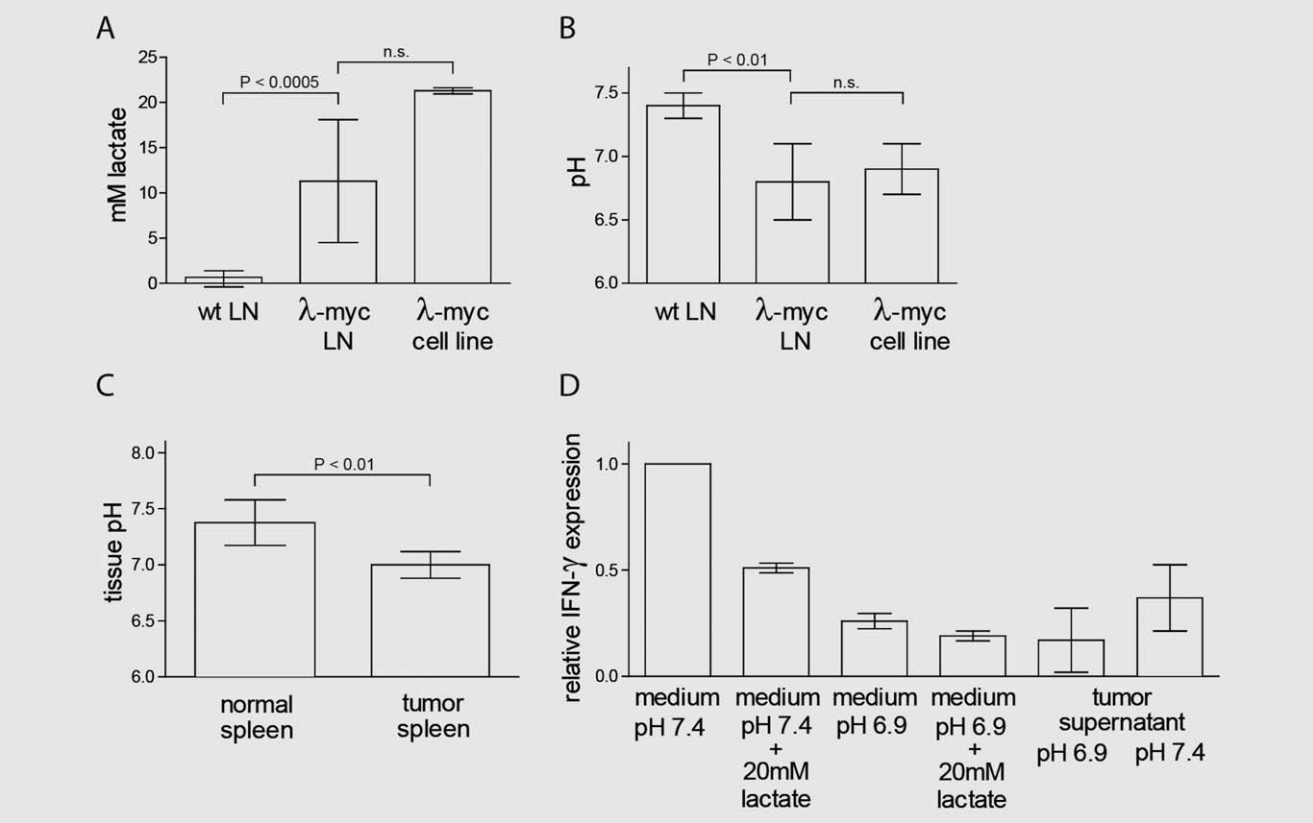


Figure 2. Role of lactate acidosis for IFN-γ expression by NK cells. (*a*) Lactate concentrations in supernatants generated from normal or tumor-infiltrated LN suspensions and from a λ-myc cell line. (*b*) pH values of cell supernatants. (*c*) pH values determined in normal and tumor-infiltrated spleen tissue. (*d*) IFN-γ expression in wt NK cells after culture in medium or tumor supernatant, which were or were not adjusted with respect to lactate concentration and/or pH value. All differences between the columns 2 to 6 are not statistically significant. The columns show mean values and standard deviations from 3 to 5 independent experiments.

measured in a Packard TopCount counter (Perkin-Elmer). Specific lysis was determined as [(specific release – spontaneous release)/(maximum release – spontaneous release)] × 100%.

Measurement of phosphorylated signaling molecules

To measure the phosphorylation status of proteins of the mitogen-activated protein kinase (MAPK) family, splenocytes were stimulated with PMA/ionomycin for 5 to 60 min, fixed (Cytofix; BD Pharmingen, Heidelberg, Germany) and then permeabilized (0.5x Perm Buffer IV; BD Pharmingen). Intracellular pp38, pJNK and pERK were detected by using unlabeled primary Abs (CST, Cambridge, UK) and Alexa-Fluor 488- or 647-conjugated secondary Abs (Life Technologies, Carlsbad). NK cells were defined as $NK1.1^+$ $CD3^-$ by counterstaining for NK1.1 and CD3 and analyzed by flow cytometry.

Statistics

Survival times of λ-myc mice were compared using the log-rank test. To assess differences between expression levels or cell numbers, the Mann–Whitney test was applied.

Results

Functional impairment of NK cells in the microenvironment of λ-myc lymphoma

Transgenic λ-myc mice constitutively express the *c-myc* proto-oncogene under the control of an Igλ enhancer and develop B-cell lymphomas, which closely mimic human Burkitt lymphoma.[25] Due to tumor growth, mice have to be euthanized by Week 10 to 20 after birth. NK cells from spleens and LNs show phenotypic signs of activation[17,23] and preceding degranulation[17] even at very early disease stages. However, their capability of lysing target cells and producing IFN-γ is progressively abolished during the course of disease development.[23]

To shed light on the impact of the tissue microenvironment on IFN-γ production by NK cells, we performed *in vivo* transfer experiments. At first we injected highly enriched, fluorescence-labeled normal NK cells into λ-myc animals bearing visible tumor burdens. As shown before,[24] these cells are readily recruited to the lymphoid organs on transfer. When the cells were recovered from tumor LNs and spleens, their IFN-γ expression was substantially reduced three days following injection, as compared to NK cells that were given to healthy wt mice (Figs. 1*a* and 1*b*). Thus, the

kinetics of functional anergization in the microenvironment of an established tumor seems to be rather fast. Conversely, we transferred enriched, fluorescence-labeled NK cells derived from λ-myc tumor spleens to normal animals. Surprisingly, IFN-γ expression of these cells, which was severely impaired before transfer, recovered in the normal LN milieu already 18 hrs after *in vivo* transfer (Fig. 1*c*).

To dissect the underlying mechanisms, we established an *in vitro* system. NK cells from normal wt or tumor-bearing λ-myc animals were exposed to a normal or to a "tumor" milieu. To this end, highly enriched, fluorescence-labeled NK cells were co-cultivated with NK cell-depleted cell fractions from either normal or tumor-infiltrated spleens. Whereas wt NK cells rapidly lost IFN-γ expression in a "tumor environment" *in vitro* but not in the presence of non-NK cells from normal spleens, λ-myc NK cells with impaired IFN-γ expression remained compromised in the *in-vitro* "tumor environment" and were fully restored in the presence of normal non-NK cells already after 18 hrs. This was also seen when λ-myc NK cells were left in normal medium (Fig. 1*d*). Thus, the cocultivation reflects the results obtained in the *in vivo* transfer experiments although a quantitative comparison is not possible between the *in vitro* and the *in vivo* situation. As IFN-γ expression of wt NK cells was equally suppressed by tumor non-NK cells in a transwell system (not shown), this effect appeared to be mediated by (a) soluble factor(s).

The role of acidosis for NK-cell function in λ-myc tumors

Since lactate acidosis may be a factor contributing to the loss of NK-cell functionality in λ-myc tumors, we determined pH values and lactate concentrations in tumor supernatants. Compared to supernatants from normal organs, an accumulation of lactate and a decreased pH were observed (Figs. 2*a* and 2*b*). Acidosis was also found when the tissue pH in the spleens of mice that developed tumors was measured using a pH microsensor (Fig. 2*c*).

The question whether the acidic milieu is sufficient to suppress IFN-γ expression in NK cells was then addressed *in vitro*. NK cells derived from wt mice were maintained in medium with decreased pH and/or augmented lactate concentrations. Each, acidic pH or lactate supplementation alone significantly suppressed IFN-γ expression, as it was also seen after culturing NK cells in supernatants of λ-myc lymphoma cells (Fig. 2*d*). IFN-γ expression was also diminished after incubation in λ-myc supernatants whose acidic pH was neutralized (Fig. 2*d*, sixth column). This result was predictable because in this situation, lactate was still present and lactate alone was also able to suppress IFN-γ even at physiologic pH (second column).

We previously showed that not only IFN-γ expression in NK cells becomes compromised during lymphoma growth *in vivo*, but also NK-cell cytotoxicity.[17] The finding of acidosis-mediated IFN-γ suppression raised the question whether loss of cytotoxicity is also dependent on the acidotic milieu. Although the cytolytic activity of wt NK cells against NK-susceptible target cells was significantly decreased after an

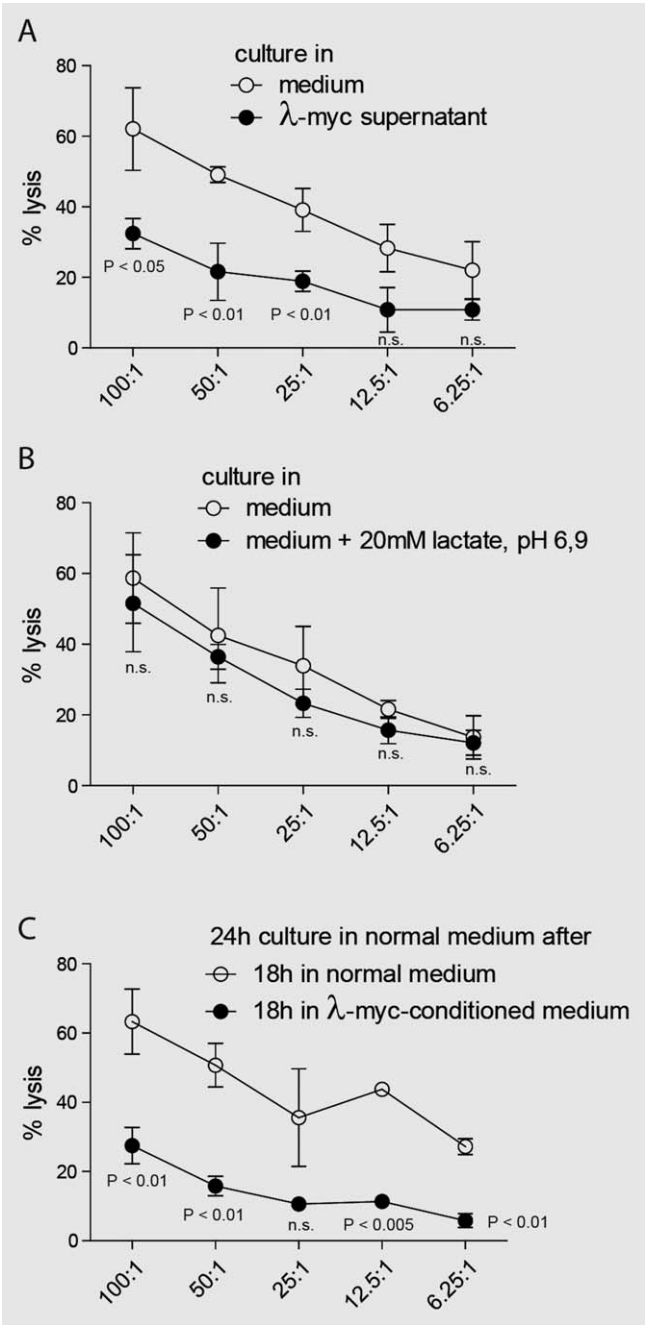
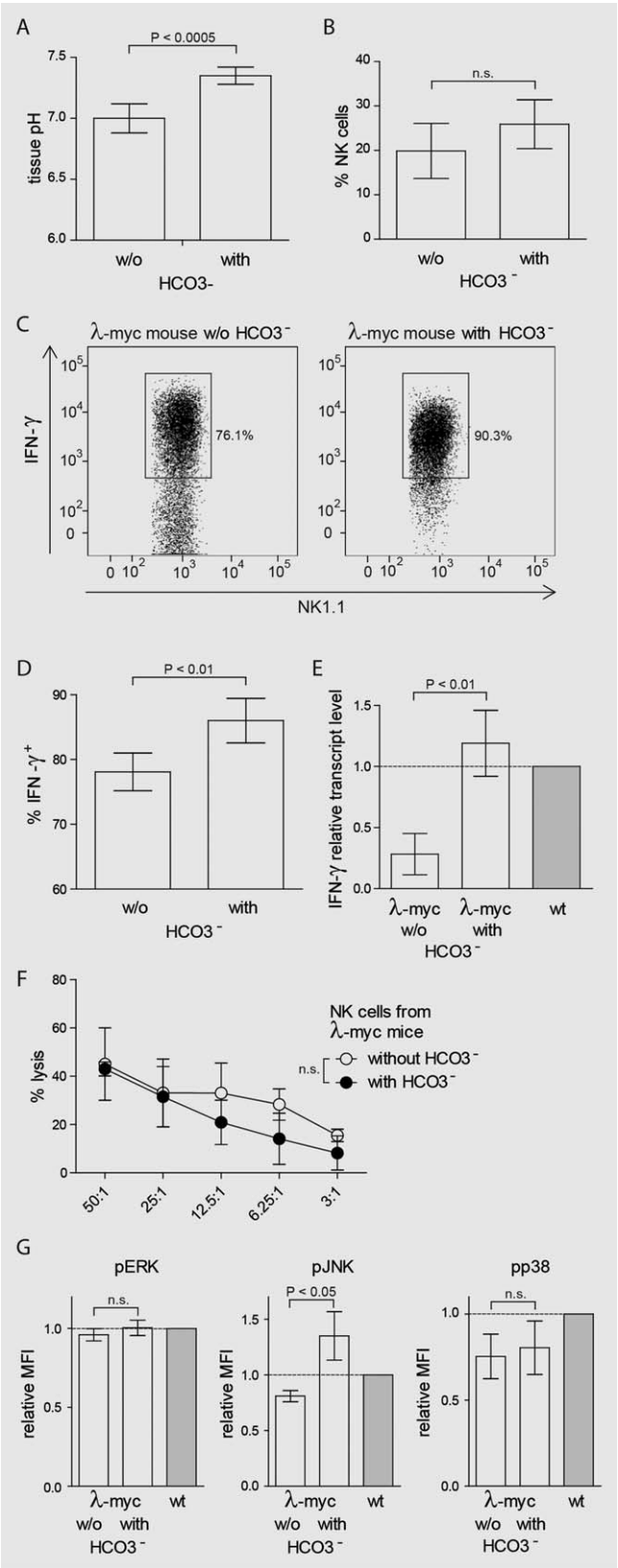


Figure 3. Abrogation of NK-cell cytotoxicity by soluble tumor-derived factors but not by lactate acidosis alone. (*a, b*) Highly enriched NK cells from wt animals were tested for cytotoxicity against YAC-1 cells in a standard chromium release assay after an 18-hrs culture in medium (*a, b*), λ-myc tumor supernatant (*a*) or acidified medium containing lactate (*b*). (*c*) NK cells were maintained in medium or λ-myc tumor supernatant for 18 hrs and then transferred to normal medium. The lytic potential was tested 24 hrs later. After 48 or 72 hrs, similar results were obtained. Each point represents the mean from three measurements. Standard deviations are indicated.

18-hrs incubation in tumor-conditioned supernatants (Fig. 3*a*), this effect was not achieved by decreasing the pH and increasing the lactate concentration in normal medium (Fig.

3*b*). Furthermore, it was not possible to restore the cytotoxicity of functionally compromised NK cells by maintaining such cells in a nonacidotic milieu (Fig. 3*c*). This is in contrast to the recovery of IFN-γ production, which was observed after transfer of cells into a normal milieu (Fig. 1*d*).



Systemic buffering ameliorates IFN-γ expression of NK cells *in vivo*

In the next step, we examined whether expression of IFN-γ by NK cells could also be upregulated *in vivo* by systemic buffering of the tissue milieu. To this end, λ-myc animals received drinking water supplemented with 200 mM sodium bicarbonate as of Day 40 after birth until the development of visible tumor burdens. To establish that this treatment leads to an alkalization of the tissue milieu, we determined the pH in spleens of mice that were or were not supplied with bicarbonate water. Oral administration of bicarbonate indeed raised the pH in the spleens significantly (Fig. 4*a*).

We then analyzed NK cells from spleens of λ-myc mice with incipient disease that had received alkalized water. At this disease stage, where IFN-γ expression is not yet as severely compromised as in the final stage, the functional state of the NK cells is a more reliable predictive parameter for NK cell-mediated retardation of tumor growth *in vivo* (C.D.B., unpublished). NK-cell numbers (Fig. 4*b*) as well as IFN-γ expression by NK cells (Figs. 4*c* and 4*d*) were increased after bicarbonate treatment. The upregulation of IFN-γ was also seen on the transcript level (Fig. 4*e*). In contrast, NK-cell cytotoxicity remained unaltered (Fig. 1*f*), as predicted by the aforementioned *in vitro* experiments.

Figure 4. Impact of systemic buffering on tissue milieu and NK-cell functions *in vivo*. (*a*) Tissue pH values measured in spleens of λ-myc mice that did ($n = 14$) or did not ($n = 10$) receive bicarbonate-containing drinking water. Mice were treated with bicarbonate as outlined in Materials and Methods. The difference is significant with $p < 0.0005$ (Mann–Whitney). (*b*) Relative NK-cell numbers in spleens of λ-myc mice, which were or were not supplied with alkalized water. Groups of 5 or 4 mice were included. Alkalization increased NK-cell numbers albeit with a significance of $p = 0.1$. (*c*) Upregulated IFN-γ expression in NK cells *in vivo* after bicarbonate treatment. Typical result from five individual mice. (*d*) Compilation of all experiments showing increased IFN-γ production in NK cells after systemic alkalization. The same individual mice as shown in panel (*b*) were analyzed. The difference is significant with $p < 0.01$. (*e*) Relative levels of IFN-γ transcripts in highly enriched NK cells derived from spleens of λ-myc animals that received bicarbonate ($n = 3$) or were left untreated ($n = 4$). The transcript levels were normalized to those detected in wt NK cells. All columns show means and standard deviations. (*f*) Failure of bicarbonate to enhance NK-cell cytotoxicity in λ-myc mice *in vivo*. Animals were treated as described in (*a*). NK cells were then isolated from spleens and examined in terms of their lytic potential against YAC-1 target cells in standard chromium release assays ($n = 5$). (*g*) Phosphorylation of ERK, JNK and p38 in NK cells from tumor-developing mice that were or were not treated with bicarbonate. The columns represent the x-fold change of the mean fluorescence intensities (MFI) related to wt controls after 5 min of PMA/I stimulation. Each column shows the mean value and the standard deviation from 4 to 5 mice.

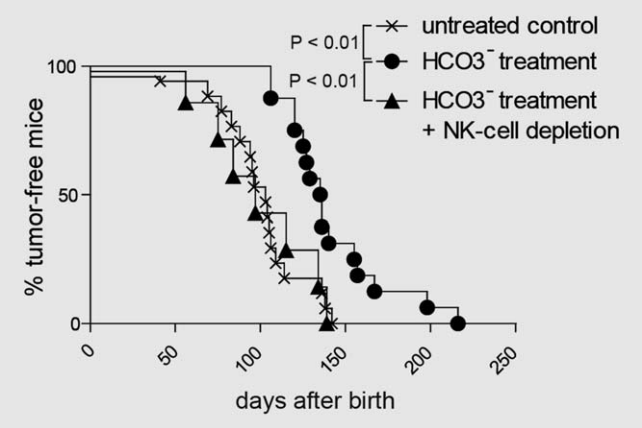


Figure 5. Kinetics of tumor growth in λ-myc mice that did or did not receive bicarbonate water. The difference between the two groups is significant with $p < 0.01$ (logrank). The tumor-suppressive effect of systemic alkalization is completely abrogated when mice are depleted of NK cells during bicarbonate treatment.

To investigate how intracellular signaling cascades in λ-myc NK cells are affected in NK cells of λ-myc mice, we determined the phosphorylation of the MAPK molecules p38, JNK and ERK, which are involved in the regulation of IFN-γ expression and degranulation, respectively. While pERK levels were only marginally reduced, JNK and p38 showed a markedly decreased phosphorylation in NK cells from developing tumors, which was slightly (pp38) or significantly (pJNK) increased by bicarbonate treatment (Fig. 4*g*).

Systemic buffering delays tumor growth in an NK cell-dependent manner

We earlier showed that NK cell-derived IFN-γ is critical for tumor suppression *in vivo*.[4,39] To evaluate the impact of the bicarbonate-induced IFN-γ upregulation in NK cells on lymphoma control *in vivo*, hence the clinical outcome, λ-myc animals received bicarbonate water until mice had to be euthanized because of lymphoma growth. It turned out that alkalization of the tissue milieu led to a significant delay of tumor growth (Fig. 5). To examine whether NK cells were necessary for this effect, mice were depleted of NK cells during bicarbonate treatment. Importantly, the survival benefit induced by alkalization was completely abrogated when NK cells were eliminated (Fig. 5). NK-cell depletion in untreated mice did not promote tumor growth compared to untreated NK cell-bearing controls (not shown).

Discussion

Orally delivered bicarbonate has been shown to effectively reverse acidity in tumors.[32,33] In several mouse models, alkalization of the tumor milieu inhibited invasive tumor growth and formation of metastases.[32–34,40] Systemic buffering was even able to suppress malignant growth in a model of autochthonous prostate carcinoma.[40] In aggressively growing cancers like the B16 melanoma, however, no tumor inhibition was observed.[32,34] In this model, pH neutralization had a beneficial effect only when combined with immune checkpoint inhibitors or adoptive cell transfer.[34] As the λ-myc lymphoma used in our study is a very aggressive tumor, the protective effect of alkalization alone was therefore an unpredicted result.

Another surprising finding was the strict dependence of bicarbonate-induced tumor suppression on NK cells (Fig. 5). We already showed by using other treatment regimens that NK cells are capable of controlling λ-myc lymphomas. Thus, NK-cell activation *in vivo* by a Toll-like receptor agonist or exogenous dendritic cells gave rise to prolonged survival.[23,41] It is well known that intratumoral lactate acidosis functionally compromises immune cells like T lymphocytes and antigen-presenting cells.[34–38] Likewise, NK cells were shown to be affected by lactic acid *in vitro*, and decreased production of lactic acid by carcinoma cells *in vivo* correlated with ameliorated NK-cell function.[42] The latter study, however, was done by using transplanted cancer cells with all *caveats* emerging from the rapid IFN-γ induction in NK cells following the injection of high numbers of tumor cells.[4,24] The λ-myc lymphoma used in our article, by contrast, rather generates an immunosuppressive micromilieu,[24,43] as it is expected in endogenously arising tumors. Thus, expression of IFN-γ, which is mainly derived from NK cells, declines during disease development in the autochthonous host.

We show in this article that a factor inhibiting NK cells in λ-myc lymphomas is lactate acidosis, which suppressed IFN-γ expression *in vitro* (Fig. 2*d*). By contrast, cytotoxicity of NK cells was reduced in the presence of tumor supernatants but not of lactic acid alone (Figs. 3*a* and 3*b*). It is unclear whether this effect was due to inhibitory factors present in the supernatants or to consumption of medium components by the tumor cells. A decreased cytotoxicity had also been found in NK cells from wt animals inoculated with λ-myc tumor cells.[44] The downregulation of NK cell-derived IFN-γ in λ-myc lymphomas correlated with reduced phosphorylation of the MAPK molecules p38 and JNK (Fig. 4*g*), which are involved in signaling cascades regulating cytokine expression.[45] For the protein ERK, however, only a marginal decline of phoshorylation was detected.

As shown by transfer and *in vitro* experiments, exposure to a normal milieu was associated with recovery of IFN-γ expression in previously silenced NK cells (Figs. 1*c* and 1*d*). Similarly, systemic buffering in tumor-developing λ-myc mice positively influenced the IFN-γ production by NK cells (Fig. 4*d*). The upregulation of IFN-γ transcripts induced by alkalization *in vivo* (Fig. 4*e*) strongly correlated with enhanced phosphorylation of the MAPK JNK, while there was a moderate, not significant increase of pp38 (Fig. 4*g*). Furthermore, alkalization had no impact on pERK levels. Since pERK is also involved in regulating the release of cytotoxic granules, the results are in accordance with the failure of alkalization to restore cytotoxicity (Figs. 3*c* and 4*f*). Thus, the signaling cascades regulating NK-cell cytotoxicity in λ-

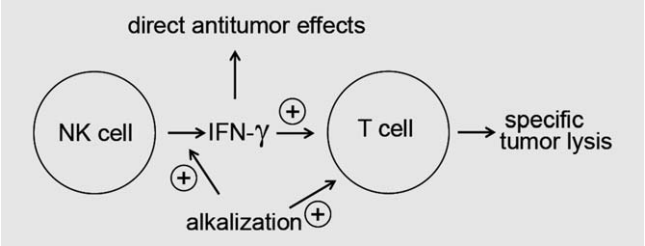


Figure 6. Hypothetical pathways leading to alkalization-induced NK cell-dependent tumor suppression. For details see text.

myc mice remain unclear for the time being. For intratumoral T cells, a similar interference of acidosis with signaling pathways has been reported.[36]

Since NK-cell depletion completely reversed the bicarbonate-induced retardation of tumor growth (Fig. 5) and NK-cell cytotoxicity could not be increased by counteracting acidosis *in vitro* nor *in vivo* (Figs. 3*c* and 4*f*), we suggest that a critical factor enabling NK cells to mediate tumor suppression in the λ-myc model is their enhanced IFN-γ expression. The mechanisms of IFN-γ-mediated tumor control are manifold and include nonimmunological pathways such as inhibition of angiogenesis[46] or senescence induction in tumor cells.[7] Increased levels of IFN-γ furthermore result in enhanced recruitment of NK cells to the tumor site. This may explain the slightly augmented NK-cell numbers in spleens of bicarbonate-treated animals (Fig. 4*b*), because an enhanced proliferation was not observed (data not shown).

Of course, other pathways apart from the above-mentioned direct effects of NK cell-derived IFN-γ may also be involved. We earlier showed that IFN-γ secreted by *in-vivo* activated NK cells induces tumor-specific T-cell responses in transplanted tumors[4,39] and even in the endogenous λ-myc lymphoma.[41] Thus, T cells stimulated by activated NK cells may also contribute to the tumor-protective effect in the present setting. Indeed, experiments indicate that the tumor suppression effected by alkalization is not only abolished by depletion of NK cells but also by T-cell ablation (manuscript in preparation). Furthermore, improved T-cell functions may not only be a consequence of NK-cell activation but may also be a direct effect of alkalization, as was reported earlier.[34–36] The interactions between NK and other immune cells in this model have to be elucidated in ongoing work. Taken together, the mechanisms leading to bicarbonate-induced tumor suppression may involve a network where NK cells and IFN-γ play a central role (Fig. 6).

Acidosis-induced downregulation of IFN-γ is not the only mechanism leading to immunosuppression in the λ-myc tumor microenvironment.[43] With regard to NK cell-mediated surveillance in B-cell lymphoma, several mechanisms of tumor escape have been identified, for example, contact-dependent downregulation of NKG2D[23] or loss of NKG2D-ligands on lymphoma cells.[17] Our results document the role of tissue acidosis as another factor involved in tumor immune evasion and indicate that pH neutralization may counteract IFN-γ deregulation in NK cells. The data support the concept of systemic buffering as an endorsement for future cancer immunotherapies.

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