Non-Invasive Bioluminescence Imaging to Monitor the Immunological Control of a Plasmablastic Lymphoma-Like B Cell Neoplasia after Hematopoietic Cell Transplantation

Martin Chopra1,2, Sabrina Kraus1,2, Stefanie Schwin1,2, Miriam Ritz1,2, Katharina Mattenheimer1,2, Anja Mottok3, Andreas Rosenwald3, Hermann Einsele1, Andreas Beilhack1,2*

1 Department of Internal Medicine II, University Hospital Würzburg, Würzburg, Germany, 2 Center for Interdisciplinary Clinical Research, Würzburg University, Würzburg, Germany, 3 Institute of Pathology, Würzburg University, Würzburg, Germany

Abstract

To promote cancer research and to develop innovative therapies, refined pre-clinical mouse tumor models that mimic the actual disease in humans are of dire need. A number of neoplasms along the B cell lineage are commonly initiated by a translocation recombining c-myc with the immunoglobulin heavy-chain gene locus. The translocation is modeled in the C.129S1-Ighatm1(Myc)Janz/J mouse which has been previously engineered to express c-myc under the control of the endogenous IgH promoter. This transgenic mouse exhibits B cell hyperplasia and develops diverse B cell tumors. We have isolated tumor cells from the spleen of a C.129S1-Ighatm1(Myc)Janz/J mouse that spontaneously developed a plasmablastic lymphoma-like disease. These cells were cultured, transduced to express eGFP and firefly luciferase, and gave rise to a highly aggressive, transplantable B cell lymphoma cell line, termed IM380. This model bears several advantages over other models as it is genetically induced and mimics the translocation that is detectable in a number of human B cell lymphomas. The growth of the tumor cells, dissemination, and response to treatment within immunocompetent hosts can be imaged non-invasively in vivo due to their expression of firefly luciferase. IM380 cells are radioresistant in vivo and mice with established tumors can be allogeneically transplanted to analyze graft-versus-tumor effects of transplanted T cells. Allogeneic hematopoietic stem cell transplantation of tumor-bearing mice results in prolonged survival. These traits make the IM380 model very valuable for the study of B cell lymphoma pathophysiology and for the development of innovative cancer therapies.

Introduction

To assess the efficacy of novel anti-cancer therapies, refined pre-clinical mouse models that mimic the actual disease in humans need to be developed [1]. Translocations recombining C-MYC at 8q24 with the immunoglobulin heavy-chain gene locus, IGH, at 14q32 (8;14)(q24;q32) in B cells are seen as the initiating genomic event in Burkitt’s lymphoma [1–5] and this translocation is also observed in other aggressive B cell tumors such as acute lymphoblastic leukemia [6,7] and plasmacytoma/multiple myeloma [8,9]. The (8;14)(q24;q32) translocation is modeled in the C.129S1-Ighatm1(Myc)Janz/J mouse [10]. Tumor-free heterozygous mice exhibit increased B cell proliferation and apoptosis and have enlarged lymph nodes and spleen due to follicular hyperplasia. More than two thirds of these mice develop mature B cell tumors between the age of 6 and 21 months resembling human endemic Burkitt’s lymphoma or plasmacytoma [10].

To reliably evaluate the response of deep-tissue tumors dynamically, non-invasive imaging techniques have gained widespread acceptance, and among these, bioluminescence imaging has proven a valuable tool to assess tumor growth in vivo [11–14].

In the current study, we have isolated tumor cells from the spleen of a C.129S1-Ighatm1(Myc)Janz/J mouse that spontaneously developed a plasmablastic lymphoma-like neoplasia. The cells were transduced to stably express eGFP and firefly luciferase and gave rise to a highly aggressive, transplantable B cell lymphoma cell line, termed IM380. Exploiting in vivo bioluminescence imaging, we could assess homing of lymphoma cells to lympho-hematopoietic compartments and their response to allogeneic stem cell transplantation.

Materials and Methods

Ethics statement

All experiments were performed according to the German regulations for animal experimentation. The study was approved
by the Regierung von Unterfranken as the responsible authority (Permit Number 55.2-2531.01-103/11). All procedures were performed under esketamine/xylazine anesthesia, and all efforts were made to minimize suffering.

Animals
BALB/c and C57Bl/6 mice were obtained from Charles River (Sulzfeld, Germany). C.129S1-Igha1m1/mmy;Janc/J [in BALB/c/H-2d background] mice expressing mouse c-myc under the control of the endogenous immunoglobulin heavy-chain 2 C-alpha locus [10] were initially obtained from Jackson Laboratories (Bar Harbor, ME, USA). Female BALB/c and C57Bl/6 mice were used for experiments between 8 and 12 weeks of age. All mice were kept within the specified pathogen-free animal facility of the Center for Experimental Molecular Medicine at the Würzburg University Hospital receiving rodent chow and autoclaved drinking water ad libitum.

Isolation of bone marrow cells and splenic T cells from C57Bl/6 mice
Bone marrow cells were isolated by flushing femur and tibia bones with phosphate buffered saline (PBS). The cell suspension was filtered through a 70 μm cell strainer (BD, Heidelberg, Germany). Spleens were directly filtered through a 70 μm cell strainer into erythrocyte lysis buffer (168 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM ethylenediaminetetraacetic acid (EDTA)), incubated for 2 minutes, and 2 volumes of PBS were added to the single cell suspension. The cells were spun down; the cell pellet was resuspended in PBS and filtered through a new 70 μm cell strainer before being spun down again. The resulting pellet was filtered through a 70 μm cell strainer (BD, Heidelberg, Germany). Spleens were directly filtered through a 70 μm cell strainer into erythrocyte lysis buffer (168 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM ethylenediaminetetraacetic acid (EDTA)), incubated for 2 minutes, and 2 volumes of PBS were added to the single cell suspension. The cells were spun down; the cell pellet was resuspended in PBS and filtered through a new 70 μm cell strainer, before being spun down again. The resulting pellet was resuspended in PBS and cells were used for further experiments. T cells were enriched from splenocytes using the Dynal Mouse T cell Negative Isolation Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer’s instructions.

Generation of a luciferase-expressing malignant plasmablastic lymphoma-like B cell line
C.129S1-Igha1m1/mmy;Janc/J mice exhibit B cell hyperproliferation and develop B cell and plasma cell neoplasms, the incidence of which increases with age [10]. Splenocytes from a five months old female moribund mouse (internal number 380) were isolated and activated with anti-CD3 (2C11, BD, Heidelberg, Germany) and embedded in paraffin for further processing. Picture...
Alexa647 (RI-2), CD54-PE (YN1/1.7.4), H-2kd-Biotin (SF1-1.1), I-A^D^Biotin (39-10-8). Biotinylated antibodies were detected with Streptavidin-Alexa647 (Invitrogen). All experiments were analyzed on a BD FACS Canto II (BD) and sample data was recorded using BD FACS Diva software and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistics
The number of animals is indicated in the figure legends. All data are shown as mean ± standard error of mean (S.E.M.). Figures were prepared with GraphPad Prism 5 software (La Jolla, CA, USA) and Adobe Photoshop 7 (San Jose, CA, USA). All groups were compared to the respective control group by two-tailed unpaired student’s t-test with GraphPad InStat 3 software. Data reaching statistical significance are indicated as: * p≤0.05, ** p≤0.01.

Results and Discussion
We isolated splenocytes from an enlarged spleen of a moribund 5 months old heterozygous C.129S1-Igha<sup>tm1(Myc)Janz</sup>/J mouse and subcultured these cells for six weeks. The primary tumor in the spleen and as an abdominal tumor mass showed a starry sky-like appearance indicative of widespread apoptosis and infiltrating macrophages often associated with Burkitt’s lymphoma [17] but also not uncommon in plasmablastic lymphomas and advanced-stage plasmacytomas [18,19] (Figure 1A). The cultured IM380 cells doubled each 20.1±6.1 hours, they produced IgM antibodies with the κ light chain (data not shown). To characterize the immunophenotype of IM380 cells, we assessed the expression of a number of B cell markers and activation-associated surface proteins (Figure 1B). The cells expressed the pan-B cell markers B220 and CD19, but were only slightly positive for the plasma cell marker CD138. They expressed BCL6 but were negative for CD10, two markers commonly expressed by Burkitt’s lymphoma marker CD138. They expressed BCL6 but were negative for CD10, two markers commonly expressed by Burkitt’s lymphoma [20]. IM380 cells stained negative for the B cell marker CD20, negative for CD27 which is expressed on plasma cells, and positive for the activation markers CD29, CD38, CD44, CD49d, and CD54. Loss of CD20 expression is rare in B cell lymphomas and associated with higher aggressiveness [21,22]. Burkitt’s lymphomas usually do not express CD138 [23–25] or CD44 [26,27]. Lymphoblastic lymphoma usually co-expresses CD10 and CD19 but show variable expression of CD20 [28,29]. In summary, the immunophenotype of IM380 cells indicates them to be of an origin intermediate of mature B cells and plasma cells.

We next tested the responsiveness of IM380 cells towards a panel of chemotherapeutic drugs in vitro by the use of Annexin V/propidium iodide staining (Figure 1C). The cells displayed a spontaneous cell death rate of about 10–25% and were found to be especially sensitive towards treatment with vincristine, bortezomib, panobinostat, etoposide, decitabine, and doxorubicin. The cells were positive for H-2-K<sup>d</sup> (MHC class I) but negative for I-A<sup>d</sup> (MHC class II). Pre-activated, but not naive allogeneic C57BL/6 T cells effectively killed IM380 cells in vitro (Figure 1D and data not shown).

To determine the in vivo homing and growth characteristics of the new IM380 cell line, we transduced the cells to stably express eGFP and firefly luciferase. Subsequently, we injected syngeneic, immunocompetent BALB/c mice with 10<sup>7</sup> IM380 cells i.v. and monitored lymphoma homing and progression by non-invasive bioluminescence imaging (Figure 2A and B). IM380 lymphoma cells grew in the bone marrow compartment and within secondary lymphatic organs (spleen and lymph nodes). Tumor cell signals could be detected in vivo as early as two days after injection (data not shown). Over the course of the experiment, additional tumor foci could be detected which speaks for tumor dissemination of the IM380 cells as they spread from their initial sites of growth (Figure 2C). Ex vivo bioluminescence imaging of internal organs and hind legs (Figure 2D) showed strong tumor cell infiltration of secondary lymphatic organs (spleen>ILN+>mLN>ILN), bone marrow, lungs, thymus, and pancreas. Involvement of spleen and bone marrow could also be confirmed by histology (Figure 2E).

To address whether the IM380 cell line is suitable for graft-versus-tumor studies, we transplanted BALB/c mice with pre-existing tumors with allogeneic C57BL/6 bone marrow and enriched T cells. Lethal irradiation as a pre-transplantation conditioning regimen of tumor-bearing mice resulted in tumor regression that lasted only for about four days after which the tumor relapsed and progressed. The same held true for mice that were irradiated and transplanted with allogeneic bone marrow cells. Transplantation with allogeneic T cells on the other hand resulted in efficient tumor eradication (Figure 3A and B) and prolonged survival when compared to the other groups (Figure 3C) (median survival: irradiation+bone marrow: 17 days, irradiation+bone marrow+T cells: 36.5 days; p<0.0001 as assessed by Log-rank (Mantel-Cox) test). Half of the mice (5/10) that were transplanted with allogeneic T cells nevertheless eventually succumbed to the tumor within 40 days after allogeneic transplantation owing to its high aggressiveness. Whereas activated allogeneic T cells are capable of killing IM380 cells in vitro, the tumor cells appear to develop immune escape variants in vivo resulting in tumor relapse [30,31].

The IgH-myc translocation has been mimicked in mouse models before. The challenge there is that the arising tumors are very heterogeneous and furthermore, the time to onset of disease varies enormously both within and between the different mouse models [10,32]. Whereas xenogenic tumor models bear relevance in terms of responsiveness of human cancer to therapy, they completely ignore the contribution of a functional immune system to tumor control on the one hand, and tumor-induced immune suppression on the other. These facts very much limit the applicability of such models in preclinical drug testing. Inoculated IM380 tumors behave in a highly reproducible manner and they arise in immunocompetent host mice which makes the IM380 cell...
line very valuable in the development of efficient anti-tumoral therapies. A number of drugs are currently successfully used for the treatment of aggressive B cell lymphomas [33,34]. In vitro drug testing revealed several of these drugs also to be functional in killing IM380 cells (e.g. vincristine, methotrexate, doxorubicine, and etoposide). We have furthermore identified a number of drugs that are promising as cytotoxic agents for these cells (e.g. bortezomib and panobinostat).

Figure 2. Non-invasive assessment of *in vivo* tumor growth and dissemination. **A**: $10^5$ luciferase-transgenic IM380 tumor cells were injected i.v. into the lateral tail vein into syngeneic BALB/c mice. Tumor growth was assessed by non-invasive *in vivo* BLI at the indicated time points. **B**: Representative BLI pictures of tumor-bearing mice. **C**: Tumor dissemination was determined by counting individual light-emitting tumor foci. **D**: Upper panel: Representative *ex vivo* BLI picture of a tumor bearing mouse (lu: lung, cLN: cervical lymph nodes, thy: thymus, hea: heart, ki: kidney, iLN: inguinal lymph nodes, li: liver, fc: femur, ti: tibia, sb: small bowel, lb: large bowel, mLN: mesenteric lymph nodes, st: stomach, cae: caecum, spl: spleen). Lower panel: Evaluation of tumor cell infiltration in individual organs. **A–D**: (Mean ± SEM; n = 5; shown is one representative experiment out of two). **E**: Representative eosin and hematoxylinstainings of organs from tumor bearing mice shown in 200× magnification.

doi:10.1371/journal.pone.0081320.g002
Other B cell lymphoma cell lines, be they of human or murine origin, have arisen either spontaneously or are the result of unspecific genotoxic insults [35]. The novel IM380 cell line results from a single genetic event, namely the IgH-myc translocation and thereby allows for the study of secondary mutations and clonal evolution [36].

In summary, this new murine tumor cell model has a number of advantages over other models [35]: 1. It is genetically induced and mimics the translocation that is responsible for the majority of human Burkitt’s lymphoma as well as other B cell neoplasias. 2. The tumors arising from IM380 inoculation in vivo are highly homogenous and reproducible. 3. Growth, dissemination, and response to treatment can be imaged non-invasively in vivo due to the expression of firefly luciferase by the tumor cells. 4. Established tumors can be allogeneically transplanted to study graft-versus-tumor effects of transplanted T cells as well as tumor cell immune escape. Nevertheless, the IM380 cell line is highly aggressive in vivo as mice succumb to it within less than three weeks following i.v. inoculation, established tumors are radiation-resistant and allogeneic transplantation is not sufficient to eradicate the tumors in all mice. These traits make the IM380 model highly valuable for the study of B cell lymphoma pathophysiology, disease progression in immunocompetent hosts and their response to treatment, and particularly for the development of innovative cancer immunotherapies.

Acknowledgments

We greatly appreciate the helpful discussion with Franziska Jundt and the members of the Beilhack lab.
Author Contributions
Conceived and designed the experiments: MC SK SS MR KM AM. Analyzed the data: MC SS AM. Wrote the paper: MC SK AM AR HE AB.

References
1. Cheon DJ, Orsulic S (2011) Mouse models of cancer. Annu Rev Pathol 6: 95–119.
2. Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, et al. (1982) Human c-myc oncogene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci U.S.A. 79: 7624–7627.
3. Taub R, Kirsch I, Morton C, Lenin G, Swan D, et al. (1982) Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci U.S.A. 79: 7837–7841.
4. Kovalchuk AL, Qi CF, Torrey TA, Taddesse-Heath L, Feigenbaum L, et al. (2004) Insertion of c-myc into the immunoglobulin heavy chain locus in human Burkitt lymphoma cells. Blood 104: 3009–3020.
5. Navid F, Mosijczuk AD, Head DR, Borowitz MJ, Carroll AJ, et al. (1999) Acute lymphoblastic leukemia with the 8;14(q24;q32) translocation and FAB L3 morphology associated with a B-precursor immunophenotype: the Pediatric Oncology Group experience. Leukemia 13: 133–141.
6. Mussolin L, Pillon M, Center V, Pigione M, Lo Nigro L, et al. (2007) Prognostic role of minimal residual disease in mature B-cell acute lymphoblastic leukemia of childhood. J Clin Oncol 25: 5254–5261.
7. Gould J, Alexanian R, Goodacre A, Pathak S, Hecht B, et al. (1981) Plasma cell karyotype in multiple myeloma. Blood 61: 435–436.
8. Dib A, Gatib A, Glebov OK, Bergpael PL, Kuehl WM (2008) Characterization of MYC translocations in multiple myeloma cell lines. JNCI Monographs 39: 25–31.
9. Park SS, Kim JS, Tesarollo L, Owens JD, Peng L, et al. (2005) Inversion of c-myc into IgH induces B-cell and plasma-cell neoplasms in mice. Cancer Res 65: 1311–1315.
10. Contag CH, Jenkins D, Contag PR, Negrin RS (2000) Use of reporter genes for optical measurements of neoplastic disease in vivo. Neoplasia 2: 41–52.
11. Choy G, O'Connor S, Diehn FE, Costouros N, Alexander HR, et al. (2003) CD138 (Syndecan-1), a plasma cell marker. Am J Clin Pathol 121: 254–263.
12. Cali C, Newell J, Cairo MS, Tripp SR, Cairo MS, et al. (2004) Comparative immunohistochemical analysis of pediatric Burkitt lymphoma and diffuse large B-cell lymphoma. Am J Pathol 121: 384–392.
13. Van-Proick F, Pinkus JL, Pinkus GS (2004) CD138 (Syndecan-1), a plasma cell marker. Am J Pathol 121: 254–263.
14. McClure RF, Remstein ED, Macon WR, Dews WH, Habermann TM, et al. (2005) Adult B-cell lymphomas with Burkitt-like morphology are phenotypically and genotypically heterogeneous with aggressive clinical behavior. Am J Surg Pathol 29: 1652–1660.
15. Attarbaschi A, Mann G, Schumich A, Konig M, Pich WF, et al. (2007) CD44 deficiency is a consistent finding in childhood Burkitt's lymphoma and leukemia. Leukemia 21: 1110–1113.
16. Schmidlerian SD, Li S, Saxe DF, Lechowicz MJ, Lee KL, et al. (2010) A novel flow cytometric antibody panel for distinguishing Burkitt lymphoma from CD10+ diffuse large B-cell. Am J Pathol 135: 718–726.
17. Liu P, Jones D, Dorfman DM, Medeiros LJ (2009) Precocious B-cell lymphoblastic lymphoma: a predominantly extranodal tumor with low propensity for leukemic involvement. Am J Surg Pathol 24: 1400–1490.
18. Cortelazzio S, Pozzoni M, Ferreri AJ, Hoelzer D (2011) Lymphoblastic lymphoma. Crit Rev Oncol Hematol 79: 330–343.
19. God JM, Haque A (2011) Immune evasion by B-cell lymphoma. J Clin Immunol: e103.
20. Prochazka V, Jaroseva M, Proussova Z, Novakova R, Papajik T, et al. (2012) Immune escape mechanisms in diffuse large B-cell lymphoma. ISRN Immunology: 208903.
21. Mori S, Rempel RE, Chang JT, Yao G, Laspou AS, et al. (2008) Utilization of pathway signatures to reveal distinct types of B lymphoma in the Emicro/myc model and human diffuse large B-cell lymphoma. Cancer Res 68: 8535–8544.
22. Niimi S (2010) Current treatment strategy of diffuse large B-cell lymphomas. Int J Hematol 92: 230–237.
23. Calvareza JL, Dalal SM (2012) Diffuse large B-cell lymphoma: current strategies and future directions. Cancer Control 19: 204–213.
24. Domon S, Galand C, Touitou V, Sautès-Fridman C, Fabry Z, et al. (2012) Murine models of B-cell lymphomas: promising tools for designing cancer therapies. Adv Hematol 701704.
25. Greaves M, Maley CC (2012) Clonal evolution in cancer. Nature 481: 306–313.