Deregulated JAK3 mediates growth advantage and hemophagocytosis in extranodal nasal-type natural killer/T-cell lymphoma

Advanced extranodal, nasal-type natural killer/T-cell lymphoma (NKTCL) is an aggressive malignancy with dismal prognosis, typically associated with hemophagocytic syndrome that worsens the prognosis.[1,2] Hemophagocytic syndrome results from excessive production of interferon-γ, although the mechanisms leading to interferon-γ overproduction in NKTCL cells remain unclear. A deregulation of Janus kinase 3 (JAK3) protein is a general oncogenic event in NKTCL, with up to 80% of patients harboring constitutive phosphorylation in the activating Y980 residue, and 7%-35% of patients displaying acquired activating mutations, mainly in the pseudokinase domain, which account for its constitutive activation.[3-6] Here we demonstrate that constitutively activated JAK3 confers natural killer (NK) cell hypersensitivity to interleukin-2 (IL-2), leading to growth advantage and excessive production of interferon-γ through activation of downstream substrates. We also show that the expression of constitutively activated JAK3^{A573V} in hematopoietic progenitors leads to NK-cell expansion in mice. Finally, using an original *in vivo* murine model based on the expression of JAK3^{A573V} in primary NK cells from *Rag2*$^{-/-}$ mice, we observed that transplanted wild-type recipients reproduce the typical features of NKTCL including NK-cell expansion and hemophagocytic syndrome.

To get insights into the role of JAK3 in NKTCL pathophysiology, we hypothesized that JAK3 deregulation could lead to an excessive sensitivity to cytokines. In preliminary experiments, we treated control NK cells and the NK-cell line MEC04, derived from a NKTCL (harboring the JAK3^{A573V} mutation), and NKL and KHYG-1 cell lines, derived from NK-cell leukemias, all harboring a constitutively phosphorylated JAK3 on Y980 residue,[3] with recombinant IL-2 (rIL-2) as a representative cytokine that stimulates a receptor involving the common γc/JAK3 axis. Cells were exposed to 100 U/mL of rIL-2 for 2 days and counted daily using the trypan blue exclusion assay. A rapid and significant increase in the number of viable cells was observed throughout time when compared to control NK cells isolated from healthy donors or to MEC04 cells cultured without rIL-2 (*Online Supplementary Figure S1A*). After 2 days of culture, there was a more than 4-fold increase in the number of MEC04 cells in comparison with normal NK cells (P<0.0001). Similar results were observed for NKL and KHYG1 cells (*Online Supplementary Figure S1*). Strikingly, this proliferative effect observed after exposure to rIL-2 was totally abrogated when cells were exposed to the JAK3 inhibitor CP-690550 (*Online Supplementary Figure S1A*), providing further evidence that JAK3 signaling is important in NKTCL oncogenesis.

To confirm the importance of JAK3 signaling in NKTCL, MEC04 cells were stimulated with rIL-2 for 30 minutes and subjected to western-blot analysis for the phosphorylation of the tyrosine 705 (Y705) residue of Signal transducer and activator of transcription 3 (STAT3) oncogene.[3,7] We found that before stimulation with rIL-2, Y705-STAT3 showed constitutive baseline phosphorylation in MEC04 cells when compared to control NK cells. After stimulation with rIL-2, Y705-STAT3 phosphorylation was increased in MEC04 cells. This phenomenon was abrogated when cells were exposed to CP-690550 before rIL-2 stimulation (*Online Supplementary Figure S1B*). Collectively, these results provide evidence for a synergistic role between JAK3 deregulation and IL-2 signaling in the growth of NK-cell lines.

To explore the involvement of deregulated JAK3 in the hypersecretion of interferon-γ, we used NKL and KHYG-1 cells as they produce the highest amounts of interferon-γ. Cells were cultured in the presence or absence of rIL-2, and interferon-γ in the supernatant was measured after 48 h of culture. The two cell lines spontaneously secrete low levels of interferon-γ in the absence of rIL-2. In the presence of rIL-2, the amount of secreted interferon-γ/10^6 viable cultured cells increased dramatically. This effect was almost completely abrogated when cells were concomitantly cultured with CP-690550 (*Online Supplementary Table S1*). Accordingly, the phosphorylation of target proteins STAT3, AKT and ERK1/2 was decreased in the presence of JAK3 inhibitors (*Online Supplementary Figure S1C*). To confirm further the role of JAK3 signaling in interferon-γ secretion, we selectively knocked-down *JAK3* in NKL cells using specific siRNA. The decreased expression of JAK3 induced a rapid decrease in cell viability (*Online Supplementary Figure S1D*), with dephosphorylation of target proteins (*Online Supplementary Figure S1E*), as well as a major decrease in interferon-γ secretion after 2 days of cell culture (27±2.1 pg/mL/10^6 viable cells in the presence of JAK3-targeting siRNA *vs.* 127±3.4 pg/mL/10^6 viable cells in the presence of scrambled siRNA, P<0.001). Furthermore, the phosphorylation of AKT and ERK, two substrates downstream of JAK3, was inhibited by GDC-0941 and UO126, respectively, as assessed after culture

for 48 h in the presence of rIL-2 (*Online Supplementary Figure S1F*). We found that both inhibitors were able to inhibit mostly (UO126) or completely (GDC-0941) the secretion of interferon-γ after 48 h of culture (*Online Supplementary Table 1*). Taken together, these results provide evidence that deregulated JAK3 and its corresponding downstream substrates are involved in the excessive secretion of interferon-γ in NKTCL.

To provide further evidence that deregulated JAK3 is oncogenic in NKTCL, we cultured mouse NK cells expressing the JAK3^{A573V} oncogenic protein. For this, Lin^{-}, Sca^{hi}, Kit^{hi} (LSK) progenitors obtained from C57Bl/6 mice were transduced with retroviral vectors encoding the wild-type form of human JAK3 (JAK3^{WT}) or JAK3^{A573V}, as well as with an empty vector.[8] Transduced cells were cultured on MS5 stromal cells in the presence of mouse stem cell factor, mouse thrombopoietin, human Fms-like tyrosine kinase 3-ligand (hFlt3-L), human IL-7, and mouse IL-15 for NK-cell differentiation (Figure 1A). After 10 days, we observed a three-fold increase in the number of NK cells expressing JAK3^{A573V} as compared to cells transduced with empty vector, and an intermediate number of cells transduced with JAK3^{WT} vector (Figure 1B), consistent with the view that deregulated JAK3 confers a growth advantage to NK cells. Accordingly, we found maximal phosphorylation of the JAK3 substrates Y705-STAT3, Y594-STAT5, Y202/204-ERK1/2 and S473-AKT by flow cytometry in NK cells harboring JAK3^{A573V} (Figure 1C).

JAK3 activating mutations have been shown to induce a rapid-onset T-cell lymphoproliferation in mouse bone marrow transplantation assays.[9] Since NK cells represent a minor lymphoid population, we hypothesized that T-cell proliferation either masked or inhibited the development of a NK-cell disorder. Hence, we assessed the transforming ability of JAK3^{A573V} in a T-cell deficient bone marrow transplantation assay by using donor *Rag2*$^{-/-}$ mice (Figure 2A). Transplantation of JAK3^{A573V}-transduced bone marrow cells from *Rag2*$^{-/-}$ mice into wild-type C57Bl/6 recipients resulted in a lymphoproliferative disease characterized by an expansion of eGFP^{+} CD3^{-} NK1.1^{+} NK cells in blood (Figure 2B) leading to the death of all animals within 7 months. Conversely, mice transplanted with empty vector

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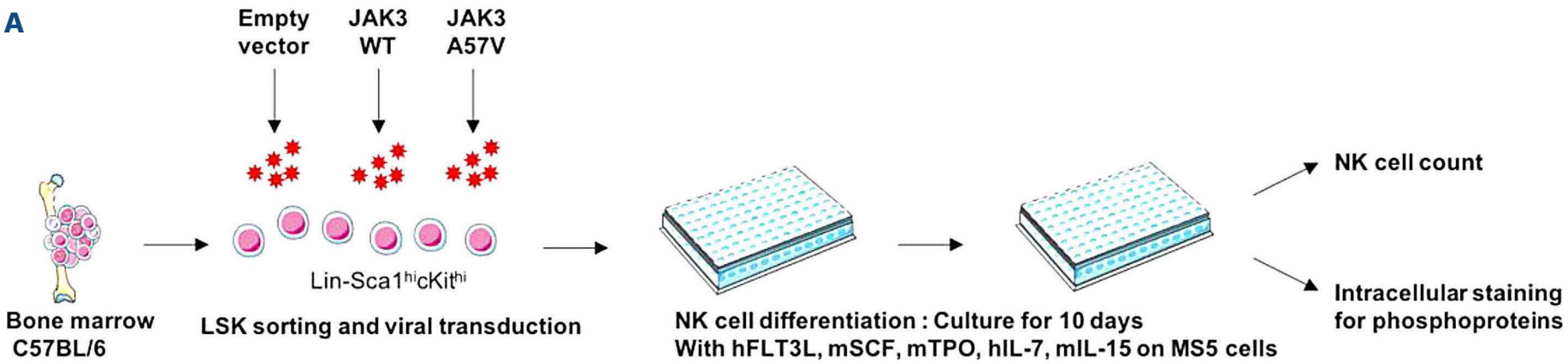

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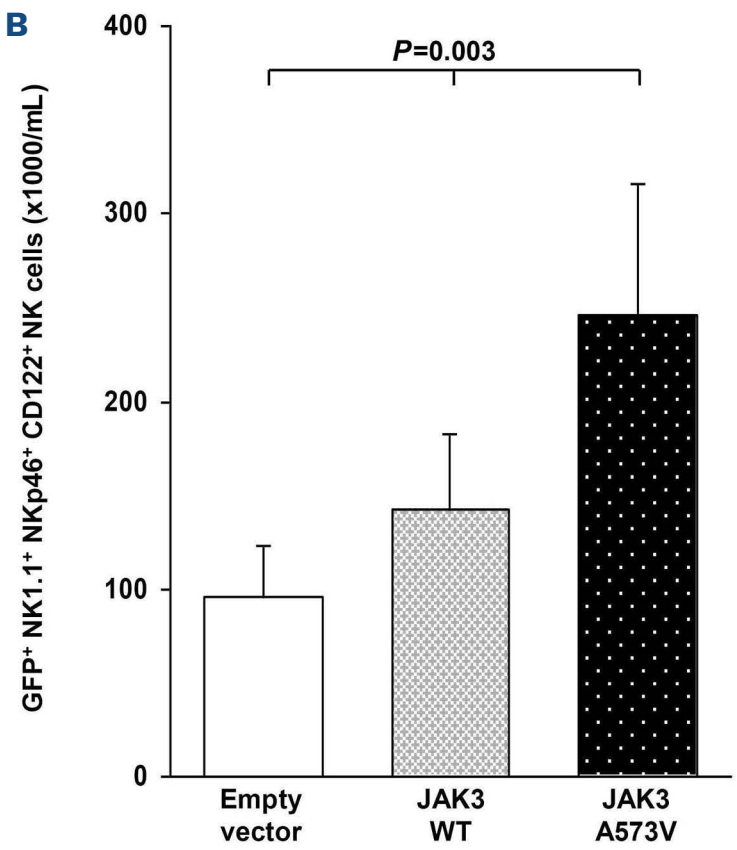

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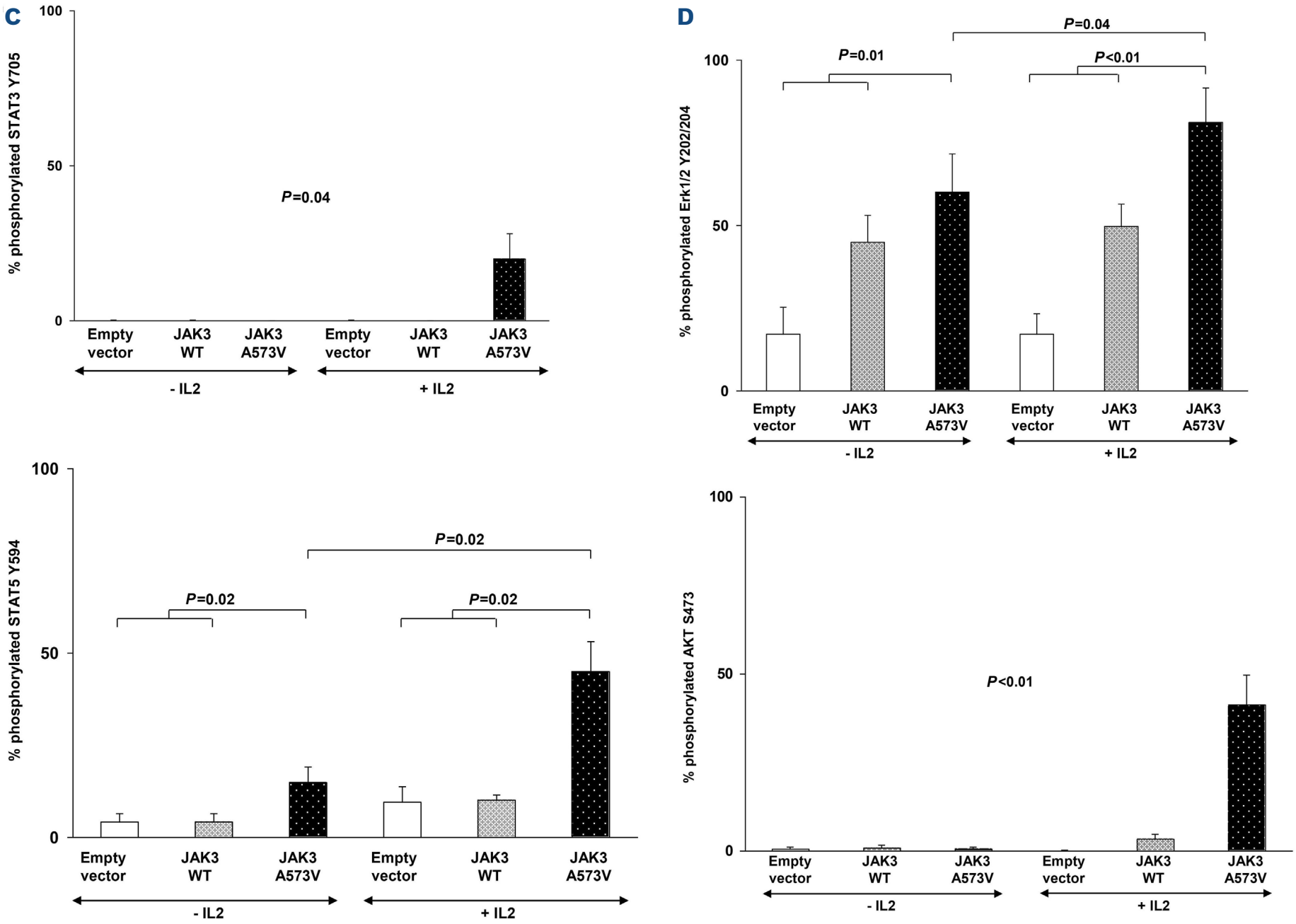
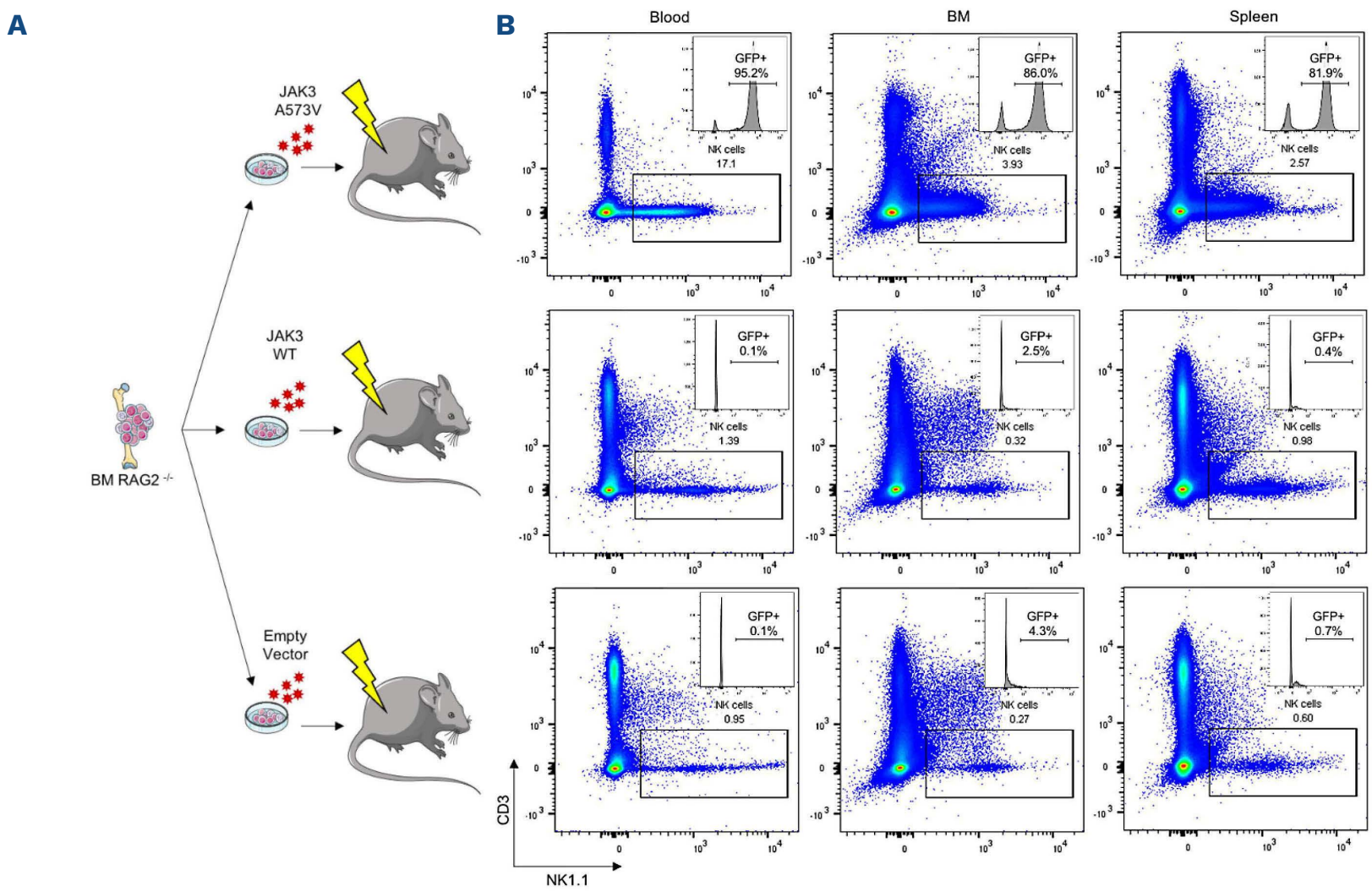


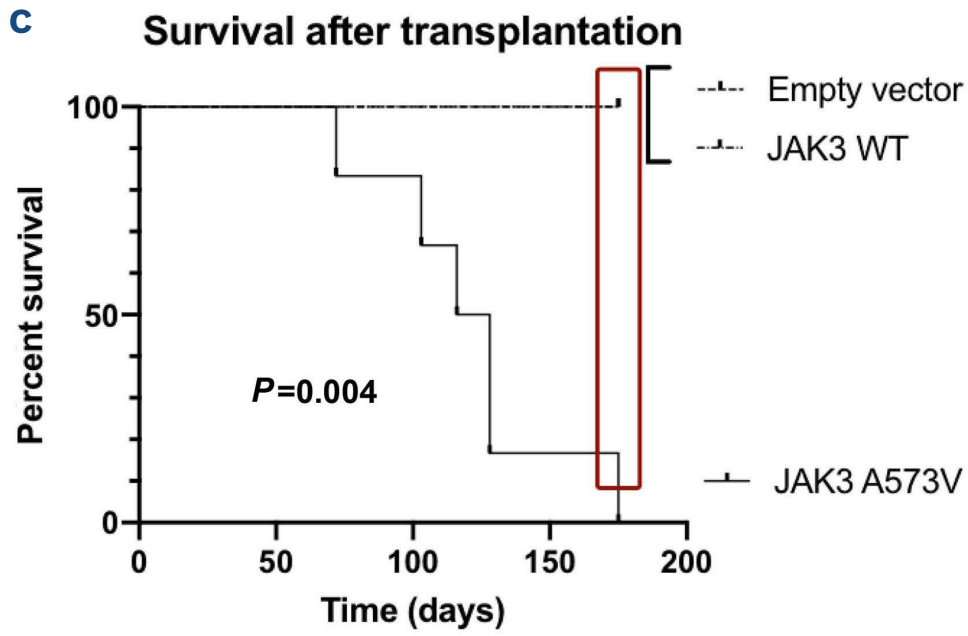
Figure 1. Generation of murine natural killer cells from LSK progenitors. (A) Experimental procedure. Five 6- to 10-week-old C57BL/6H mice were pooled in each experiment. Freshly isolated bone marrow cells were treated with Fc-block (CD16/CD32) and stained with biotin-conjugated lineage antibodies (CD3 [145-2C11], Gr-1 [RB6-8C5], B220 [B-220], and TER-119 [TER-119]). Lineage-positive cells were first depleted by magnetic-activated cell separation using Streptavidin Microbeads (BD Biosciences, Le Pont de Claix, France). Cells were then stained with anti-CD3-APC/Cy7, anti-Gr1-APC/Cy7, anti-B220-APC/Cy7, anti-TER119-APC/Cy7, anti-Sca1-FITC (E13-161.7), and anti-c-Kit-PerCP/Cy5.5 (2B8) and LSK cells were sorted with a Beckton Dickinson FACSInflux. All antibodies were purchased from Ozyme (Saint Quentin en Yvelines, France), except for biotin-conjugated lineage antibodies and the anti TER119-APC/Cy7, which were purchased from BD Biosciences (Le Pont de Claix, France). LSK cells were then transduced with $JAK3^{A573V}$, $JAK3^{WT}$ or empty vector as previously described[8,9] and cultured in 96-well plates on MS5 stromal cells for 10 days in Dulbecco modified Eagle medium with murine stem cell factor (25 ng/mL), murine thrombopoietin (10 ng/mL), human interleukin-7 (10 ng/mL), murine interleukin-15 (50 ng/mL) (all from PeproTech, Rocky Hill, NJ, USA) and human Fms-like tyrosine kinase 3-ligand (10 ng/mL) (Celldex Therapeutics, Inc., Needham, MA, USA), for natural killer (NK)-cell differentiation. Cells were then stained with anti-CD3-APC, anti-NK1.1-PerCp/Cy5.5, anti-NKp46-PECy7, as well as intracellular Alexa Fluor® 647 fluorochrome-conjugated antibodies for PY-STAT3 (Y705), PY-STAT5 (Y594) or PY-ERK 1/2 (Y202/204) or phospho-AKT (S473) after permeabilization with Perifix expose purchased from Beckman Coulter (Villepinte, France), according to the manufacturer's instructions. (B) The bar chart shows that the number of mature murine NK cells ($CD3^-$, $NK1.1^+$, $NKp46^+$) obtained from 10^4 LSK cells transduced with $JAK3^{A573V}$, $JAK3^{WT}$ or empty vector, after 10 days of culture. Results are the mean ± standard deviation of three independent experiments. The statistical significance was calculated by a Kruskal-Wallis test. (C) Analysis of intracellular staining for Y705-STAT3, Y594-STAT5, Y202/204-ERK1/2, and S573-AKT. Results are provided in percentages from $CD3^-$ $NK1.1^+$ $NKp46^+$ NK cells. LSK: Lin^-, Sca^{hi}, Kit^{hi}; WT: wild-type; mSCF: murine stem cell factor; mIL-3: murine interleukin-3; hFlt3-L: human Fms-like tyrosine kinase 3-ligand; hIL-7: human interleukin-7; mIL-15: murine interleukin-15; hIL-2: human interleukin-2; NK: natural killer; GFP: green fluorescent protein.

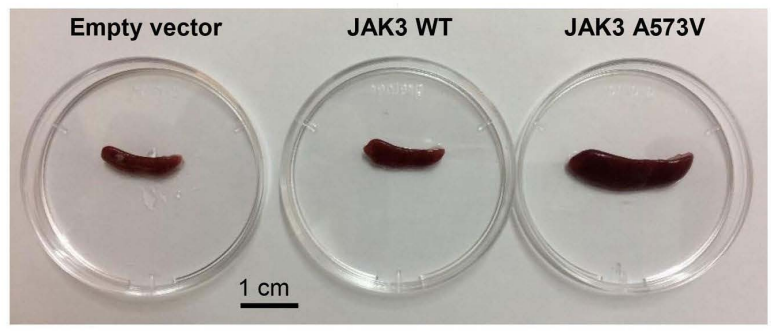
or $JAK3^{WT}$ remained alive and disease-free (log-rank test, *P*=0.004) (Figure 2C). Moreover, the blood cell count of two sick $JAK3^{A573V}$ mice before autopsy showed pancytopenia, with the lymphopenia and thrombocytopenia being particularly pronounced (*Online Supplementary Table S2*), a hallmark of hemophagocytic syndrome.[10] Autopsy of these two mice was remarkable for the spleen enlargement (492±285 mg *vs.* 86±9 mg and 82±9 mg in mice trans-

planted with bone marrow cells transduced with JAK3^{WT} and with empty vector, respectively) (Figure 2D). Histopathological analysis of the spleen, liver, bone marrow and lung showed massive and destructive infiltration by malignant cells (Figure 2E). Immunostaining performed on spleen and lung, which were the most massively infiltrated tissues, was positive for NK1.1 and for cytoplasmic CD3ε, consistent with a NK-cell proliferation. We verified that JAK3 is constitutively phosphorylated in the activating Y980 residue in these tumors. In contrast, we found no tumoral infiltration in empty vector- and JAK3^{WT}-transplanted mice. In liver biopsies, shown as illustrative tissues, we only observed small non-tumoral (probably autologous) CD3^{+} lymphocytes on portal tracts, suggesting mild inflammation (Figure 2F).

Lastly, multiple images of hemophagocytosis were recognizable on both spleen and lung samples (Figure 3A) and were unequivocally detected in the cytoplasm of CD68^{+} activated macrophages (Figure 3B). Malignant cells expressed interferon-γ (Figure 3C), whereas macrophages expressed tumor necrosis factor-α (Figure 3D), consistent with the histopathological pictures of hemophagocytosis. Here we took advantage of the identification of JAK3^{A573V} mutation as a model of JAK3 deregulation to explore the







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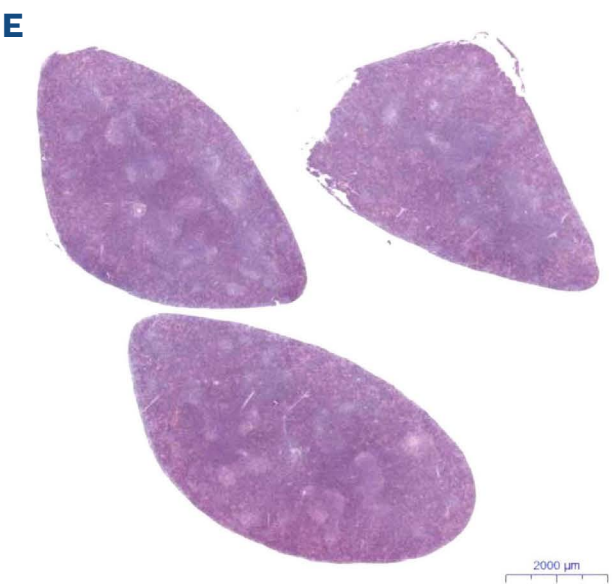

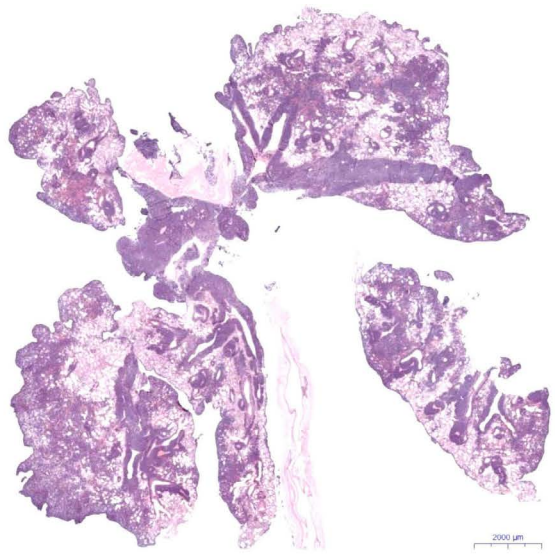

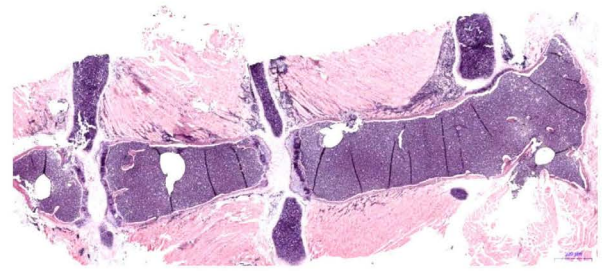

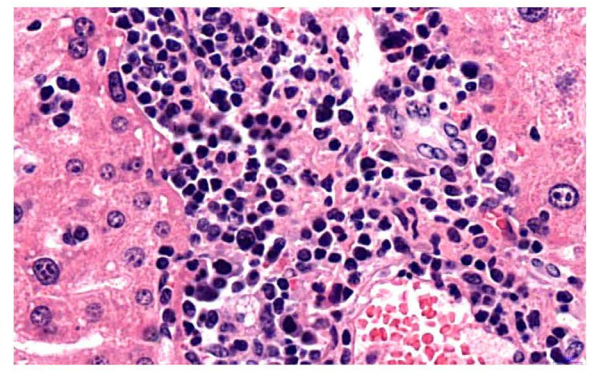

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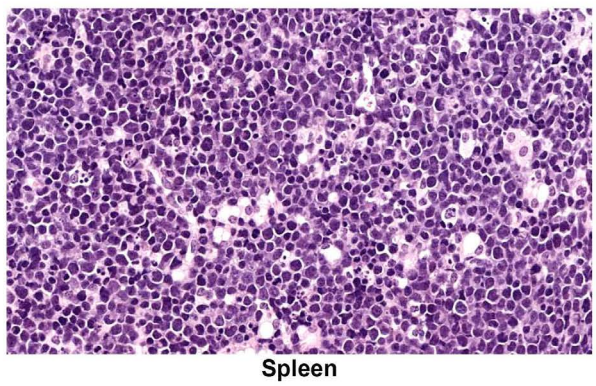

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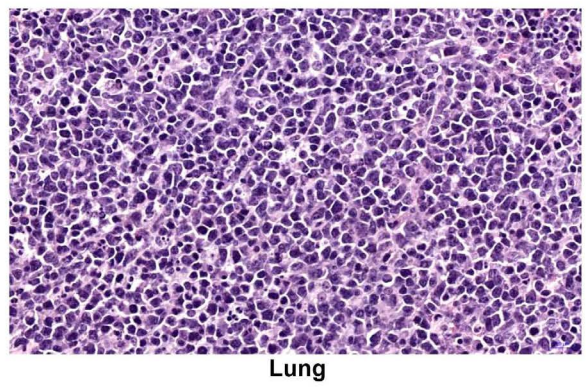

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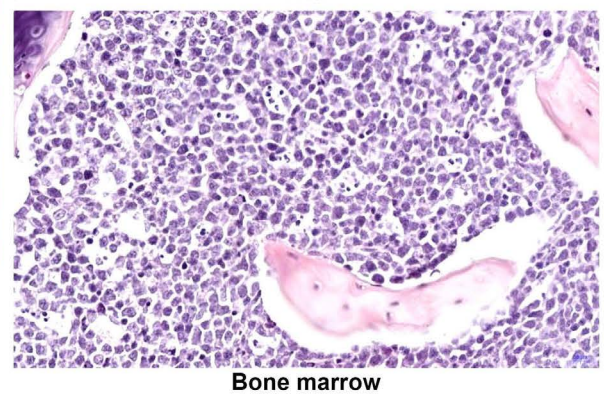

Bone marrow

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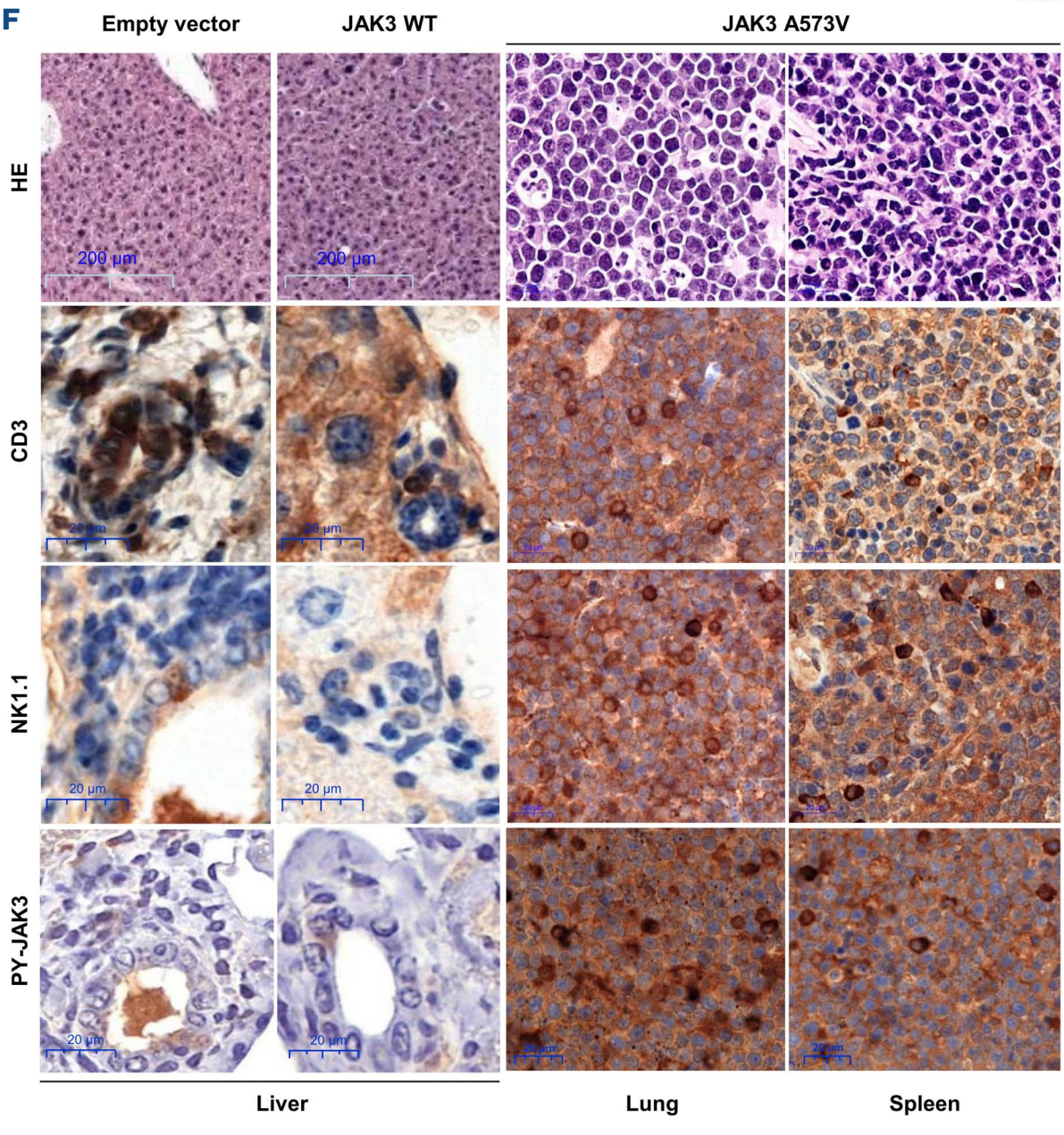

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Figure 2. Natural killer cells expressing JAK3^{A573V} generate a massive lymphoproliferative disease in mice. (A) Eight- to 10-week-old Rag2$^{-/-}$ donor mice were injected intraperitoneally with 150 mg/kg 5-fluorouracil (5-FU; Sigma-Aldrich, St Louis, MO, USA) 5 days prior to bone marrow collection from iliac bones, femora and tibiae. Bone marrow cells transduced with JAK3^{A573V}, JAK3^{WT} or empty vector were administered intravenously to sublethally irradiated C57BL/6 recipient mice (N=6 for each condition). (B) NK1.1^{+}/CD3^{-} eGFP^{+} cells from the peripheral blood, bone marrow or spleen of mice. Cells were harvested at the time of autopsy. (C) Survival of wild-type C57Bl/6 mice transplanted with JAK3^{A573V}-, JAK3^{WT}- and empty vector-transduced bone marrow cells from Rag2$^{-/-}$ mice. The red box represents the time of cell harvesting for immunophenotyping analysis and autopsy of the last diseased JAK3^{A573V}-transplanted mouse as well as healthy JAK3^{WT}- and empty vector-transplanted mice. Log-rank empty vector or JAK3^{WT} *vs*. JAK3^{A573V}, *P*=0.004. (D) Comparison of spleen size between conditions. (E) Hematoxylin & eosin staining showing the infiltration of spleen, lung, bone marrow (sternum), and liver in peri-portal spaces. (F) Hematoxylin & eosin staining and immunostaining with CD3ε (primary antibody: clone SP7, ThermoFisher Scientific SAS, Illkirch, France), NK1.1 (clone PK136, ThermoFisher Scientific SAS, Illkirch, France) and PY-JAK3 (D44E3, Cell Signaling Technology, Danvers, MA, USA) of spleen, lung and liver tissue. Specimens were counterstained with the corresponding secondary antibodies. BM: bone marrow; eGFP: enhanced green fluorescent protein. Rag2: recombination activating gene 2; LSK: Lin^{-}, Sca^{hi}, Kit^{hi}. HE: hematoxylin & eosin; PY-JAK3: phosphorylated JAK3.

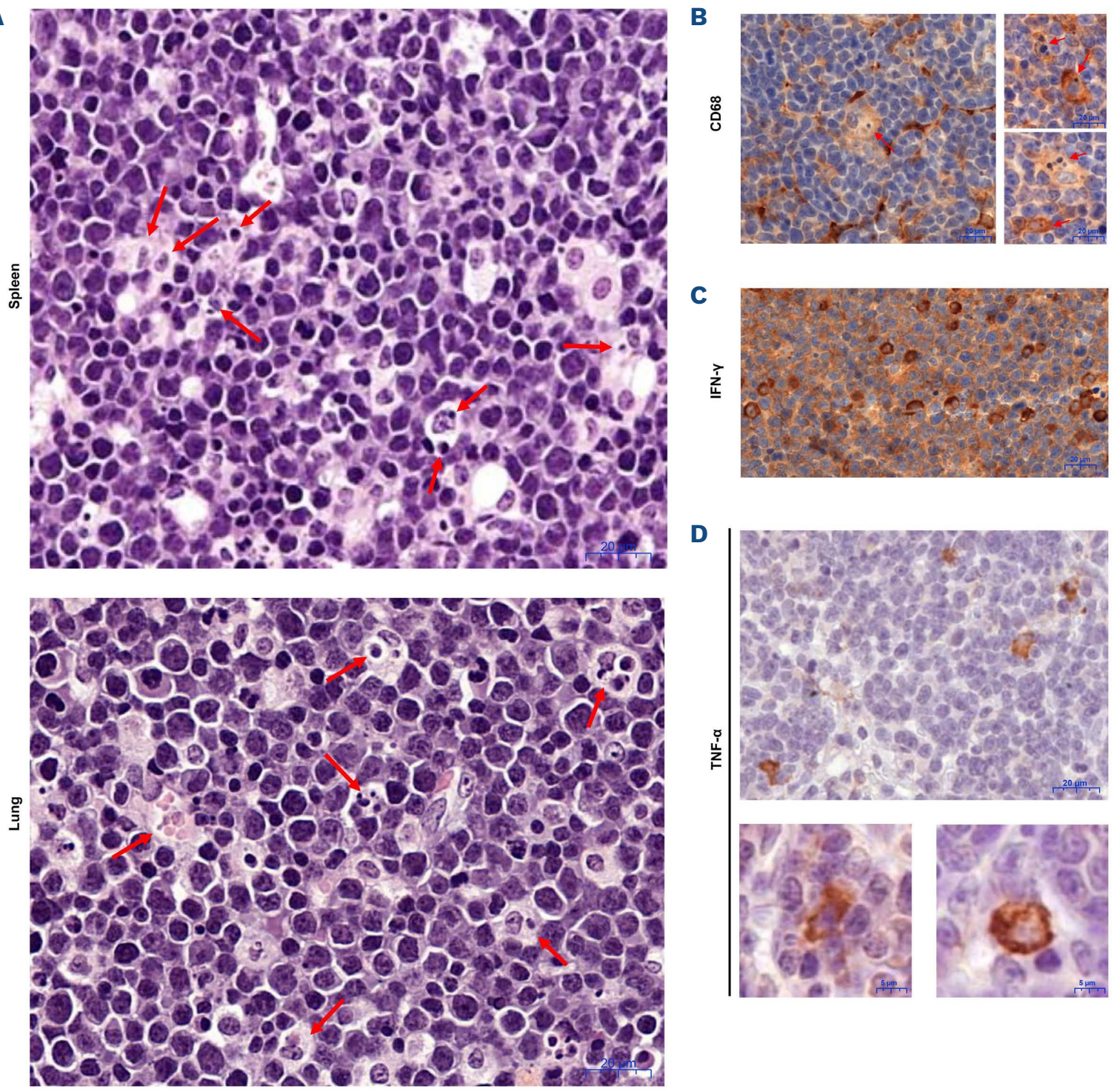

Figure 3. Mice with JAK3^{A573V}-induced lymphoproliferative disease develop hemophagocytosis. (A) Macrophages phagocytosing cells (red arrows) in spleen and lung tissue. (B) CD68 stain (clone 514H12, Leica Biosystems, Nanterre, France) shows numerous macrophages as large, irregularly shaped CD68^{+} cells. Detail of CD68 stain shows active phagocytosis by CD68^{+} macrophages of lymphocytes (red arrows). (C) Interferon-γ stain (clone ab216642, Abcam, Paris, France) shows interferon-γ production by malignant cells. (D) Tumor necrosis factor-α (TNF-α) stain (AF410-NA, R&D System, Minneapolis, MN, USA) shows TNF-α production by macrophages, identified as large irregularly shaped cells with an abundant cytoplasm. A detailed morphology of TNF-α-secreting macrophages is provided. HE: hematoxylin & eosin; IFN-γ: interferon-γ. TNF-α: tumor necrosis factor-α.

consequences of a constitutively activated JAK3 in NK-cell neoplasms, and its role in the occurrence of hemophagocytic syndrome. By reproducing an animal model of aggressive NKTCL with the features of hemophagocytic syndrome, we found evidence that JAK3 deregulation in NKTCL provides a growth advantage to malignant cells,[3,4] but also leads to an excessive production of interferon-γ, accounting for the systemic manifestations associated with NKTCL, such as hemophagocytic syndrome.[11,12] Given that deregulated JAK3 is a common feature in NKTCL, our findings provide an explanation for the high prevalence of hemophagocytic syndrome reported in advanced NKTCL.[13] Notably, the physiological regulation of interferon-γ secretion was related to multiple signaling proteins, including those in the STAT family and PI3-kinase and MAP-kinase pathways,[14,15] and their constitutive activation in the presence of deregulated JAK3 is in accordance with our model. An excessive production of interferon-γ, in turn, activates macrophages that release tumor necrosis factor-α. The conjunction of both cytokines accounts for the usual features of hemophagocytic syndrome, including fever and wasting, acute cytopenia, hyperferritinemia, hypertriglyceridemia, hyponatremia and hypofibrinogenemia.[10]

The crucial role of deregulated JAK3 in the pathophysiology of NKTCL, accounting for both growth advantage and excessive interferon-γ secretion, but also invasiveness through an amoeboid, matrix metalloproteinase-independent mechanism,[3] makes this oncogenic protein targetable with specific inhibitors already approved for the treatment of inflammatory diseases.

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Disclosures

No conflicts of interest to disclose.

Contributions

AP performed experiments on mice and wrote the first version of the manuscript. SM, GM and MA performed experiments *in vitro*. MW, YZ, HL and FB performed experiments on mice. PCe performed histopathological analyses. ES, FL and PCo designed experiments and supervised the work.

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Data-sharing statement

Data from the current work are available on request.

References

1. Bigenwald C, Fardet L, Coppo P, et al. A comprehensive analysis of lymphoma-associated haemophagocytic syndrome in a large French multicentre cohort detects some clues to improve prognosis. Br J Haematol. 2018;183(1):68-75.
2. Wang H, Fu BB, Gale RP, Liang Y. NK-/T-cell lymphomas. Leukemia. 2021;35(9):2460-2468.
3. Bouchekioua A, Scourzic L, de Wever O, et al. JAK3 deregulation by activating mutations confers invasive growth advantage in extranodal nasal-type natural killer cell lymphoma. Leukemia. 2014;28(2):338-348.
4. Koo GC, Tan SY, Tang T, et al. Janus kinase 3-activating mutations identified in natural killer/T-cell lymphoma. Cancer Discov. 2012;2(7):591-597.
5. Lee S, Park HY, Kang SY, et al. Genetic alterations of JAK/STAT cascade and histone modification in extranodal NK/T-cell lymphoma nasal type. Oncotarget. 2015;6(19):17764-17776.

6. Sim SH, Kim S, Kim TM, et al. Novel JAK3-activating mutations in extranodal NK/T-cell lymphoma, nasal type. Am J Pathol. 2017;187(5):980-986.
7. Coppo P, Gouilleux-Gruart V, Huang Y, et al. STAT3 transcription factor is constitutively activated and is oncogenic in nasal-type NK/T-cell lymphoma. Leukemia. 2009;23(9):1667-1678.
8. Malinge S, Ragu C, Della-Valle V, et al. Activating mutations in human acute megakaryoblastic leukemia. Blood. 2008;112(10):4220-4226.
9. Cornejo MG, Kharas MG, Werneck MB, et al. Constitutive JAK3 activation induces lymphoproliferative syndromes in murine bone marrow transplantation models. Blood. 2009;113(12):2746-2754.
10. Fardet L, Galicier L, Lambotte O, et al. Development and validation of the HScore, a score for the diagnosis of reactive hemophagocytic syndrome. Arthritis Rheumatol. 2014;66(9):2613-2620.
11. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. Blood. 2004;104(3):735-743.
12. Zoller EE, Lykens JE, Terrell CE, et al. Hemophagocytosis causes a consumptive anemia of inflammation. J Exp Med. 2011;208(6):1203-1214.
13. Jia J, Song Y, Lin N, et al. Clinical features and survival of extranodal natural killer/T cell lymphoma with and without hemophagocytic syndrome. Ann Hematol. 2016;95(12):2023-2031.
14. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. Adv Immunol. 2007;96:41-101.
15. Souza-Fonseca-Guimaraes F, Parlato M, de Oliveira RB, et al. Interferon-gamma and granulocyte/monocyte colony-stimulating factor production by natural killer cells involves different signaling pathways and the adaptor stimulator of interferon genes (STING). J Biol Chem. 2013;288(15):10715-10721.