Letter to Blood

Chronic Borrelia burgdorferi infection triggers NKT lymphomagenesis

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*Borrelia burgdorferi* sensu lato complex (*Bb sl*) is a group of *Borrelia* species responsible for Lyme disease that is transmitted to humans by *Ixodes* tick bites. Lyme borreliosis is an emerging zoonosis and the most important vector-borne disease of the northern hemisphere with reported 30,000 to 300,000 cases per year in the United States\(^1\) and 65,000 cases per year in Europe\(^2\). The association between chronic bacterial infections and lymphomas has been suggested for a long time, but with the exception of a few pathologies (i.e., gastric B-cell lymphomas and *Helicobacter pylori* infection\(^3\)), clear demonstrations of causative links are missing. Although various *Borrelia* strains have been associated with primary cutaneous lymphomas, the results have been almost exclusively reported as case studies or small retrospective series of cutaneous lymphomas\(^4,5,6,7\), and remain controversial\(^8,9\). *Bb sl* DNA has been detected in 10-42% of patients with cutaneous mucosa-associated lymphoïd tissue (MALT) B-cell lymphomas, in 15-26% of cutaneous follicular and diffuse large B-cell lymphomas\(^5,6\), and in 18% of mycosis fungoides diagnosed in some European countries\(^7,10\). Taken together, there is insufficient evidence to show a definitive association between *Bb sl* and lymphomas. Therefore, the development of animal models to show the direct link between *Borrelia*-driven infection and lymphomagenesis is required.

To explore the role of *Bb sl* in T-cell lymphomagenesis, we used *p53*\(^{-/-}\) mice that develop, in addition to the well-known immature thymic T-cell lymphomas\(^11,12\), spontaneous peripheral T-cell lymphomas (PTCL) originating from NKT cells\(^13\). All animal studies and procedures were performed in accordance with European Union guidelines and approved by the Animal Ethics Evaluation Committee (CECCAPP).
Injections of p53-/- mice with live Borrelia afzelii IBS39 (n=34) or with control medium (Barbour-Stoenner-Kelly medium; BSK-H) (n=39) were carried out intradermally to mimic tick bites. Of the 34 mice injected with B. afzelii, 29 tested positive for anti-Borrelia IgG 15- and 30-days post-injection, thus confirming the infection. The median survival of 158 days was not significantly different from uninfected p53-/- mice (median survival = 168 days) (Figure 1A). Among the tumors developed in Borrelia-infected mice, almost 50% developed CD19-, CD3+, Thy1+ PTCL compared to only 32% in the uninfected group (Figure 1B and Supplemental Figure 1).

Macroscopically, these PTCL were characterized by hepatomegaly and splenomegaly (Figure 1C) with a 58- and 4-fold increase in lymphocytes in liver and spleen, respectively, compared to healthy mice (Figure 1D). The architecture of spleen and liver from PTCL mice was nodular and diffuse, with massive cell infiltration leading to the effacement of the normal structure (Figure 1E). The infiltrate in the liver was massively perivascular but also intrasinusoïdal, the preferential localization of normal NKT cells in the liver (Figure 1E). Since Borrelia-expressed glycolipids enable direct NKT cell activation and expansion14, we studied the NKT cell origin of these PTCL using αGalactosylceramide (αGalCer) loaded CD1d tetramer. A set of PTCL was characterized by positive CD1d tetramer staining, defining NKT lymphomas (NKTL), whereas another set of PTCL was negative, indicating conventional PTCL (Figure 1F, Supplemental Figure 1 and Supplemental Table 1).

As shown in Figure 1G, Borrelia infection significantly increased the incidence of NKTL (94% in Borrelia-infected group vs 61% in uninfected group). The NKT cell origin of these PTCL was further validated by the expression of the invariant Vα14-Jα18 rearrangement of the TCRα chain and by the rearrangement of the TCRβ chain with only Vβ7, Vβ8.2 and Vβ8.3 rearrangements (Supplemental Table 1). In the
*Borrelia*-infected group, NKTL were clonal with a clone productive frequency of the TCRβ chain rearrangement between 84.9% to 99.8% (Supplemental Table 1).

To further understand the role of chronic infection by *Bb* sl in the NKTL development, we studied NKTL incidence in different experimental conditions, as well as the persistence of anti-*Borrelia* IgG, since these Abs play an important role in immune responses against spirochetes. *p53*−/− mice infected with live *B. afzelii* produced high levels of anti-*Borrelia* IgG that persisted for more than 120 days (Figure 2A), and developed significantly more NKTL, compared to BSK-H-injected mice (Figure 2B). Conversely, mice injected with HK-*Borrelia* or *Borrelia*-infected mice treated with antibiotics to eradicate bacteria, did not produce long-term persisting anti-*Borrelia* IgG (Figure 2A) and showed no significant increase in NKTL frequency (Figure 2B). These results demonstrated the importance of live *Borrelia* persistence (i.e., chronic infection) in NKTL development. NKTL showed downregulated NK1.1 and increased PD-1 expression, two characteristics of chronically activated NKT lymphocytes (Figure 2C). The role of chronic TCR stimulation in NKT lymphomagenesis was further demonstrated by the increase of NKTL after chronic injection with αGalCer (Figure 2D).

Normal NKT cells persist *in vivo* in the absence of TCR/CD1d interactions. Conversely, during chronic infections the survival of antigen-specific memory T cells relies on the persistence of cognate antigens through repeated TCR engagements. To investigate the importance of CD1d-mediated chronic TCR engagement in NKTL survival, we transferred NKTL cells into *CD1d*−/− recipient mice. All WT recipients succumbed to lymphoma, whereas all *CD1d*−/− remained healthy (Figure 2E). Liver cellularity increased significantly in WT recipient mice with NKTL cells representing
92 to 95% of total liver cells, whereas they were undetectable in \(CD1d^{-}\) recipient mice (Figure 2F). Such addiction to CD1d/TCR interaction strongly suggests that NKTL development is driven by chronic TCR stimulation.

In summary, we described for the first time that chronic \(Bb\) sl infection increases the incidence of PTCL originating from NKT cells in \(p53^{-/-}\) mice. These results reinforce previous data demonstrating the role of TCR in NKT lymphomagenesis\(^{13}\). In addition to transforming viruses such as EBV\(^{19}\) and HTLV-1\(^{20}\), implicated in several non-Hodgkin lymphoma subtypes, chronic immune stimulation by pathogens, such as \(Helicobacter pylori\)\(^3\) and HCV\(^{21}\), has been reported to trigger lymphomagenesis. This study constitutes a novel illustration of the association between bacterial infections, chronic antigen receptor stimulation and lymphoma development and a formal demonstration that \(Bb\) sl infection drives T-cell lymphomagenesis. Whether \(Bb\) infection also triggers NKTL in humans needs to be further investigated, since our \(p53^{-/-}\) model may not recapitulate the pathophysiological genetic events of mycosis fungoides or other PTCL associated with \(Bb\) infection. However, recent data from whole-exome sequencing have pointed out TP53 mutations and copy number alterations as the most prevalent genetic abnormalities in cutaneous T-cell lymphoma\(^{22,23}\), suggesting that TP53 alterations might be the genetic driver events associated with chronic TCR stimulation in cutaneous T-cell lymphomagenesis associated with \textit{Borrelia} infection. In addition, the transcription factor PLZF, a key transcription factor in NKT cell differentiation, is overexpressed in some human T-cell lymphomas/leukemias, such as MF and SS\(^{24,25}\), suggesting that at least some SS and/or MF may be NKTL.
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Authorship Contributions

R.R. designed and performed experiments, analyzed data, made figures and wrote the manuscript. S.Ca., M.U., S.Ch., E.Bar. and D.C. performed experiments and analyzes data. E.Bac, A.T.-G., P.N.M., G.S. and B.J. designed and analyzed data. L.G. designed research, analyzed data, made figures and wrote the manuscript.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.
References


Figure Legends

**Figure 1:** *Borrelia* infection increases the frequency of PTCL arising from NKT cells.

(A) Survival curves of *p53*-/- mice infected with 5.10⁴ spirochetes (black box, n=29) or control medium (white box, n=39). (B) Histograms show the tumor spectrums developed in infected and control *p53*-/- mice. Other tumors include solid tumors and thymic lymphomas. (C) PTCL are characterized by large hepatosplenomegaly. Macroscopic images of livers and spleens from PTCL-bearing and healthy *p53*-/- mice. (D) PTCL show a significant increase in cells in liver and spleen (p<0.0001). P-values were determined by Mann-Whitney U tests. Data show mean ± SD. (E) HPS staining of paraffin-embedded organ sections from healthy and PTCL-bearing *p53*-/- mice. Arrows in middle panel indicate perivascular infiltrates of lymphoma cells. Arrow in right panel shows intrasinusoidal infiltrate of lymphoma cells. Bars: 200 µm (left) and 50 µm (right). (F) *p53*-/- mice develop two different entities of PTCL. Flow cytometry analysis of PTCL (gated on CD3⁺ Thy1.2⁺ population) stained with αGalCer-loaded CD1d tetramers. Left histograms are representative of two different PTCL types: CD1d-tetramer negative PTCL in grey (PTCL) and CD1d-tetramer positive PTCL in red (PTCL-NKT). Right histogram shows CD1d tetramer staining of all PTCL compared to normal T and NKT cells. (G) *Borrelia* infection increases PTCL-NKT frequency. PTCL and PTCL-NKT frequency among all PTCLs in control and *Borrelia*-infected *p53*-/- mice with significantly different PTCL spectrums (p<0.02). P value was determined by χ² test.
Figure 2: PTCL-NKT are driven by chronic TCR activation.

(A) *Borrelia* induces chronic infection in *p53*−/− mice. Kinetics of anti-*Borrelia* IgG titer in *p53*−/− mice sera by ELISA test. Mice (n=8 to 10/group) were intradermally injected with control medium (white line), heat-killed (HK)-*Borrelia* (gray line) or live *Borrelia*, treated (blue line) or not (red line) with ceftriaxone (antibiotics; ATB) at 25 mg/kg, twice a day for five days, starting 10 days post-infection, to eradicate the bacteria after short-term infection. Data show mean ± SEM. P=0.031 for kinetics between the live *Borrelia* and live *Borrelia* + ATB was determined by Kruskal-Wallis test. (B) Chronic infection is required to increase PTCL-NKT frequency. Lymphoma spectrum developed in control medium-, live *Borrelia-*-, HK-*Borrelia-* and live *Borrelia* + ATB-injected *p53*−/− mice. Only live *Borrelia* injection induced significantly more PTCL-NKT compared to control mice (p=0.023). Of note, the number of tumors exceeded the total number of mice due to some animals bearing several tumors (i.e., a solid tumor and a lymphoma). P-values were determined by χ² test. Values in the middle refer to the total numbers of lymphomas. TL represents thymic lymphomas. (C) PTCL-NKT show features of chronically activated NKT cells. Flow cytometric analyses of NK1.1 and PD-1 expression on PTCL-NKT, showing a significant loss of NK1.1 (p<0.0001) and overexpression of PD-1 (p=0.002). P values were determined by Mann-Whitney U tests. (D) NKT-activating glycolipids drive PTCL-NKT. Lymphoma spectrums developed in PBS- and αGalCer-injected *p53*−/− mice. Mice were intraperitoneally injected every week with PBS or 4 µg of αGalCer. αGalCer injection induced significantly more PTCL-NKT compared to control mice (p=0.044). P-value was determined by χ² test. (E) PTCL-NKT depend on TCR-CD1d interaction for engraftment and survival in vivo. Survival curves of WT and *CD1d*−/− mice (n=5) iv-injected with 10⁶ PTCL-NKT cells, showing absence of lymphoma development in
CD1d<sup>-/-</sup> recipient mice. All mice alive 100 days post-infection were sacrificed. P-value was determined by log-rank test. (F) Histogram shows the number of total liver NKT cells at sacrifice. P value was determined by Mann-Whitney U test.
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