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The protooncogene Vav1 regulates murine leukemia virus-induced T cell leukemogenesis

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Key words: Vav, T cell, leukemia, murine leukemia virus, retrovirus

Vav1 is expressed exclusively in hematopoietic cells and is required for T cell development and activation. Vav1-deficient mice show thymic hypopcellularity due to a partial block during thymocyte development at the DN3 stage and between the double positive (DP) and single positive (SP) transition. Vav1 has been shown to play a significant role in several non-hematopoietic tumors but its role in leukemogenesis is unknown. To address this question, we investigated the role of Vav1 in retrovirus-induced T cell leukemogenesis. Infection of Vav1-deficient mice with the Moloney strain of murine leukemia virus (M-MuLV) significantly affected tumor phenotype without modulating tumor incidence or latency. M-MuLV-infected Vav1-deficient mice showed reduced splenomegaly, higher hematocrit levels and hypertrophic thymi. Notably, Vav1-deficient mice with M-MuLV leukemias presented with markedly lower TCRβ/cD3 levels, indicating that transformation occurred at an earlier stage of T cell development than in WT mice. Thus, impaired T cell development modulates the outcome of retrovirus-induced T cell leukemias, demonstrating a link between T cell development and T cell leukemogenesis.

Introduction

Vav1, the first member of the Vav family to be described, is expressed exclusively in hematopoietic and trophoblast cells. Vav-family proteins share a tandem arrangement of a Dbl-homology (DH) domain followed by a pleckstrin homology (PH) domain. The DH domains of these proteins possess guanine nucleotide exchange factor (GEF) activity, which mediates GDP exchange for GTP on Rho-family GTPases, thereby causing activation of these GTPases. Some Rho proteins are overexpressed and hyperactivated in a number of human tumors.1 As gain-of-function mutations in Rho family GTP binding proteins, such as those found in ras, are very rare in human cancers, inhibition of GTPase-activating proteins (GAPs) or expression of GEFs may account for the hyperactivation of Rho family signaling in cancer.2 In fact, Vav1, similarly to other GEFs, was originally isolated as an oncogene3 and has been shown to play a role in tumorigenesis of different origins.4 Vav1 is aberrantly expressed in 53% of primary pancreatic adenocarcinomas due to demethylation of the gene promoter, and is associated with decreased patient survival.5 Moreover, it contributes to the tumorigenic properties of neuroblastoma,6 melanoma7 and pancreatic cancer cells.8 In the latter, the role of Vav1 is mediated by its GEF activity with a subsequent activation of Rac1, PAK1, Nfkb and an upregulation of cyclin D1. Azathioprine, used in the treatment of lymphocytic leukemias, is an inhibitor of nucleotide biosynthesis but also blocks Vav1-induced Rac activation.8,9

Vav1−/− mice are viable and fertile,10 but they present with an abnormal hematopoiesis: the overall size of the thymus is reduced by 50% due to a reduction in the number of double positive (DP) thymocytes, albeit with normal numbers of double negative (DN) thymocytes. Analyses of DN thymocytes have demonstrated that there is a block between the DN3 (CD44+CD25+) and DN4 (CD44−CD25−) stages, at the β-selection checkpoint. This observation strongly suggests that in the absence of Vav1, pre-TCR signaling is impaired. Other steps though are impaired as well since single positive (SP) cells are reduced by approximately 10-fold in Vav1−/− mice and this block between DP to SP cells is exacerbated in the absence of other Vav family proteins, Vav2 and Vav3.10,11 Furthermore, peripheral T cells derived from Vav1-deficient mice show a strong defect in cytoskeleton rearrangement and lipid raft organization, leading to impaired function.12-15

Vav1−/−, but not Vav2−/− or Vav3−/−, mice are prone to developing CD3+ lymphoblastic lymphoma-like tumors with increasing age, with 30% of mice dying as a consequence of this disease before 1 y.16 Retroviruses of the murine leukemia virus (MuLV) family promote leukemogenesis mainly by insertion activation of proto-oncogene expression.17 Viral enhancers act on proximal as well as distal promoters with the latter mediated via interactions facilitated by chromatin looping (reviewed in ref. 18).
Thus, integrated proviruses can affect many genes\(^1\) and induce cell leukemogenesis, we inoculated newborn Vav1\(^+/+\) mice to investigate the role of Vav1 in T cell development with M-MuLV inoculation. Despite similar infection, all mice had developed leukemia. In neonatal WT Vav1\(^+/+\) and Vav1\(^+/-\) mice, which under normal conditions have reduced thymic cellularity due to a lower number of DP cells,\(^1\) M-MuLV-induced leukemia resulted in a massive increase in the size of the thymus (Fig. 2B and D). The thymi of Vav1\(^+/-\) infected mice often invaded the entire thoracic cavity and indeed, these mice often suffered from respiratory problems during the latter stages of disease.

Distinct thymocyte profiles in Vav1\(^+/-\) and Vav1\(^+/-\) mice with M-MuLV-induced leukemia. M-MuLV-induced T cell leukemogenesis is often associated with the expansion and accumulation of thymocytes with an immature phenotype.\(^2\) To study the phenotype of thymocytes accumulating in the different mice cohorts, we first assessed the CD4/CD8 profiles. While profiles of thymocytes from infected mice showed some variability, they differed significantly from those of healthy mice. CD4/CD8 staining from individual mice is presented in Figure 3A. The majority of lymphomagenic thymi from leukemic Vav1\(^+/-\) mice harbored a high percentage of CD4-expressing cells, as also reported following infection with the radiation leukemia virus (RadLV \(^2\)\(^6\)). In healthy Vav1\(^+/-\) mice, the percentages of DN and SP thymocytes were modulated, with significantly higher levels of the former (p < 0.05) and lower levels of the latter (p < 0.05; Fig. 3A and B) and reviewed in ref. 11. Leukemia development in Vav1\(^+/-\) mice was associated with higher variability; approximately half the mice harbored >40% single positive CD4 cells (SP4) while the remainder harbored <20% SP4 cells (Fig. 3A and B). To study the differentiation state of these SP4 cells, we monitored TCR\(^\beta\) expression. In agreement with previously published data in reference 27, thymi of healthy Vav1\(^+/-\) mice had a significantly lower level of TCR\(^\beta\)-hi cells (Fig. 3C and Table 1), indicative of an immature stage of thymopoiesis as compared with WT mice where the majority of cells were mature TCR\(^\beta\)-hi SP4 thymocytes (49% and 73%, respectively). While TCR\(^\beta\) expression remained elevated on SP4 thymocytes in leukemic Vav1\(^+/-\) mice, it was undetectable on the vast majority of SP4 thymocytes from Vav1\(^+/-\) lymphomagenic thymi. Altogether, our results indicate that lymphomagenic thymocytes from Vav1\(^+/-\) mice have a more immature phenotype than those present in the WT counterpart.
given that M-MuLV infection targets proliferating cells, it was of interest to assess the cell cycle entry of different thymocyte populations. The percentage of DP thymocytes slightly decreased in WT mice between 3 d of age (the time point of M-MuLV inoculation) and 2 mo of age (Fig. 5A) whereas it was not statistically different in Vav1−/− mice. While the presence of SP4 and SP8 thymocytes augmented with age, they remained significantly lower in Vav1−/− than WT mice. The entry of thymocytes into cell cycle was monitored as a function of Ki67 expression, a protein upregulated during mid G1 phase. The percentage of Ki67 + cells within the DP thymocyte subset was not modulated by either age or the presence of Vav1 (Fig. 5A). The percentages of Ki67 + DN cells decreased with age but this phenomenon was observed in both WT and Vav1−/− mice. Notably, within the SP8 population, the relative proportion of Ki67− cells was higher during the newborn period, with greater than 50% of this subset expressing Ki67 in neonatal Vav1−/− mice. However, the total numbers of Ki67− SP8 thymocytes were still lower in Vav1−/− mice due to the relative paucity of this population. Almost identical data were obtained in the SP4 population. Importantly, the increased

Furthermore, there was a significant increase in DN thymocytes in lymphomagenic thymi from both WT and Vav1+/− mice (Fig. 3). As Vav1+/− mice have a partial block between DN3 and DN4 thymocyte stages (Fig. 4 and reviewed in ref. 11), it was of interest to determine whether leukemogenesis would result in the accumulation of distinct DN populations in WT and Vav1−/− mice. The majority of DN cells in leukemic WT mice had a CD44+CD25− (DN1) phenotype, while the DN profile in leukemic Vav1−/− mice revealed significantly increased percentages of CD44+CD25− (DN3) and CD44−CD25− (DN4) cells. Thus, early stages of thymopoiesis are disrupted by M-MuLV-induced leukemia, in both WT and Vav1−/− mice, but the absence of Vav1 significantly affects the development of lymphomagenic thymi.

Age- and leukemia-mediated changes in the cell cycle entry of Vav1+/− and Vav1−/− thymocytes. While the differentiation state of WT and Vav1+/− thymocytes in adult mice have long been known to be distinct,10 the partition of thymocytes of different developmental stages during the newborn period is not known. This is of importance in the context of the present studies because the mice were infected with M-MuLV at 3 d of age. Furthermore, given that M-MuLV infection targets proliferating cells, it was of interest to assess the cell cycle entry of different thymocyte populations. The percentage of DP thymocytes slightly decreased in WT mice between 3 d of age (the time point of M-MuLV inoculation) and 2 mo of age (Fig. 5A) whereas it was not statistically different in Vav1−/− mice. While the presence of SP4 and SP8 thymocytes augmented with age, they remained significantly lower in Vav1−/− than WT mice.

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subpopulation, which is the largest population present in both genotypes. Interestingly though, we did not observe any increase in DP cells in lymphomagenic thymi of adult mice (Fig. 3), suggesting that the virus did not target this population, or, alternatively, that infected cells underwent differentiation during the transformation process. SP4 cells, which represented the most abundant population in lymphomagenic thymi from both genotypes, were only rarely Ki67+ (Fig. 5B). Given that MuLV infection requires mitosis, these data strongly suggest it is not the SP4 subpopulation, which is the largest population present in both genotypes. Interestingly though, we did not observe any increase in DP cells in lymphomagenic thymi of adult mice (Fig. 3), suggesting that the virus did not target this population, or, alternatively, that infected cells underwent differentiation during the transformation process. SP4 cells, which represented the most abundant population in lymphomagenic thymi from both genotypes, were only rarely Ki67+ (Fig. 5B). Given that MuLV infection requires mitosis, these data strongly suggest it is not the SP4

percentages of Ki67+ cells within the DN and immature SP4 and SP8 thymocyte populations of Vav1−+/− mice were associated with the less mature phenotype of leukemic cells in M-MLV-induced mice as compared with their wild-type counterpart (Figs. 3 and 4).

Newborn Vav1−+/− mice showed a higher percentage of Ki67+ DN cells as compared with WT mice, a feature that was also observed in adult mice (Fig. 5B and reviewed in ref. 27). However, the majority of Ki67+ cells were found in the DP subpopulation, which is the largest population present in both genotypes. Interestingly though, we did not observe any increase in DP cells in lymphomagenic thymi of adult mice (Fig. 3), suggesting that the virus did not target this population, or, alternatively, that infected cells underwent differentiation during the transformation process. SP4 cells, which represented the most abundant population in lymphomagenic thymi from both genotypes, were only rarely Ki67+ (Fig. 5B). Given that MuLV infection requires mitosis, these data strongly suggest it is not the SP4

Table 1. Low surface TCRβ expression on CD4+ thymocytes in Vav1−+/− mice with M-MuLV-induced leukemias

<table>
<thead>
<tr>
<th>TCRβ</th>
<th>Control (Vav1+/+)</th>
<th>M-MuLV (Vav1−−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 low</td>
<td>27.1 ± 4.7</td>
<td>17.7 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>58.1 ± 13.8</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>CD4 high: average ± SEM (%)</td>
<td>79.8 ± 15.7</td>
<td>64.1 ± 8.3</td>
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Thymocytes from control or M-MuLV infected mice were stained as described in Figure 3. Within the SP4 gate (CD4−/CD8−), TCRβ expression was assessed. The mean percentages ± SEM of SP4 cells expressing TCRβ are presented. Data were evaluated using Student’s t-test and TCRβ expression within the SP4 thymocyte subset in infected Vav1−− mice was significantly lower than that detected in uninfected WT mice, uninfected Vav1−− mice and WT infected mice. p < 0.05 compare with non infected WT mice (1), compare with WT mice (2) and compare with non infected Vav1−− mice.
In this report, we investigated whether a protein that plays an essential role in lymphopoiesis also influences leukemogenesis. Vav1 strongly affects the T cell compartment without modulating other lineages and as such, it was of interest to selectively induce T cell leukemias. To this end, we used the M-MuLV retrovirus that lacks oncogenes and induces T cell lymphomas with a latency of 3–4 mo. Here, we report that the presence of Vav1 regulates M-MuLV-induced T cell leukemogenesis.

Vav1+/+ mice infected with M-MuLV develop the typical disease, characterized by a decrease in hematocrit, a modest enlargement of the thymus and a significant splenomegaly (Figs. 1 and 2 and reviewed in ref. 17 and 20–24). At the cellular level, leukemic cells predominantly expressed markers associated with T cell maturation such as CD3 and TCRβ (Figs. 3 and 4). Notably, in the absence of Vav1, infection with M-MuLV resulted in leukemias characterized by higher hematocrits, massive thymus enlargement and a more modest splenomegaly. Furthermore, the leukemic phenotypes in the thymus as well as the periphery differed; with a significantly more immature phenotype in Vav1-/- as compared with WT mice.

Induction of tumors by M-MuLV presumably results from infection of a target cell and a series of subsequent tumor progression events. Target cells for M-MuLV are early cells committed to the T cell lineage and they differentiate to various stages in T-cell differentiation before the final transforming event.21-23 The identity of the infected cells during the early stages of leukemogenesis remains unknown. The most population itself that was targeted by the virus but a cell at an earlier stage of differentiation.

**Phenotype of peripheral leukemic cells in Vav1+/+ and Vav1-/- mice.** M-MuLV-induced leukemias are first produced in the thymus but there is also a peripheral involvement during later stages of the disease.17,21-23 In order to characterize the phenotype of lymphomagenic cells in the periphery, we studied the expression of several cell markers in splenocytes from M-MuLV-inoculated mice. In WT as well as Vav1-/- leukemic mice, there was an expected decrease in the percentage of CD19+ B cells due to the presence of leukemic cells. As Vav1+ mice have decreased percentages of peripheral T cells under normal conditions, the relative percentage of CD19+ cells filling this niche was initially increased. Somewhat surprisingly though, there was a decreased percentage of CD3+ cells in leukemic Vav1+ mice (Fig. 6A). As leukemic cells generally lacked TCRβ expression Vav1+ mice (Fig. 3), we assessed whether these peripheral TCRβ CD3+ cells expressed CD4 or CD8 lineage markers. Indeed, there were more than 4-fold increases in both CD3+CD4+ and CD3+CD8+ cells in leukemic Vav1+ mice (from >5% to 15–20% of all splenocytes, Fig. 6B). Interestingly, the increase in these immature CD3+ cells was modest in WT mice with leukemias (2–4% to approximately 8%; Fig. 6B). Furthermore, mature CD3+CD4+ and CD3+CD8+ were not significantly increased in leukemic WT mice and in fact, were decreased in the Vav1+ mice. Thus, in Vav1+ mice with leukemias, splenocytes as well as thymocytes harbor an immature CD3+ T cell phenotype. Altogether, CD3+ leukemias were significantly decreased in Vav1+ mice as compared with WT mice following infection with M-MuLV.

**Discussion**

In this report, we investigated whether a protein that plays an essential role in lymphopoiesis also influences leukemogenesis. Vav1 strongly affects the T cell compartment without modulating other lineages and as such, it was of interest to selectively induce T cell leukemias. To this end, we used the M-MuLV retrovirus that lacks oncogenes and induces T cell lymphomas with a latency of 3–4 mo. Here, we report that the presence of Vav1 regulates M-MuLV-induced T cell leukemogenesis. Vav1+/- mice infected with M-MuLV develop the typical disease, characterized by a decrease in hematocrit, a modest enlargement of the thymus and a significant splenomegaly (Figs. 1 and 2 and reviewed in ref. 17 and 20–24). At the cellular level, leukemic cells predominantly expressed markers associated with T cell maturation such as CD3 and TCRβ (Figs. 3 and 4). Notably, in the absence of Vav1, infection with M-MuLV resulted in leukemias characterized by a decrease in hematocrits, massive thymus enlargement and a more modest splenomegaly. Furthermore, the leukemic phenotypes in the thymus as well as the periphery differed; with a significantly more immature phenotype in Vav1+ as compared with WT mice.
immature population in the thymus is the DN subset, which is subdivided into different stages: CD44+CD25- (DN1), the CD44+CD25+ (DN2) stage during which TCRβ rearrangements are initiated, the CD44-CD25+ (DN3) stage associated with initial CD3 expression and selection for productive TCRβ rearrangements, followed by the CD44-CD25- (DN4) stage of the TCRβ locus. The expression of a functional pre-TCR complexes leads to the DN4 stage and DP cells, expressing the CD4 and CD8 coreceptors, undergo TCRα rearrangements and express a mature TCRαβ complex. After positive and negative selection these cells become SP thymocytes with the maximal expression of CD3. The CD4+ thymocyte population increased in mice developing M-MuLV-induced leukemias. Importantly though, in Vav1-/- mice, leukemogenesis resulted in a massive accumulation in DN cells. Moreover, the DN population was altered in both mice strains, suggesting that these cells may represent a preleukemic state. As DN4 cells were the major DN population in infected Vav1-/- thymi, it is likely that leukemic cells overpassed the Vav1 requirement at the DN3 to DN4 transition (Fig. 4) or alternatively, were infected at that stage as they show a proliferative burst.30,31

Because the thymocyte population that increased in lymphomagenic thymi was CD4+, we compared the expression of two T cell differentiation markers in leukemic Vav1+/+ and Vav1-/- CD4+ cells; CD3 and TCRβ. We found that CD4+ cells derived from leukemic Vav1-/- mice lacked expression of TCRβ (Fig. 3 and Table 1), even if CD4 was expressed at high levels. In agreement with the thymus data, the vast majority of peripheral leukemic cells in Vav1-/- mice were CD3+. We favor a model in which, in the absence of Vav1, the pre-leukemic T cells fail to properly differentiate keeping their immature phenotype.

Vav1+/+, but not Vav2+ or Vav3+, mice are prone to developing lymphoblastic lymphoma-like tumors during aging.16 It is possible that in old animals, exhaustion of T cell activity...
the majority of M-MuLV-induced leukemias were of the CD4 phenotype. Some ATL, as well as mice harboring the Tax gene of HTLV-1, present with an immature CD4-CD8- phenotype. It will be of interest to assess Vav1 expression in these leukemias, which could correlate with the developmental status of the leukemic cells.

All together our data suggest that the proteins involved in T cell differentiation also regulate the development of T cell leukemias. Induction of tumors by a retrovirus results from infection of a target cell and a series of subsequent tumor progression events. The data presented here suggest that the target cells wherein M-MuLV promotes tumor formation differ in the presence and absence of Vav1. In Vav1 deficient mice, impaired T cell development during the selection processes favors the emergence of a different tumor phenotype.

Materials and Methods

Mice. Vav1-/- mice in the 129 SV background, a generous gift from Dr. V. Tybulewicz, have been previously described in reference 12, and were maintained under the same conditions as 129 SV mice. All animal experiments were performed according to the guidelines and regulations of the Centre National de la Recherche Scientifique.

Virus inoculation and clinical evaluation of mice. Vav1-/-, Vav1+/+ and 129 SV mice, 18 to 72 h old, were inoculated intraperitoneal with 10<sup>5</sup> foci forming units (FFU) of M-MuLV in 50 μL as previously described in reference 20. Mice were monitored for gross organ enlargement by palpation under Forene® (isoflurane, Abbott France) anesthesia. Hematocrits, expressed as the percentage of erythrocytes in the blood volume, were measured in retroorbital vein blood samples collected in heparinized capillary tubes. Fifty days after infection, hematocrits were monitored regularly at approximately 20-d intervals. After sacrifice of moribund animals, spleens were weighed and thymic size was scored from normal, to clearly enlarge (medium), to invading the whole thoracic cavity (large).

...and increased pressure for T cells to divide could be responsible for the increase in tumorigenesis. However, the phenotype of “spontaneous” lymphomas in aging Vav1-/- mice is similar to that detected in aging wt mice. This might suggest that it is the increased homeostatic-drive proliferation occurring in lymphopenic Vav1-/- mice that contributes to leukemia formation in a spontaneous setting. In contrast, under the conditions reported here, M-MuLV clearly favored the transformation of a more immature T cell precursor. Compare with our results (Fig. 2), aging leukemic Vav1-/- mice do not show massive thymic enlargement. It could be that initial leukemic cells are produced in a more mature phenotype in aging mice, which do not localize in the thymus. An alternative explanation is that aging thymi lose partially their functions, allowing tumor cells to exit the thymus even at the first stages of the disease.

Vav1 is misexpressed and plays a significant role in different kind of non-hematopoietic tumors, including melanoma cell invasion. However, gain-of-function mutations in the Vav1 gene have not yet been found in human tumors of hematopoietic origin. Perhaps primary cells select against such mutations. In fact, a recent study on mice has shown that oncogenic Vav1 expression inhibits hematopoietic stem cell engraftment and cell expansion in vitro by inducing apoptosis. Studies of M-MuLV-induced leukemia have provided insights into the multistep process of leukemogenesis. The fact that the disease occurs with a moderate, predictable latency has allowed identification of the virological and physiological steps that occur during preleukemic stages and during tumor progression. Some of the principles uncovered are likely to be applicable to the development of neoplasms in other species, including humans. This model is complex and often involves activation of more than a specific oncogenic pathway. Human T cell leukemia virus (HTLV-1), the etiologic agent of adult T cell leukemia (ATL), can be transmitted by breastfeeding but only leads to the development of the disease in 2–5% of the infected infants and only after latency periods of 20 to 60 y. Transformed ATL cells are generally CD4+ and it is interesting to note that in our WT mice, the majority of M-MuLV-induced leukemias were of the CD4 phenotype. Some ATL, as well as mice harboring the Tax gene of HTLV-1, present with an immature CD4-CD8- phenotype. It will be of interest to assess Vav1 expression in these leukemias, which could correlate with the developmental status of the leukemic cells.

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Figure 6. Analysis of splenocyte populations in M-MuLV infected mice. (A) Expression of CD3, CD19, CD4 and CD8 in splenocytes (Vav1<sup>++</sup> control (n = 8); Vav1<sup>-/-</sup> control (n = 8); Vav1<sup>++</sup> M-MuLV (n = 36); Vav1<sup>-/-</sup> M-MuLV (n = 46)) were assessed by flow cytometry and bar graphs presenting the mean percentages ± SEM of splenocytes expressing CD3 and CD19 are shown. (B) Graphs presenting the mean percentages ± SEM of splenocytes expressing CD4 and CD8 in the absence or presence of surface CD3 are shown. Data were evaluated using Student’s t-test: *p < 0.05; **p < 0.01; ***p < 0.001.
Immunophenotyping and flow cytometry analyses. Splenocytes and thymocytes were stained with the appropriate conjugated 
αCD8, αCD4, αCD3, αCD25, αCD44, Ki-67, TCRβ (PharMingen, San Diego, CA), αCD3 and αCD19 (Immunotech, Marseille, France) mouse monoclonal Abs, as indicated. Stained cells were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA) and analyses were performed with CellQuest (Becton Dickinson) or FlowJo (Tree Star, Ashland, Oregon) software as previously described in reference 36 and 37.

Statistical analysis. All results were analyzed by an unpaired Student’s t-test.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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