Table 3. Results of a Logistic Regression Analysis

		Univariate		Multivariate (stepwise)	
Characteristics		Odds Ratio (95% Confidence Interval)	P-Value	Odds Ratio (95% Confidence Interval)	P-Value
Age		1.000 (0.954-1.048)	.988		
Sex	male versus female	1.799 (0.464-6.982)	.396		
Underlying disease	lymphoid malignancy versus others	1.312 (0.388-4.443)	.662		
Disease status	nonearly versus early	1.385 (0.393-4.876)	.612		
Conditioning regimen	Reduced intensity versus myeloabrative	2.491 (0.707-8.777)	.155		
Stem cell source	non-CB	1(referrence)	–	1(referrence)	–
	CB (previous HSCT −)	1.052 (0.119-9.313)	.964	1.052 (0.119-9.313)	.964
	CB (previous HSCT +)	13.58 (3.447-53.52)	<.001	13.58 (3.447-53.52)	<.001
HLA	mismatched versus matched	1.933 (0.542-6.889)	.309		
GVHD prophylaxis	CI alone	1(referrence)	–		
	CI plus sMTX	0.197 (0.035-1.107)	.065		
	CI plus MMF	2.800 (0.427-18.38)	.283		

GVHD indicates graft-versus-host disease; CB, cord blood; HSCT, hematopoietic stem cell transplantation; MTX, methotrexate; MMF, mycophenolate mofetil; CI, calcineulin-inhibitor.

maintained at a high concentration, whereas several cases of CIPS have been reported for patients undergoing HSCT [18,19,36-39]. Moreover, in our study, 11 of 13 patients with HHV6-associated encephalitis/myelitis developed CIPS-like dysesthesia and/or pruritus. Although CI concentrations in these patients were maintained within the targeted ranges and reduction in concentrations was ineffective, HHV6 DNA was directly detected in CSF samples and administration of antiviral agents improved their symptoms, indicating the relation of the symptoms to the reactivation of HHV6. We found that 7 cases that developed CIPS following HSCT have been reported to date [18,19,36-39]; 3 developed CIPS after UCBT, and 4 developed CIPS after a second HSCT: 2 for UCBT and 2 for BMT, respectively. In addition, Kida et al. [38] showed that no possible cases of CIPS were observed among 189 allogeneic BMT and 65 allogeneic PBSCT recipients, whereas 2 of 34 cases that underwent UCBT developed CIPS. As shown in our study, patients who receive UCBT or a second HSCT have a high risk of occurrence of HHV6-associated encephalitis/myelitis. Based on these observations, we hypothesize that HHV6 reactivation might contribute to aggravation of CIPS, and therefore, CIPS might be one of the early manifestations of HHV6-associated encephalitis/myelitis. HHV6 reactivation, localized in the posterior horn of the spinal cord, where signals for both itch and pain sensations are transmitted via c-fibers or Aδ-fibers [40], may cause distinctive manifestations like CIPS. Indeed, all 4 of the cases who only developed CIPS-like dysesthesia and/or pruritus had positive results for HHV6 in their CSF samples, but not for head MRI (limbic encephalitis), and only 1 case with MRI scanning of the spine (case #13) showed multiple abnormal T2-high lesions. Because HHV6-associated encephalitis/myelitis and CIPS can often occur at 2 to 4 weeks posttransplantation, it is important to make a differential diagnosis of these 2 syndromes.

In summary, our results have clearly shown that 2 or more RIC-UCBT presents a high risk for the development of HHV6-associated encephalitis/myelitis, although there are the limitations of the retrospective single center analysis. Earlier diagnosis of and intervention for this complication with combination antiviral therapy may provide prolonged survival and neurologic benefits. Therefore, transplantation physicians should be aware that CIPS-like dysesthesia and pruritus might be early manifestations that correlate with the reactivation of HHV6, especially for patients who develop myelitis. These "high-risk" recipients can be potential candidates for prophylactic administration of antiviral agents in future investigations.

ACKNOWLEDGMENTS

REFERENCES

1. Yamanishi K, Okuno T, Shiraki K, et al. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. *Lancet.* 1988;1:1065-1067.
2. Drobyski WR, Knox KK, Majewski D, Carrigan DR. Brief report: fatal encephalitis due to variant B human herpesvirus-6 infection in a bone marrow-transplant recipient. *N Engl J Med.* 1994;330:1356-1360.
3. Bleggi-Torres LF, de Medeiros BC, Werner B, et al. Neuropathological findings after bone marrow transplantation: an autopsy study of 180 cases. *Bone Marrow Transplant.* 2000;25: 301-307.
4. Chik KW, Chan PK, Li CK, et al. Human herpesvirus-6 encephalitis after unrelated umbilical cord blood transplant in children. *Bone Marrow Transplant.* 2002;29:991-994.
5. Sostak P, Padovan CS, Yousry TA, Ledderose G, Kolb HJ, Straube A. Prospective evaluation of neurological complications

after allogeneic bone marrow transplantation. *Neurology*. 2003; 60:842-848.

6. Kishi Y, Miyakoshi S, Kami M, et al. Early central nervous system complications after reduced-intensity stem cell transplantation. *Biol Blood Marrow Transplant*. 2004;10:561-568.

7. Tanaka M, Taguchi J, Hyo R, et al. Human herpesvirus-6 encephalitis after unrelated cord blood transplantation. *Leuk Lymphoma*. 2005;46:561-566.

8. Mori T, Mihara A, Yamazaki R, et al. Myelitis associated with human herpes virus 6 (HHV-6) after allogeneic cord blood transplantation. *Scand J Infect Dis*. 2007;39:276-278.

9. Seeley WW, Marty FM, Holmes TM, et al. Post-transplant acute limbic encephalitis: clinical features and relationship to HHV6. *Neurology*. 2007;69:156-165.

10. Tasaka T, Matsuhashi Y, Sadhira K, et al. Diabetes insipidus following HHV-6 encephalitis after cord blood transplantation in acute myeloid leukemia. *Leuk Res*. 2009;33:202-204.

11. Zerr DM, Gupta D, Huang ML, Carter R, Corey L. Effect of antivirals on human herpesvirus 6 replication in hematopoietic stem cell transplant recipients. *Clin Infect Dis*. 2002; 34:309-317.

12. Sashihara J, Tanaka-Taya K, Tanaka S, et al. High incidence of human herpesvirus 6 infection with a high viral load in cord blood stem cell transplant recipients. *Blood*. 2002;100:2005-2011.

13. Kishi Y, Kami M, Miyakoshi S, et al. Early immune reaction after reduced-intensity cord-blood transplantation for adult patients. *Transplantation*. 2005;80:34-40.

14. Yamane A, Mori T, Suzuki S, et al. Risk factors for developing human herpesvirus 6 (HHV-6) reactivation after allogeneic hematopoietic stem cell transplantation and its association with central nervous system disorders. *Biol Blood Marrow Transplant*. 2007;13:100-106.

15. Wang FZ, Linde A, Hagglund H, Testa M, Locasciulli A, Ljungman P. Human herpesvirus 6 DNA in cerebrospinal fluid specimens from allogeneic bone marrow transplant patients: does it have clinical significance? *Clin Infect Dis*. 1999;28:562-568.

16. Ogata M, Kikuchi H, Satou T, et al. Human herpesvirus 6 DNA in plasma after allogeneic stem cell transplantation: incidence and clinical significance. *J Infect Dis*. 2006;193:68-79.

17. Muta T, Fukuda T, Harada M. Human herpesvirus-6 encephalitis in hematopoietic SCT recipients in Japan: a retrospective multicenter study. *Bone Marrow Transplant*. 2009;43:583-585.

18. Kida A, Ohashi K, Tanaka C, Kamata N, Akiyama H, Sakamaki H. Calcineurin-inhibitor pain syndrome following haematopoietic stem cell transplantation. *Br J Haematol*. 2004; 126:288.

19. Nishikawa T, Okamoto Y, Tanabe T, et al. Calcineurin-inhibitor-induced pain syndrome after a second allogeneic bone marrow transplantation for a child with aplastic anemia. *Pediatr Transplant*. 2009;13:641-644.

20. Saliba RM, de Lima M, Giralt S, et al. Hyperacute GVHD: risk factors, outcomes, and clinical implications. *Blood*. 2007;109: 2751-2758.

21. Gordon B, Lyden E, Lynch J, et al. Central nervous system dysfunction as the first manifestation of multiple organ dysfunction syndrome in stem cell transplant patients. *Bone Marrow Transplant*. 2000;25:79-83.

22. Fujimaki K, Mori T, Kida A, et al. Human herpesvirus 6 meningoencephalitis in allogeneic hematopoietic stem cell transplant recipients. *Int J Hematol*. 2006;84:432-437.

23. Narimatsu H, Terakura S, Matsuo K, et al. Short-term methotrexate could reduce early immune reactions and improve outcomes in umbilical cord blood transplantation for adults. *Bone Marrow Transplant*. 2007;39:31-39.

24. Uchida N, Wake A, Takagi S, et al. Umbilical cord blood transplantation after reduced-intensity conditioning for elderly patients with hematologic diseases. *Biol Blood Marrow Transplant*. 2008;14:583-590.

25. Takagi S, Masuoka K, Uchida N, et al. High incidence of haemophagocytic syndrome following umbilical cord blood transplantation for adults. *Br J Haematol*. 2009;147:543-553.

26. Ogata M, Satou T, Kawano R, et al. Correlations of HHV-6 viral load and plasma IL-6 concentration with HHV-6 encephalitis in allogeneic stem cell transplant recipients. *Bone Marrow Transplant*. 45:129-136.

27. Chavanet P, Bonnotte B, Guiguet M, et al. High concentrations of intrathecal interleukin-6 in human bacterial and nonbacterial meningitis. *J Infect Dis*. 1992;166:428-431.

28. Hsieh CC, Lu JH, Chen SJ, Lan CC, Chow WC, Tang RB. Cerebrospinal fluid levels of interleukin-6 and interleukin-12 in children with meningitis. *Childs Nerv Syst*. 2009;25:461-465.

29. Fujita A, Ihira M, Suzuki R, et al. Elevated serum cytokine levels are associated with human herpesvirus 6 reactivation in hematopoietic stem cell transplantation recipients. *J Infection*. 2008;57: 241-248.

30. Takatsuka H, Takemoto Y, Yamada S, et al. Complications after bone marrow transplantation are manifestations of systemic inflammatory response syndrome. *Bone Marrow Transplant*. 2000; 26:419-426.

31. Nunoi H, Mercado MR, Mizukami T, et al. Apoptosis under hypercytokinemia is a possible pathogenesis in influenza-associated encephalopathy. *Pediatr Int*. 2005;47:175-179.

32. Lucas VP, Ponge TD, Plougastel-Lucas ML, Glemain P, Hourmant M, Soulillou JP. Musculoskeletal pain in renal-transplant recipients. *N Engl J Med*. 1991;325:1449-1450.

33. Grotz WH, Breitenfeldt MK, Braune SW, et al. Calcineurin-inhibitor induced pain syndrome (CIPS): a severe disabling complication after organ transplantation. *Transpl Int*. 2001;14:16-23.

34. Stevens JM, Hilson AJ, Sweny P. Post-renal transplant distal limb bone pain. An under-recognized complication of transplantation distinct from avascular necrosis of bone? *Transplantation*. 1995;60:305-307.

35. Malat GE, Dupuis RE, Kassman B, et al. Tacrolimus-induced pain syndrome in a pediatric orthotopic liver transplant patient. *Pediatr Transplant*. 2002;6:435-438.

36. Fujii N, Ikeda K, Koyama M, et al. Calcineurin inhibitor-induced irreversible neuropathic pain after allogeneic hematopoietic stem cell transplantation. *Int J Hematol*. 2006;83:459-461.

37. Takashima S, Numata A, Miyamoto T, et al. Acute lymphoblastic leukemia presenting with calcineurin-inhibitor induced pain syndrome after a second allogeneic bone marrow transplantation. *Rinsho Ketsueki*. 2006;47:1372-1376.

38. Kida A, Ohashi K, Kobayashii T, et al. Incapacitating lower limb pain syndrome in cord blood stem cell transplant recipients with calcineurin inhibitor. *Pathol Oncol Res*. 2004;10:204-206.

39. Lavoratore SR, Navarro OM, Grunebaum E, et al. Cyclosporine-induced pain syndrome in a child undergoing hematopoietic stem cell transplant. *Ann Ppharmacother*. 2009;43:767-771.

40. McGlone F, Reilly D. The cutaneous sensory system. *Neurosci Biobehav Rev*. 34:148–159.

JAK2 V617F uses distinct signalling pathways to induce cell proliferation and neutrophil activation

Seido Oku,[1] Katsuto Takenaka,[1] Takuro Kuriyama,[1] Kotaro Shide,[2] Takashi Kumano,[1] Yoshikane Kikushige,[1] Shingo Urata,[1] Takuji Yamauchi,[1] Chika Iwamoto,[1] Haruko K. Shimoda,[2] Toshihiro Miyamoto,[1] Koji Nagafuji,[3] Junji Kishimoto,[4] Kazuya Shimoda[2] and Koichi Akashi[1,5]

[1]Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, [2]Faculty of Medicine, Department of Gastroenterology and Haematology, Miyazaki University, Miyazaki, [3]Department of Medicine, Division of Haematology, Kurume University School of Medicine, [4]Digital Medicine Initiative, Kyushu University, and [5]Centre for Cellular and Molecular Medicine, Kyushu University Hospital, Fukuoka, Japan

Summary

The acquired *JAK2* V617F mutation is observed in the majority of patients with *BCR-ABL1* negative chronic myeloproliferative neoplasms (MPN). *BCR-ABL1* negative MPN displays myeloproliferation with an elevated leucocyte alkaline phosphatase (LAP) activity, a neutrophil activation marker. We tried to separate the downstream signalling of JAK2 V617F to stimulate myeloproliferation and LAP activity. NB4, a myeloid lineage cell line, was transduced with *Jak2* V617F mutation or wild-type *Jak2*. We found that *Jak2* V617F mutation, but not wild-type *Jak2* enhanced LAP expression in NB4-derived neutrophils and proliferation of NB4 cells. JAK2 V617F induces constitutive phosphorylation of STAT3 and STAT5, and uses signalling targets such as Ras/MEK/ERK and PI3K/Akt pathways. By using MEK1/2 inhibitor U0126, PI3K inhibitor LY294002, and STAT3 or STAT5 siRNAs, JAK2 V617F was found to specifically use the STAT3 pathway to enhance LAP expression, while STAT5, Ras/MEK/ERK and PI3K/Akt, but not STAT3 pathways, were able to stimulate cell proliferation. These data strongly suggest that JAK2 V617F uses distinct signalling pathways to induce typical pathological features of MPN, such as high LAP activity and enhanced cell proliferation.

Keywords: myeloproliferative neoplasm, JAK2 V617F, STAT3, STAT5, leucocyte alkaline phosphatase.

The *BCR-ABL1* negative chronic myeloproliferative neoplasms (MPN) include polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) (Dameshek, 1951). The G-to-T point mutation at position 617 (V617F) in Janus kinase 2 (JAK2) that results in substitution of phenylalanine for valine has been reported in patients with *BCR-ABL1* negative MPN. This mutation is seen in approximately 95% of the patients with PV and in about 50% of the patients with ET or PMF (Baxter et al, 2005; James et al, 2005; Jones et al, 2005; Kralovics et al, 2005; Levine et al, 2005). JAK2 is a member of the non-receptor tyrosine kinase family that signals between type I cytokine receptors and downstream pathways, such as STAT3 (signal transducers and activators of transcription 3) and STAT5 (Witthuhn et al, 1993; Parganas

et al, 1998). These pathways are critical for cell growth and differentiation. The *JAK2* V617F mutation induces constitutive activation of the downstream signalling pathways such as STAT3/STAT5, Ras/MEK/ERK and PI3K/Akt: the expression of JAK2 V617F in cytokine-dependent cell lines confers cytokine-independent growth by constitutively activating STAT3, STAT5, Akt and ERK (James et al, 2005; Levine et al, 2005; Levine & Wernig, 2006; Shide et al, 2007). The enforced expression of JAK2 V617F in murine bone marrow cells *in vivo* resulted in erythrocytosis and subsequent myelofibrosis in recipient mice (James et al, 2005; Lacout et al, 2006; Wernig et al, 2006; Zaleskas et al, 2006). Therefore, it is possible that the *JAK2* V617F mutation directly induces pathogenic features of *BCR-ABL1* negative MPN.

Leucocyte alkaline phosphatase (LAP) is a monophosphoesterase that is the product of the gene encoding the liver/bone/kidney-type alkaline phosphatase (ALP) isoforms (Hayhoe & Quaglino, 1958; Weiss et al, 1988; Gianni et al, 1994; Sato et al, 1994). In neutrophils, LAP is stored in unique secretory vesicles, and is exported to the plasma membrane by chemotactic agents (Borregaard et al, 1987, 1992). Neutrophils play a critical role in host defence by phagocytozing and digesting microorganisms. This task is accomplished by mobilization of several types of neutrophil granules, including azurophilic (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules and secretory vesicles. These granules contain potent antimicrobial compounds, proteolytic enzymes and also many membrane proteins. The azurophilic granules are formed during the promyelocytic stage, while specific granules and gelatinase granules are formed in more matured cells. Secretory vesicles appear in segmented neutrophils, and constitute a reservoir of membrane protein needed for neutrophil-mediated inflammatory response. Alkaline phosphatase is located at the luminal face of the membrane in secretory vesicles. Acquisition of LAP enzymatic activity is a very late event during myeloid maturation, and indicates that mature neutrophils acquire secretory vesicles. Thus, LAP is an indispensable marker of functionally mature neutrophils (Stewart, 1974; Garattini & Gianni, 1996; Borregaard & Cowland, 1997; Falanga et al, 2000; Faurschou & Borregaard, 2003). In clinics, the level of LAP in neutrophils is scored to evaluate neutrophil activation, which was classically designated as the neutrophil alkaline phosphatase (NAP) score (Kaplow, 1955, 1963; Shibata et al, 1985). Evaluation of the LAP score has been useful to distinguish chronic myeloid leukaemia (CML) from BCR-ABL1 negative MPN, or from infection- or inflammation-associated leucocytosis: untreated CML patients usually have a low LAP score, whereas it is elevated in patients with PV, PMF and reactive leucocytosis.

In CML patients, the release of premature granulocytes from the bone marrow into the peripheral blood might result in low LAP scores (Dotti et al, 1999). It is, however, unclear why the LAP scores in patients with PV or PMF are elevated. Recent reports have shown that patients with BCR-ABL1 negative MPN carrying the JAK2 V617F mutation display higher LAP scores as compared to those carrying the wild-type JAK2 alleles (Passamonti et al, 2006; Basquiera et al, 2007; Kondo et al, 2008). This finding suggests that an elevated LAP score in these diseases could be due to constitutive activation of the JAK2 signalling via the JAK2 V617F mutation.

Neutrophil activation is the key feature of MPN as well as myeloproliferation. To understand the developmental mechanisms of MPN, we tried to separate the downstream signalling of JAK2 V617F in each pathway, and we used LAP expression as a neutrophil maturation/activation marker. The present study showed that the JAK2 V617F signalling stimulates LAP expression in neutrophils through specifically the activation of STAT3-dependent signalling pathway, whereas its stimulation of cell proliferation is dependent upon STAT5 activation.

Thus, the acquisition of JAK2 V617F at the haematopoietic stem cell (HSC) stage might induce at least two independent signals to alter myelopoiesis, including myeloproliferation and in elevation of the LAP score at the neutrophil stage, both of which are the pathological features specific to MPN.

Materials and methods

Cell lines and culture conditions

A human acute promyelocytic leukaemia (APL) cell line NB4, which retains t(15;17), was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). NB4 cells were cultured in RPMI 1640 medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (ICN, Osaka, Japan). NB4 cells were differentiated into neutrophil-like cells after they were cultured with all-trans retinoic acid (ATRA) (10 μmol/l) (Sigma-Aldrich, St. Louis, MO, USA) and granulocyte colony-stimulating factor (G-CSF) (10 ng/ml) (Sigma-Aldrich) for 4 d (Gianni et al, 1994). Cells were counted by trypan blue dye exclusion staining.

Preparation of neutrophils from patients and healthy donors

This study was approved by Institutional Review Board of Kyushu University and all patients gave informed consent. Heparinized peripheral blood samples were collected from 15 healthy volunteer donors, 15 patients with PV, 21 patients with ET, and nine patients with PMF according to the World Health Organization criteria. Neutrophils were isolated by dextran sedimentation, hypotonic lysis of contaminating erythrocytes, and centrifugation with lymphocyte separation medium (LSM) (MP Biomedicals, Irvine, CA, USA) as described previously (Sullivan et al, 1984).

Mutation analysis and DNA sequencing of JAK2

DNA from patient's neutrophils was extracted using the Blood and Cell Culture DNA Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. To examine the putative JAK2 V617F mutation, exon 14 of JAK2 was amplified using 5'-TATAGTCATGCTGAAAGTAGG-3' and 5'-TAACT-GAATAGTCCTACAGTG-3' primers as described previously (Shide et al, 2007). The polymerase chain reaction (PCR) products were sequenced directly using an ABI3130XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). Furthermore, when the JAK2 V617F mutation was not detected by direct sequencing, we used the three-primer allele-specific PCR assay as described previously (Baxter et al, 2005).

Evaluation of LAP expression

Leucocyte alkaline phosphatase expression was evaluated by LAP score, flow cytometric analysis, or ALP enzyme assay.

Leucocyte alkaline phosphatase scores were measured by the LAP staining kit (Muto, Tokyo, Japan) according to the manufacturer's instructions (Sakamoto, 1966; Shibata *et al*, 1985).

Flow cytometric analysis was used for the analysis of cell surface expression of LAP (sLAP). Isolated neutrophils were washed once in phosphate-buffered saline (PBS) (Wako) and stained with phycoerythrin (PE)-conjugated anti-human LAP (BD PharMingen, San Diego, CA, USA) or PE-conjugated mouse $IgG_1\kappa$ (isotype-matched negative control) (BD PharMingen). The mean fluorescent intensity (MFI) of sLAP was used as the LAP expression value as described previously (Shibano *et al*, 1999).

In the ALP enzyme assay, LAP expression in 5×10^5 NB4-derived neutrophils or neutrophils was measured by the SensoLyte™ p-Nitrophenyl phosphate (pNPP) alkaline phosphatase assay kit (Ana Spec, San Jose, CA, USA) according to the manufacturer's instructions.

Jak2 lentiviral infection

Murine *Jak2* cDNA was kindly provided by Dr J. Ihle (St. Jude Children's Research Hospital, Memphis, TN, USA). Oligonucleotide-directed mutagenesis was performed to substitute phenylalanine for valine at residue 617 of *Jak2* as described previously (Shide *et al*, 2007). For the wild-type *Jak2* or the *Jak2* V617F construct, each of the cDNAs were cloned downstream of an E1Fα promoter in a third-generation lentiviral vector backbone consisting of a reporter gene encoding GFP driven by the promoter of the human gene that encodes phosphoglycerate kinase. The control vector CEP contained only the gene encoding GFP. Vector construction was confirmed by sequencing. Viruses 'pseudotyped' with the vesicular stomatitis virus G protein were produced by transient transfection of 293T cells as described previously (Takenaka *et al*, 2007) and were concentrated by ultracentrifugation. Functional titres of virus producing wild-type JAK2 or JAK2 V617F, as determined by infection of HeLa cells, were 1×10^7 or 2×10^7 infectious particles/ml, respectively. Virus, at a multiplicity of infection of 40–50, was added to 24-well tissue culture plates of NB4 cells. Infected cells were collected on day 3. The efficiency of gene transfer into cells was 20–45%, as assessed by GFP fluorescence performed using a FACSCalibur flow cytometer (Becton Dickinson [BD] Biosciences, San Jose, CA, USA). GFP⁺ cells were sorted by a FACS Aria cell sorter (BD Biosciences) and then the GFP⁺ NB4 cells were cloned at one cell per well into 96-well tissue culture plates.

Inhibitions of JAK2, Ras/MEK/ERK and PI3K/Akt pathways by protein kinase inhibitors

We investigated the effects of protein kinase inhibitors on LAP expression in neutrophils/NB4-derived neutrophils, and on proliferation of NB4 cells. Protein kinase inhibitors used to suppress JAK2 and its downstream pathways were as follows:

AG490 (Calbiochem, San Diego, CA, USA) was used as a JAK2 inhibitor at a concentration of 5–50 μmol/l, U0126 (Calbiochem) as a MEK1/2 inhibitor at a concentration of 1–20 μmol/l, and LY294002 (Calbiochem) as a PI3K inhibitor at a concentration of 5–50 μmol/l.

Leucocyte alkaline phosphatase expression was evaluated by flow cytometric analysis after neutrophils were cultured with/ without protein kinase inhibitors in the presence of G-CSF (50 ng/ml) for 24 h.

Leucocyte alkaline phosphatase expression was evaluated by ALP enzyme assay after NB4 cells were cultured with ATRA (10 μmol/l), G-CSF (10 ng/ml), and protein kinase inhibitors for 4 d.

The proliferation of NB4 cells with medium alone or protein kinase inhibitors was evaluated for 4 d.

Inhibitions of STAT3/STAT5 pathways by small interfering RNAs (siRNAs)

To investigate the role of the STAT3 and STAT5 pathways in LAP expression in NB4-derived neutrophils and in proliferation of NB4 cells, STAT3 or STAT5 expression in NB4 cells was knocked down using double-stranded siRNAs. The siRNAs to knock down human STAT3, STAT5a and STAT5b were obtained from Invitrogen (Carlsbad, CA, USA). NB4 cells were transiently transfected with siRNAs using Nucleofector technology (Amaxa Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Non-specific siRNA (Invitrogen) was used as a negative control.

Leucocyte alkaline phosphatase expression was evaluated by ALP enzyme assay after NB4 cells were cultured with ATRA (10 μmol/l) and G-CSF (10 ng/ml) for 4 d.

The proliferation of NB4 cells with medium alone was evaluated for 5 d.

Western blotting

Total cell lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride nitrocellulose membranes (Amersham, Uppsala, Sweden) as described previously (Shide *et al*, 2008). Membranes were probed using the appropriate antibodies and visualized by enhanced chemiluminescence (Amersham). Anti-phospho Tyr^{705} STAT3, anti-phospho Ser^{727} STAT3, anti-phospho Tyr^{694} STAT5, anti-phospho Thr^{202}/Tyr^{204} ERK1/2, anti-ERK1/2, anti-phospho-Ser^{473} Akt, anti-Akt and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-JAK2, anti-STAT3, anti-STAT5a and anti-STAT5b antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Real-time quantitative PCR

Total RNA was extracted from NB4 cells. Reverse transcription was performed using a high-capacity cDNA

reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Power SYBR Green Master Mix (Applied Biosystems) and subsequent primers were used for real-time quantitative PCR amplification of the murine *Jak2* cDNA, using 5′-AAGTGGAGCTTCGGGAC-CACTCT-3′ and 5′-GCTTATCTTCATAGAACTGCAGC-3′ primers on an ABI 7000 sequence detection system (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using SAS or JMP software (SAS Institute Inc., Cary, NC, USA). LAP expressions among the groups were compared by Tukey's test or Dunnett's test. For inhibitor experiments, LAP expressions were compared using the least-squares mean test or paired *t*-test. For siRNAs experiments, LAP expressions were compared by paired *t*-test. For cell proliferation experiments, data were plotted on a single logarithmic graph and approximated by the straight line from the origin for each group. The gradients of the straight lines among the groups were compared by Tukey's test. Differences were considered statistically significant when the *P*-value was <0·05.

Results

Neutrophils with JAK2 V617F mutation express high levels of LAP in all types of MPN

The presence of the *JAK2* V617F mutation was documented by direct sequencing, or by allele-specific PCR of isolated neutrophils' DNA from *BCR-ABL1* negative MPN patients ($n = 45$). Patients were judged to be homozygous for the *JAK2* V617F mutation if their mutational load exceeded 50%. *JAK2* V617F mutation was identified in 13 of the 15 PV patients, 11 of the 21 ET patients and five of the nine PMF patients. Among them, five PV patients, eight ET patients and five PMF patients carried heterozygous mutations.

We analysed LAP expression of isolated neutrophils in *BCR-ABL1* negative MPN patients ($n = 45$) and healthy volunteer donors ($n = 15$) by flow cytometric analysis (Fig 1). *JAK2* V617F positive MPN patients ($n = 29$) had a higher LAP expression than *JAK2* V617F negative MPN patients ($n = 16$) or than healthy controls ($n = 15$) ($P < 0·01$). Among *JAK2* V617F positive MPN patients, those carrying homozygous mutation ($n = 11$) had higher levels of neutrophil LAP than those carrying heterozygous mutation ($n = 18$) ($P < 0·01$). Patients carrying heterozygous mutation also displayed higher LAP expression than healthy controls ($P < 0·05$), although the difference between *JAK2* V617F negative patients and patients carrying heterozygous mutation was not statistically significant. These data suggest that the expression level of JAK2 V617F correlates with the LAP levels in MPN neutrophils.
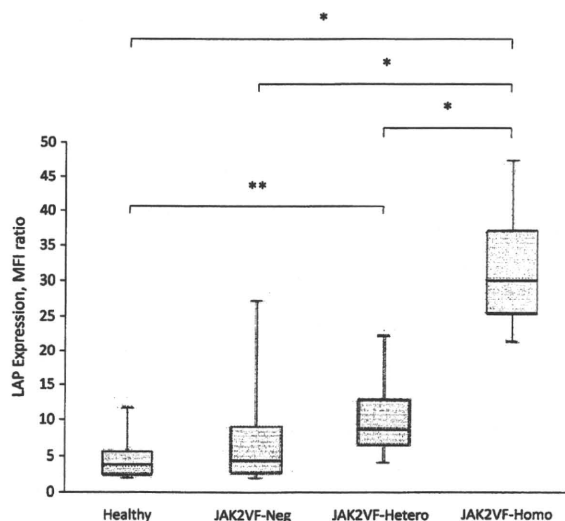

Fig 1. LAP expression of neutrophils in healthy volunteer donors ($n = 15$), *JAK2* V617F negative MPN (JAK2VF-neg, $n = 16$), *JAK2* V617F heterozygous MPN (JAK2VF-hetero, $n = 18$), and *JAK2* V617F homozygous MPN (JAK2VF-homo, $n = 11$). LAP levels were evaluated by flow cytometric analysis. LAP expression (ratio of mean fluorescence intensity, MFI) is shown in a box plot. JAK2VF-homo patients had a significantly higher level of LAP than those in the other three groups. *$P < 0·01$; **$P < 0·05$.

AG490, a JAK2 inhibitor, suppresses LAP expression in neutrophils with JAK2 V617F

To test whether the high level of LAP in *JAK2* V617F positive neutrophils is dependent upon JAK2 activation, we evaluated the effect of AG490, a JAK2 inhibitor, on LAP activity in *JAK2* V617F positive neutrophils. Neutrophils were purified from MPN patients with homozygous *JAK2* V617F, and were cultured for 24 h in the presence of AG490. AG490 significantly suppressed the LAP expression (Fig 2), confirming that the expression of LAP is dependent upon JAK2 activation in *JAK2* V617 positive neutrophils.

The enforced expression of JAK2 V617F stimulates the expression of LAP

To verify that JAK2 V617F signalling activates the expression of LAP in myeloid cells, we used NB4, a myeloid lineage cell line. NB4 was infected with lentiviral vectors harbouring wild-type *Jak2* or *Jak2* V617F mutation with GFP reporters. Infected cells were purified by positive expression of GFP by FACS. NB4 cells transfected with wild-type *Jak2* and *Jak2* V617F mutation showed an increased expression of JAK2 protein as compared to control by Western blotting, but their levels did not differ between these *Jak2*- or *Jak2* V617F-transduced NB4 clones (Fig 3A).

NB4 cells differentiated into neutrophil-like cells in the presence of ATRA and G-CSF for 4 d (Gianni *et al*, 1994). The NB4-derived neutrophils were then evaluated for LAP
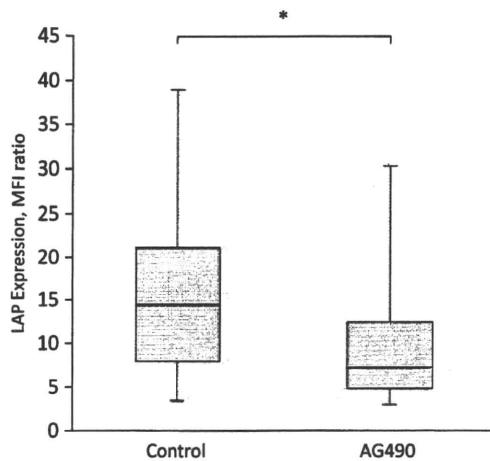
Fig 2. Effect of the AG490, a JAK2 inhibitor, on LAP expression in *JAK2* V617F positive neutrophils. Neutrophils from *JAK2* V617F positive MPN patients ($n = 16$) were cultured in medium in the presence of G-CSF (50 ng/ml), and the effect of addition of AG490 (50 μmol/l) on LAP expression was evaluated 24 h after culture on a flow cytometry. LAP expression (ratio of MFI) is shown in a box plot. AG490 suppressed LAP expression. *$P < 0.01$.

expression. We measured LAP levels in neutrophils differentiated from *Jak2* V617F clones, from wild-type *Jak2* clones, and from control clones with an empty vector by ALP enzyme assays. Representative data are shown in Fig 3A, B. Neutrophils from *Jak2* V617F clones expressed LAP at a much higher level, as compared to those from wild-type *Jak2* clones, whereas the expression levels of JAK2 in these clones were equivalent ($P < 0.01$) (Fig 3A, B). Thus, JAK2 V617F signalling is able to stimulate LAP expression in neutrophils.

STAT3 but not STAT5 signalling is required for JAK2 V617F-dependent LAP expression

JAK2 V617F mutation constitutively activates multiple JAK2 downstream signalling molecules, including STAT3/STAT5, Ras/MEK/ERK and PI3K/AKt (James *et al*, 2005; Levine *et al*, 2005; Levine & Wernig, 2006; Shide *et al*, 2007). We therefore tried to identify the signalling pathway responsible for LAP expression. We first tested the requirement of STAT3 or STAT5 for LAP expression stimulated by JAK2 V617F in *Jak2* V617F-transduced NB4 cells after inducing neutrophil lineage differentiation by ATRA and G-CSF. STAT3 and STAT5 expression were inhibited by STAT3 and STAT5 siRNAs respectively, in NB4-derived neutrophils: *Jak2* V617F-transduced NB4 cells were transduced with STAT3 or STAT5 siRNA, and cultured with ATRA and G-CSF for 4 d. As shown in Fig 4A, C, STAT3 siRNA, but not STAT5 siRNA, inhibited LAP expression ($P < 0.05$). Western blot analysis showed that STAT3 and STAT5 siRNAs specifically inhibited the expression of STAT3 and STAT5 respectively, but did not inhibit the signalling molecules on Ras/MEK/ERK and PI3K/Akt pathways
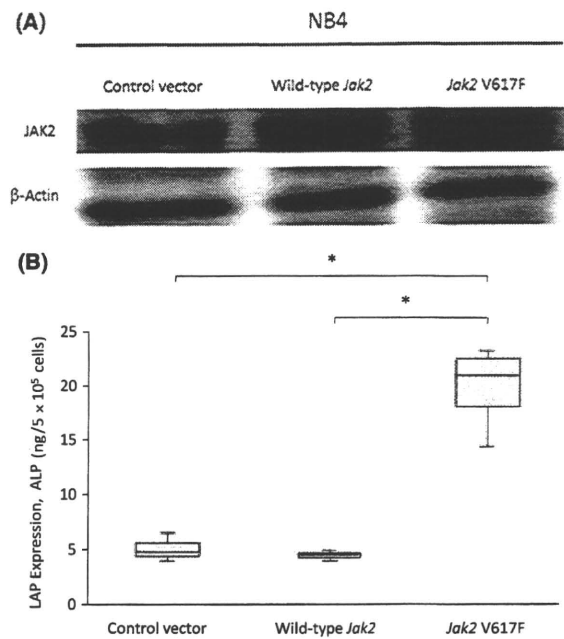

Fig 3. (A) JAK2 protein expression in NB4 cells by Western blotting. NB4 cells expressing control vector, wild-type JAK2 or JAK2 V617F were lysed and analysed by Western blotting with the indicated antibodies. Expression of JAK2 protein in wild-type *Jak2* and *Jak2* V617F-transduced NB4 cells was equivalent, but higher than that in the control vector-transduced cells. (B) Effect of the *Jak2* V617F mutation on LAP expression in NB4-derived neutrophils. NB4 cells expressing control vector, wild-type JAK2 or JAK2 V617F were cultured with ATRA (10 μmol/l) and G-CSF (10 ng/ml) for 4 d and differentiated into neutrophil-like cells. LAP expression was evaluated by ALP enzyme assay. LAP expression (ALP level) obtained from three independent experiments is shown in a box plot. *Jak2* V617F-transduced NB4-derived neutrophils had a higher LAP expression than the other two groups. *$P < 0.01$.

(Fig 4B, D). These data suggest that STAT3 is indispensable for LAP expression in JAK2 V617F signalling.

The LAP expression is dependent upon the MEK, but not the PI3K signalling pathway

We then tested the effects of MEK1/2 inhibitor (U0126) and PI3K inhibitor (LY294002) on LAP expression in *Jak2* V617F-transduced NB4 cells. LAP expression was evaluated by ALP enzyme assay. U0126 but not LY294002 significantly suppressed LAP expression in *Jak2* V617F-transduced NB4-derived neutrophils (Fig 5A, C). Further analysis of signalling molecules, such as ERK and Akt, downstream of MEK and PI3K respectively, revealed that U0126 and LY294002 efficiently inhibited phosphorylation of ERK and Akt respectively (Fig 5B, D).

Activated JAK2 transduces the signal cascade through phosphorylation of both the receptors and the major substrates, STATs. STAT3 is phosphorylated by JAK2 on a single tyrosine at position 705. This phosphorylated STAT3 is
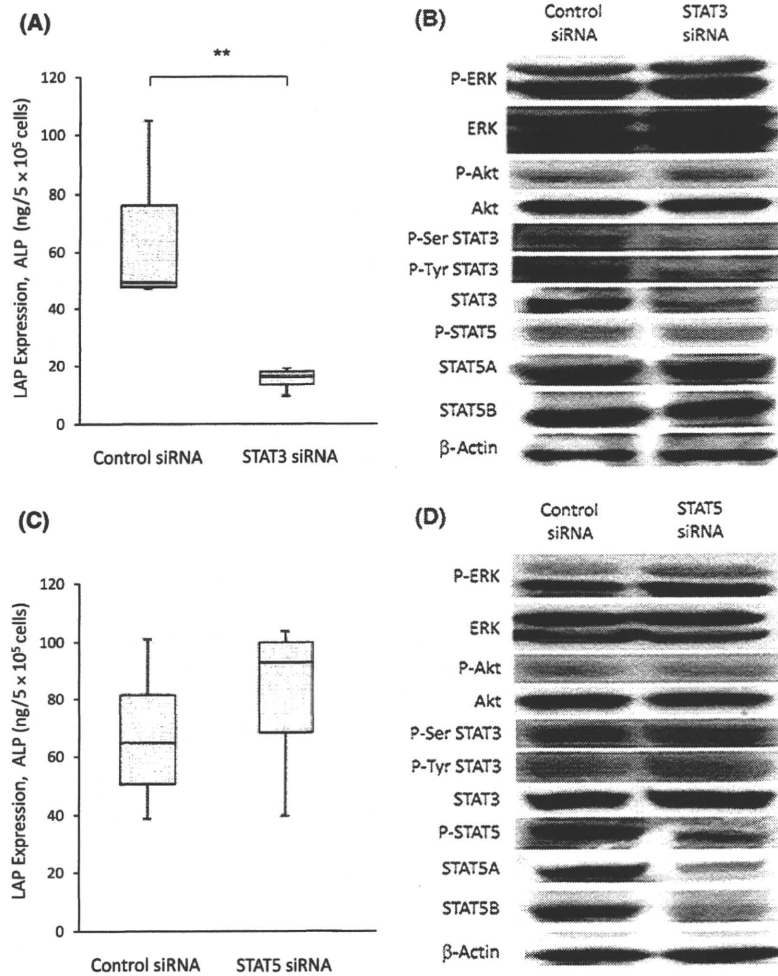
41

Fig 4. Effect of STAT3 and STAT5 siRNAs on LAP expression in NB4-derived neutrophils. *Jak2* V617F-transduced NB4 cells were transfected with control, STAT3 (A), or STAT5 (C) siRNA, and cultured with ATRA (10 µmol/l) and G-CSF (10 ng/ml) for 4 d and differentiated into neutrophil-like cells. LAP expression in NB4-derived neutrophils was evaluated by ALP enzyme assay. LAP expression (ALP level) obtained from three independent experiments is shown in a box plot. STAT3 siRNA decreased LAP expression, but STAT5 siRNA did not affect LAP expression ($P > 0.05$) **$P < 0.05$. Phosphorylation and expression of ERK, Akt, STAT3 and STAT5 proteins by Western blotting. NB4 cells expressing JAK2 V617F were transfected with control, STAT3 (B) or STAT5 (D) siRNA, and then cultured with ATRA (10 µmol/l) and G-CSF (10 ng/ml) for 2 d. Cells were then lysed and analysed by Western blotting with the indicated antibodies. STAT3 and STAT5 siRNAs inhibited only phosphorylation and expression of STAT3 and STAT5 proteins, respectively.

dimerized, migrates to the nucleus, and subsequently binds specific regulatory sequences to activate or repress transcription of target genes (Zhong *et al*, 1994; Ihle, 1995). Another phosphorylation event in a single serine at position 727 has been described that modulates transcriptional activity of STAT3. Serine phosphorylation is necessary to potentiate STAT3 transcriptional activity or negatively regulates STAT3 (Wen *et al*, 1995; Zhang *et al*, 1995; Jain *et al*, 1998; Sengupta *et al*, 1998). It has been reported that MEK/ERK mediates signalling to activate the JAK/STAT pathway through phosphorylation of STAT3 at Ser^{727} but not at Tyr^{705} (Chung *et al*, 1997). In fact, interestingly, U0126 inhibited STAT3 Ser^{727} but not Tyr^{705} phosphorylation (Fig 5B).

Among the MEK/ERK signalling cascades, signals to induce phosphorylation of STAT3 at Ser^{727} are especially required for LAP expression because STAT3 siRNA inhibited LAP expression but not ERK phosphorylation in our hands (Fig 4A, B). As STAT3 is indispensable for LAP expression (Fig 4A, B), the suppression of LAP expression by U0126 might be due to the inhibition of phosphorylation of STAT3 Ser^{727}. Importantly, LAP expression was inhibited only by U0126 also in neutrophils isolated from *JAK2* V617F positive MPN patients ($n = 10$) (Fig 5E, F). These data collectively suggest that JAK2 V617F stimulates the LAP expression also via MEK/ERK-dependent signalling pathway that phosphorylates STAT3 Ser^{727}.
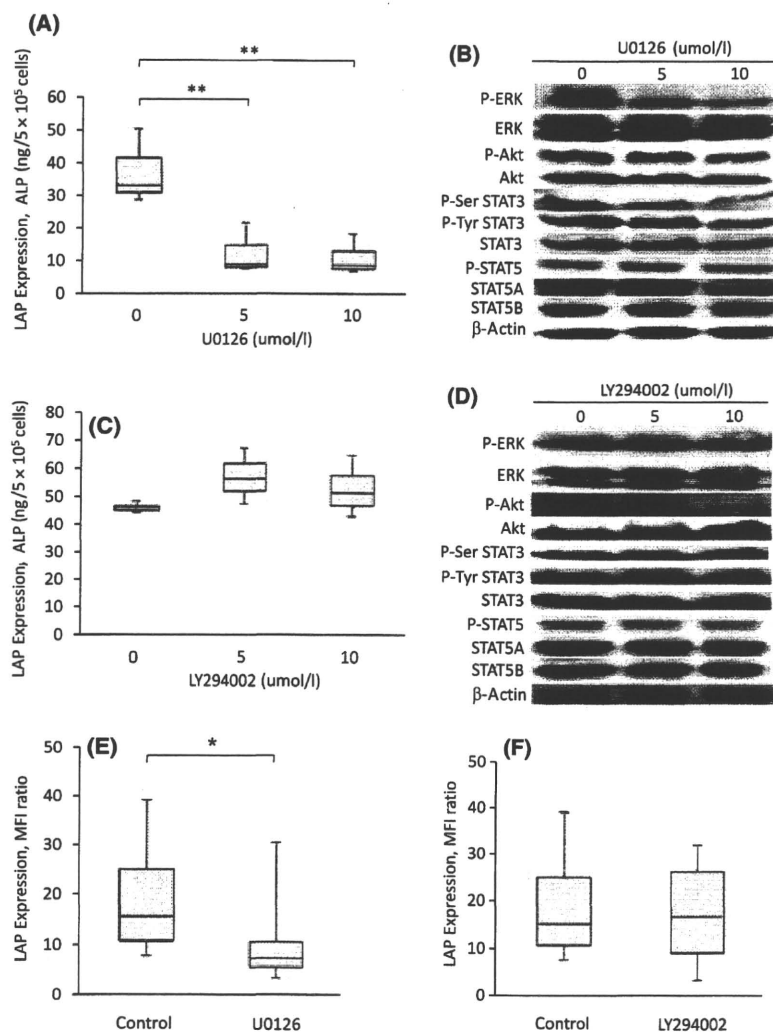
Fig 5. The effects of U0126 (MEK1/2 inhibitor) and LY294002 (PI3K inhibitor) on LAP expression in NB4-derived neutrophils. *Jak2* V617F-transduced NB4 cells were cultured with ATRA (10 μmol/l), G-CSF (10 ng/ml) and U0126 (0–10 μmol/l) (A) or LY294002 (0–10 μmol/l) (C) for 4 d and differentiated into neutrophil-like cells. LAP expression was evaluated by ALP enzyme assay. LAP expression (ALP level) obtained from three independent experiments is shown in a box plot. U0126 suppressed LAP expression in NB4-derived neutrophils, but LY294002 did not affect LAP expression ($P > 0.05$). **$P < 0.05$. Phosphorylation and expression of ERK, Akt, STAT3 and STAT5 proteins by Western blotting. *Jak2* V617F-transduced NB4 cells were cultured with ATRA (10 μmol/l), G-CSF (10 ng/ml) and U0126 (0–10 μmol/l) (B) or LY294002 (0–10 μmol/l) (D) for 24 h. Cells were then lysed and analysed by Western blotting with the indicated antibodies. The inhibitory effects of MAPK and PI3K pathways by U0126 and LY294002 were confirmed by decreased phosphorylation of ERK and Akt proteins, respectively. U0126 not only inhibited ERK phosphorylation but also STAT3 Ser[727] phosphorylation. However, LY294002 inhibited only phosphorylation of Akt and did not show any inhibition of phosphorylation of STAT3/STAT5. Neutrophils from *JAK2* V617F positive MPN patients ($n = 10$) were cultured in medium in the presence of G-CSF (50 ng/ml), and the effect of addition of U0126 (10 μmol/l) or LY294002 (50 μmol/l) on LAP expression was evaluated 24 h after culture on a flow cytometry. LAP expression (ratio of MFI) is shown in a box plot. LAP expression was inhibited only by U0126 (E) in neutrophils from *JAK2* V617F positive MPN patients, but LY294002 (F) ($P > 0.05$) *$P < 0.01$.

Jak2 V617F-induced cell proliferation requires STAT5 but not STAT3 signalling

Enforced expression of JAK2 V617F into NB4 cells significantly enhanced proliferation of NB4 cells *in vitro* (Fig 6A). To test whether stimulation for LAP expression and cell proliferation shared the signalling pathway, we tested the effect of inhibitors (U0126 and LY294002) and STAT3/STAT5 siRNAs on proliferation of NB4 cells. As shown in Fig 6B, C, both inhibitors significantly suppressed proliferation of *Jak2* V617F-transduced NB4 cells. Furthermore, in marked contrast to the LAP expression (Fig 4A, C), STAT5 siRNA but not STAT3 siRNA was able to suppress proliferation of *Jak2* V617F-transduced NB4 cells (Fig 6D, E). These data strongly suggest
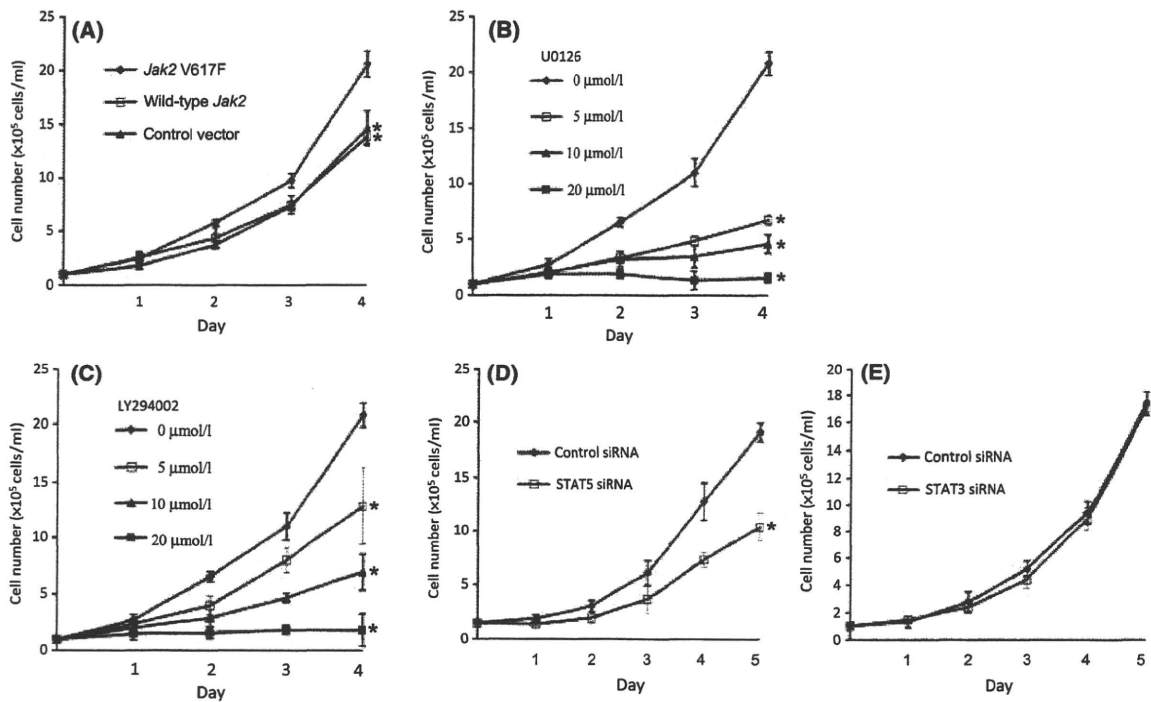
Fig 6. Effect of U0126 (MEK 1/2 inhibitor), LY294002 (PI3K inhibitor), STAT3 siRNA and STAT5 siRNA on proliferation of NB4 cells. (A) Effect of the *Jak2* V617F mutation on proliferation of NB4 cells. NB4 cells expressing wild-type JAK2 or JAK2 V617F were cultured for 4 d. The *Jak2* V617F mutation enhanced cell proliferation. *Jak2* V617F-transduced NB4 cells were cultured with U0126 (0–20 μmol/l) (B) or LY294002 (0–20 μmol/l) (C) for 4 d or cells were transfected with control, STAT5 (D) or STAT3 (E) siRNA, and then cultured with medium alone for 5 d. U0126, LY294002, STAT5 siRNA, but not STAT3 siRNA, inhibited cell proliferation. Cells were cultured at a density of 1×10^5 cells/ml and counted on the indicated days. The mean (±SD) cell numbers obtained from three independent experiments are shown in a line plot. *$P < 0.01$ compared with *Jak2* V617F (A), 0 μmol/l (B, C) or control siRNA (D).

that JAK2 V617F uses distinct pathways for stimulation of cell proliferation and neutrophil maturation represented by LAP expression; the former is dependent upon STAT5, Ras/MEK/ERK and PI3K pathways, whereas the latter is dependent upon STAT3.

Discussion

Determination of the LAP score has been a useful tool for differential diagnosis of CML and other MPN. However, the underlying mechanisms for elevation of LAP scores in *BCR-ABL1* negative MPN patients but not in CML has been unclear. We observed that patients with *JAK2* V617F homozygous mutations had a higher LAP expression than other patients (Fig 1). Similar results were reported previously (Passamonti *et al*, 2006; Basquiera *et al*, 2007; Kondo *et al*, 2008), thus suggesting that the LAP levels in neutrophils are dependent upon the level of JAK2 V617F expression. The AG490, a JAK2 inhibitor, significantly decreased LAP expression in neutrophils of *JAK2* V617F positive patients (Fig 2). In addition, the enforced expression of JAK2 V617F but not wild-type JAK2 induced a high level of LAP expression in NB4-derived neutrophils (Fig 3A, B). These data show that signalling from the JAK2 V617F can directly induce LAP activation.

It has been shown that the *JAK2* V617F mutation induces constitutive activation of downstream targets, such as STAT3, STAT5 and other signalling pathways including Ras/MEK/ERK and PI3K/Akt to alter cell proliferation, differentiation and apoptosis (James *et al*, 2005; Levine *et al*, 2005; Levine & Wernig, 2006; Shide *et al*, 2007). We investigated as to which pathway is responsible for inducing LAP expression and cell proliferation. The JAK2 V617F signalling pathways on LAP and cell proliferation based on our results is schematized in Fig 7.

Jak2 V617F mutation enhanced not only LAP expression but also cell proliferation of NB4, a myeloid lineage cell line (Fig 6A). The STAT3 pathway was specifically used when enhancing LAP expression (Fig 4A). On the other hand, STAT5, MEK/ERK and PI3K/Akt, but not STAT3 were used for cell proliferation (Fig 6B–E). The activated JAK2 kinase induces STAT3/STAT5 tyrosine phosphorylation, and then phosphorylated STATs are dimerized, enter the nucleus, and bind specific sequences to regulate transcription of target genes. This pathway is a major downstream signalling cascade of JAK/STAT, and has been considered to be critical in enhancing cell proliferation (Zhong *et al*, 1994; Ihle, 1995). On the other hand, JAK2 also phosphorylates signalling molecules in the Ras/MEK/ERK and PI3K/Akt pathways. We
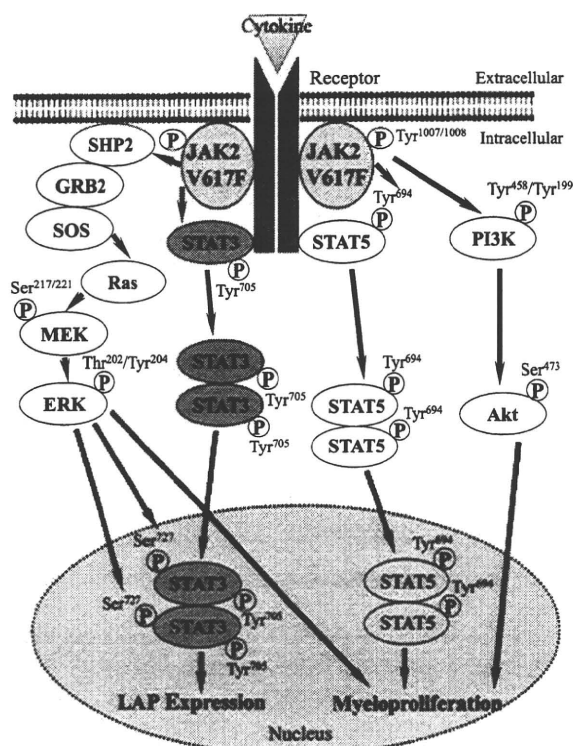
Fig 7. Schematic presentation of JAK2 V617F signalling to activate myeloproliferation and LAP expression. The activated JAK2 kinase induces STAT3/STAT5 tyrosine phosphorylation, and then phosphorylated STATs are dimerized, enter the nucleus, and bind specific regulatory sequences to regulate transcription of target genes. This pathway is a major downstream signalling cascade of JAK/STAT. Tyrosine phosphorylation of STAT5 mediates enhancing myeloproliferation. On the other hand, JAK2 also phosphorylates signalling molecules in Ras/MEK/ERK and PI3K/Akt pathways that are also responsible for cell proliferation. STAT3 has two phosphorylation sites, Tyr^{705} and Ser^{727}. Tyrosine phosphorylation is transduced by JAK2, whereas serine phosphorylation is transduced by MEK/ERK pathway. Serine phosphorylation of STAT3 probably affects the STAT3 transcriptional activation. JAK2 V617F stimulates the LAP expression also via MEK/ERK-dependent signalling pathway that phosphorylates STAT3 Ser^{727}. Thus, JAK2 V617F uses STAT3 pathway to induce LAP expression, and STAT5, Ras/MEK/ERK and PI3K/Akt pathways to stimulate myeloproliferation.

showed that both pathways are responsible for cell proliferation. STAT3 siRNA and the MEK1/2 inhibitor U0126 significantly reduced LAP expression (Figs 4A, 5A, E). Furthermore, U0126 not only inhibited ERK phosphorylation but also STAT3 Ser^{727} phosphorylation (Fig 5B). STAT3 has two phosphorylation sites, which are Tyr^{705} and Ser^{727} sites. Tyrosine phosphorylation of STAT3 is the major signalling cascade of JAK/STAT pathway described above. On the other hand, serine phosphorylation of STAT3 probably affects the STAT3 transcriptional activation, which is mainly transduced by the MEK/ERK pathway. In fact, a STAT3 S727A mutant, where Ser^{727} was replaced with an alanine, exhibited marked reduction in transcriptional activation, indicating that STAT3

Ser^{727} phosphorylation is essential for STAT3 transcriptional activation (Wen *et al*, 1995; Zhang *et al*, 1995). Consistent with this, LAP expression was suppressed significantly by STAT3 siRNA despite ERK phosphorylation (Fig 4A, B). The MEK/ERK signalling unrelated to STAT3 serine phosphorylation was not required for LAP expression. These findings show that STAT3 serine phosphorylation is mainly involved in enhancing LAP expression in Jak2 V617F signalling pathways.

JAK2 V617F mutation occurs at the HSC level (Jamieson *et al*, 2006; Kota *et al*, 2008). It is still unclear how this common mutation can induce three distinct MPN. Our data shows that the JAK2 V617F uses at least two distinct signalling pathways for enhancing LAP expression in neutrophil and cell proliferation of the myeloid lineage cell line (Fig 7). We and others have reported that the balance between constitutively activated STAT3/STAT5 or the expression level of JAK2 V617F could be a determinant for the type of MPN (Mesa *et al*, 2006; Teofili *et al*, 2007; Shide *et al*, 2008; Tiedt *et al*, 2008; Xing *et al*, 2008). To understand the developmental mechanisms of MPN, it is critical to understand the contribution of each signalling pathway toward the proliferation and lineage fate decision of HSCs in different types of MPN.

In conclusion, we obtained direct evidence that JAK2 V617F mainly induces elevation of LAP scores via the STAT3 pathway, whereas it stimulates cell proliferation via the STAT5, Ras/MEK/ERK and PI3K pathways. Thus, JAK2 V617F uses distinct signalling pathways to enhance LAP expression and myeloproliferation, typical characteristics in *BCR-ABL1* negative MPN.

Acknowledgements

References

Basquiera, A.L., Fassetta, F., Soria, N., Barral, J.M., Ricchi, B. & Garcia, J.J. (2007) Accuracy of leukocyte alkaline phosphatase score to predict JAK2 V617F mutation. *Haematologica*, **92**, 704–705.

Baxter, E.J., Scott, L.M., Campbell, P.J., East, C., Fourouclas, N., Swanton, S., Vassiliou, G.S., Bench, A.J., Boyd, E.M., Curtin, N., Scott, M.A., Erber, W.N. & Green, A.R. (2005) Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet*, **365**, 1054–1061.

Borregaard, N. & Cowland, J.B. (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood*, **89**, 3503–3521.

Borregaard, N., Miller, L.J. & Springer, T.A. (1987) Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. *Science*, **237**, 1204–1206.

Borregaard, N., Kjeldsen, L., Rygaard, K., Bastholm, L., Nielsen, M.H., Sengelov, H., Bjerrum, O.W. & Johnsen, A.H. (1992) Stimulus-

dependent secretion of plasma proteins from human neutrophils. *Journal of Clinical Investigation*, **90**, 86–96.

Chung, J., Uchida, E., Grammer, T.C. & Blenis, J. (1997) STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Molecular and Cellular Biology*, **17**, 6508–6516.

Dameshek, W. (1951) Some speculations on the myeloproliferative syndromes. *Blood*, **6**, 372–375.

Dotti, G., Garattini, E., Borleri, G., Masuhara, K., Spinelli, O., Barbui, T. & Rambaldi, A. (1999) Leucocyte alkaline phosphatase identifies terminally differentiated normal neutrophils and its lack in chronic myelogenous leukaemia is not dependent on p210 tyrosine kinase activity. *British Journal of Haematology*, **105**, 163–172.

Falanga, A., Marchetti, M., Evangelista, V., Vignoli, A., Licini, M., Balicco, M., Manarini, S., Finazzi, G., Cerletti, C. & Barbui, T. (2000) Polymorphonuclear leukocyte activation and hemostasis in patients with essential thrombocythemia and polycythemia vera. *Blood*, **96**, 4261–4266.

Faurschou, M. & Borregaard, N. (2003) Neutrophil granules and secretory vesicles in inflammation. *Microbes and Infection*, **5**, 1317–1327.

Garattini, E. & Gianni, M. (1996) Leukocyte alkaline phosphatase a specific marker for the post-mitotic neutrophilic granulocyte: regulation in acute promyelocytic leukemia. *Leukaemia & Lymphoma*, **23**, 493–503.

Gianni, M., Terao, M., Zanotta, S., Barbui, T., Rambaldi, A. & Garattini, E. (1994) Retinoic acid and granulocyte colony-stimulating factor synergistically induce leukocyte alkaline phosphatase in acute promyelocytic leukemia cells. *Blood*, **83**, 1909–1921.

Hayhoe, F.G. & Quaglino, D. (1958) Cytochemical demonstration and measurement of leucocyte alkaline phosphatase activity in normal and pathological states by a modified azo-dye coupling technique. *British Journal of Haematology*, **4**, 375–389.

Ihle, J.N. (1995) Cytokine receptor signalling. *Nature*, **377**, 591–594.

Jain, N., Zhang, T., Fong, S.L., Lim, C.P. & Cao, X. (1998) Repression of Stat3 activity by activation of mitogen-activated protein kinase (MAPK). *Oncogene*, **17**, 3157–3167.

James, C., Ugo, V., Le Couedic, J.P., Staerk, J., Delhommeau, F., Lacout, C., Garcon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A., Villeval, J.L., Constantinescu, S.N., Casadevall, N. & Vainchenker, W. (2005) A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*, **434**, 1144–1148.

Jamieson, C.H., Gotlib, J., Durocher, J.A., Chao, M.P., Mariappan, M.R., Lay, M., Jones, C., Zehnder, J.L., Lilleberg, S.L. & Weissman, I.L. (2006) The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 6224–6229.

Jones, A.V., Kreil, S., Zoi, K., Waghorn, K., Curtis, C., Zhang, L., Score, J., Seear, R., Chase, A.J., Grand, F.H., White, H., Zoi, C., Loukopoulos, D., Terpos, E., Vervessou, E.C., Schultheis, B., Emig, M., Ernst, T., Lengfelder, E., Hehlmann, R., Hochhaus, A., Oscier, D., Silver, R.T., Reiter, A. & Cross, N.C. (2005) Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. *Blood*, **106**, 2162–2168.

Kaplow, L.S. (1955) A histochemical procedure for localizing and evaluating leukocyte alkaline phosphatase activity in smears of blood and marrow. *Blood*, **10**, 1023–1029.

Kaplow, L.S. (1963) Cytochemistry of leukocyte alkaline phosphatase. Use of complex naphthol as phosphates in azo dyecoupling technics. *American Journal of Clinical Pathology*, **39**, 439–449.

Kondo, T., Okuno, N., Naruse, H., Kishimoto, M., Tasaka, T., Tsujioka, T., Matsuoka, A., Sugihara, T., Tohyama, Y. & Tohyama, K. (2008) Validation of the revised 2008 WHO diagnostic criteria in 75 suspected cases of myeloproliferative neoplasm. *Leukaemia & Lymphoma*, **49**, 1784–1791.

Kota, J., Caceres, N. & Constantinescu, S.N. (2008) Aberrant signal transduction pathways in myeloproliferative neoplasms. *Leukemia*, **22**, 1828–1840.

Kralovics, R., Passamonti, F., Buser, A.S., Teo, S.S., Tiedt, R., Passweg, J.R., Tichelli, A., Cazzola, M. & Skoda, R.C. (2005) A gain-of-function mutation of JAK2 in myeloproliferative disorders. *New England Journal of Medicine*, **352**, 1779–1790.

Lacout, C., Pisani, D.F., Tulliez, M., Gachelin, F.M., Vainchenker, W. & Villeval, J.L. (2006) JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. *Blood*, **108**, 1652–1660.

Levine, R.L. & Wernig, G. (2006) Role of JAK-STAT signaling in the pathogenesis of myeloproliferative disorders. *Hematology/the Education Program of the American Society of Hematology. American Society of Hematology. Education Program*, **2006**, 233–239.

Levine, R.L., Wadleigh, M., Cools, J., Ebert, B.L., Wernig, G., Huntly, B.J., Boggon, T.J., Wlodarska, I., Clark, J.J., Moore, S., Adelsperger, J., Koo, S., Lee, J.C., Gabriel, S., Mercher, T., D'Andrea, A., Frohling, S., Dohner, K., Marynen, P., Vandenberghe, P., Mesa, R.A., Tefferi, A., Griffin, J.D., Eck, M.J., Sellers, W.R., Meyerson, M., Golub, T.R., Lee, S.J. & Gilliland, D.G. (2005) Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*, **7**, 387–397.

Mesa, R.A., Tefferi, A., Lasho, T.S., Loegering, D., McClure, R.F., Powell, H.L., Dai, N.T., Steensma, D.P. & Kaufmann, S.H. (2006) Janus kinase 2 (V617F) mutation status, signal transducer and activator of transcription-3 phosphorylation and impaired neutrophil apoptosis in myelofibrosis with myeloid metaplasia. *Leukemia*, **20**, 1800–1808.

Parganas, E., Wang, D., Stravopodis, D., Topham, D.J., Marine, J.C., Teglund, S., Vanin, E.F., Bodner, S., Colamonici, O.R., van Deursen, J.M., Grosveld, G. & Ihle, J.N. (1998) Jak2 is essential for signaling through a variety of cytokine receptors. *Cell*, **93**, 385–395.

Passamonti, F., Rumi, E., Pietra, D., Della Porta, M.G., Boveri, E., Pascutto, C., Vanelli, L., Arcaini, L., Burcheri, S., Malcovati, L., Lazzarino, M. & Cazzola, M. (2006) Relation between JAK2 (V617F) mutation status, granulocyte activation, and constitutive mobilization of CD34+ cells into peripheral blood in myeloproliferative disorders. *Blood*, **107**, 3676–3682.

Sakamoto, S. (1966) Histochemical studies on leukocyte alkaline phosphatase activity with special reference to various types of hemolytic disorders. *Tohoku Journal of Experimental Medicine*, **89**, 387–399.

Sato, N., Takahashi, Y. & Asano, S. (1994) Preferential usage of the bone-type leader sequence for the transcripts of liver/bone/kidney-type alkaline phosphatase gene in neutrophilic granulocytes. *Blood*, **83**, 1093–1101.

Sengupta, T.K., Talbot, E.S., Scherle, P.A. & Ivashkiv, L.B. (1998) Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. *Proceedings of the*

46

National Academy of Sciences of the United States of America, **95**, 11107–11112.

Shibano, M., Machii, T., Nishimori, Y., Nakamoto, I., Ueda, E., Masuhara, K. & Kitani, T. (1999) Assessment of alkaline phosphatase on the surface membrane of neutrophils by immunofluorescence. *American Journal of Hematology*, **60**, 12–18.

Shibata, A., Bennett, J.M., Castoldi, G.L., Catovsky, D., Flandrin, G., Jaffe, E.S., Katayama, I., Nanba, K., Schmalzl, F. & Yam, L.T. (1985) Recommended methods for cytological procedures in haematology. International Committee for Standardization in Haematology (ICSH). *Clinical and Laboratory Haematology*, **7**, 55–74.

Shide, K., Shimoda, K., Kamezaki, K., Kakumitsu, H., Kumano, T., Numata, A., Ishikawa, F., Takenaka, K., Yamamoto, K., Matsuda, T. & Harada, M. (2007) Tyk2 mutation homologous to V617F Jak2 is not found in essential thrombocythaemia, although it induces constitutive signaling and growth factor independence. *Leukemia Research*, **31**, 1077–1084.

Shide, K., Shimoda, H.K., Kumano, T., Karube, K., Kameda, T., Takenaka, K., Oku, S., Abe, H., Katayose, K.S., Kubuki, Y., Kusumoto, K., Hasuike, S., Tahara, Y., Nagata, K., Matsuda, T., Ohshima, K., Harada, M. & Shimoda, K. (2008) Development of ET, primary myelofibrosis and PV in mice expressing JAK2 V617F. *Leukemia*, **22**, 87–95.

Stewart, C.A. (1974) Leucocyte alkaline phosphatase in myeloid maturation. *Pathology*, **6**, 287–293.

Sullivan, G.W., Donowitz, G.R., Sullivan, J.A. & Mandell, G.L. (1984) Interrelationships of polymorphonuclear neutrophil membrane-bound calcium, membrane potential, and chemiluminescence: studies in single living cells. *Blood*, **64**, 1184–1192.

Takenaka, K., Prasolava, T.K., Wang, J.C., Mortin-Toth, S.M., Khalouei, S., Gan, O.I., Dick, J.E. & Danska, J.S. (2007) Polymorphism in Sirpa modulates engraftment of human hematopoietic stem cells. *Nature Immunology*, **8**, 1313–1323.

Teofili, L., Martini, M., Cenci, T., Petrucci, G., Torti, L., Storti, S., Guidi, F., Leone, G. & Larocca, L.M. (2007) Different STAT-3 and STAT-5 phosphorylation discriminates among Ph-negative chronic myeloproliferative diseases and is independent of the V617F JAK-2 mutation. *Blood*, **110**, 354–359.

Tiedt, R., Hao-Shen, H., Sobas, M.A., Looser, R., Dirnhofer, S., Schwaller, J. & Skoda, R.C. (2008) Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. *Blood*, **111**, 3931–3940.

Weiss, M.J., Ray, K., Henthorn, P.S., Lamb, B., Kadesch, T. & Harris, H. (1988) Structure of the human liver/bone/kidney alkaline phosphatase gene. *Journal of Biological Chemistry*, **263**, 12002–12010.

Wen, Z., Zhong, Z. & Darnell, Jr, J.E. (1995) Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell*, **82**, 241–250.

Wernig, G., Mercher, T., Okabe, R., Levine, R.L., Lee, B.H. & Gilliland, D.G. (2006) Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. *Blood*, **107**, 4274–4281.

Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Yi, T., Tang, B., Miura, O. & Ihle, J.N. (1993) JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell*, **74**, 227–236.

Xing, S., Wanting, T.H., Zhao, W., Ma, J., Wang, S., Xu, X., Li, Q., Fu, X., Xu, M. & Zhao, Z.J. (2008) Transgenic expression of JAK2V617F causes myeloproliferative disorders in mice. *Blood*, **111**, 5109–5117.

Zaleskas, V.M., Krause, D.S., Lazarides, K., Patel, N., Hu, Y., Li, S. & Van Etten, R.A. (2006) Molecular pathogenesis and therapy of polycythemia induced in mice by JAK2 V617F. *PLoS ONE*, **1**, e18.

Zhang, X., Blenis, J., Li, H.C., Schindler, C. & Chen-Kiang, S. (1995) Requirement of serine phosphorylation for formation of STAT-promoter complexes. *Science*, **267**, 1990–1994.

Zhong, Z., Wen, Z. & Darnell, Jr, J.E. (1994) Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science*, **264**, 95–98.

47

High incidence of false-positive *Aspergillus* galactomannan test in multiple myeloma

Yasuo Mori,[1,2] Yoji Nagasaki,[1,2] Kenjiro Kamezaki,[1] Katsuto Takenaka,[1] Hiromi Iwasaki,[2] Naoki Harada,[1] Toshihiro Miyamoto,[1] Yasunobu Abe,[3] Nobuyuki Shimono,[1] Koichi Akashi,[1,2] and Takanori Teshima[2]

Invasive aspergillosis (IA) remains one of the most significant causes of morbidity and mortality in patients with hematological malignancies undergoing chemotherapy and hematopoietic stem cell transplantation (HSCT), mainly due to the difficulty in its early diagnosis. Monitoring of galactomannan (GM) antigen, an exoantigen of Aspergillus, in the blood by sandwich ELISA is a useful and noninvasive method for early diagnosis of IA. The GM test has a sensitivity of 67–100% with a specificity of 81–99% in neutropenic patients and allogeneic transplant recipients [1–3]. Although it has been widely used as a diagnostic criterion for IA [4,5], one of the major limitations of this assay is false-positivity, particularly in pediatric patients [1], patients with graft-versus-host disease (GVHD) [6,7], and those taking dietary GM [8,9] or fungus-derived antibiotics, such as piperacillin-tazobactam (PIPC/TAZ) [10–12].

Multiple myeloma results from malignant proliferation of a single clone of plasma cells, which produces a monoclonal immunoglobulin. Opportunistic infection is a major cause of death in patients with myeloma [13,14]. The risk for infection primarily resides during periods of chemotherapy-induced neutropenia or in the terminal stages of the disease. Therefore, monitoring of Aspergillus is recommended during chemotherapy-induced neutropenia.

One hundred twenty-four patients with hematological disorders hospitalized in our institution from April 2007 to September 2009 were analyzed retrospectively. The clinical characteristics of these patients are summarized in Supporting Information Table 1. Twenty-seven patients had plasma cell associated disorders (IgG type myeloma: 14, IgA type myeloma: 1, IgD type myeloma: 1, Bence-Jones type myeloma: 3, lymphoplasmacytic lymphoma/Waldenstrom macroglobulinemia: 3, plasma cell leukemia: 1, primary AL amyloidosis: 1, and POEMS syndrome: 2). The remaining 97 patients were diagnosed as acute leukemia (MDS/AML: 32, ALL: 15) or malignant lymphoma (n = 50). Out of the 124 patients, those receiving cytotoxic chemotherapy, autologous peripheral blood stem cell transplantation (PBSCT), and allogeneic HSCT were 81, 10, and 28, respectively. One hundred eleven patients received antifungal prophylaxis, mostly with FLCZ or ITCZ. Eight patients were administered PIPC/TAZ at sampling time points. Seventy patients were low risk for the development of IA, whereas the remaining 54 patients were high risk.

In 21 of the 124 (16.9%) patients, GM antigenemia was positive at least two consecutive times and their characteristics are shown in Table I. However, only seven of the 21 patients showed clinical features of IA and were diagnosed with probable IA in the lung (Cases 1–7). Clinical features were relieved with treatment with VRCZ in these patients, confirming a diagnosis of IA. All of the seven patients had received antifungal prophylaxis, but not antibiotics known to cause false-positive results, such as PIPC/TAZ [10–12], amoxicillin/clavulanic acid, or amoxicillin [15]. Four of the 7 patients were high risk for the developing IA and three were low risk. Possible IA was diagnosed in four patients in the absence of positive GM results. On the other hand, no proven or probable IA was detected in 103 patients with negative GM antigenemia.

Fourteen of the 21 (66.7%) patients with positive GM antigenemia did not satisfy the diagnostic criteria of proven or probable IA (Cases 8–21 in Table I); thus, their episodes were considered to be false-positive. None of the 14 patients were treated with antibiotics potentially causing false-positivity of GM test. Antifungal prophylaxis had been given in all 14 episodes (FLCZ in 5, ITCZ in 5, and MCFG in 4). These patients did not show any clinical features suggestive of IA. Chest CT scans did not show any abnormal findings in 10 patients, whereas four patients showed abnormalities in the lung which were not suspicious of IA. Diagnosis of these lung lesions were history of pneumoconiosis in one patient, proven bacterial pneumonia in one, and idiopathic interstitial pneumonia in the remaining two patients. These lesions were not deteriorated without antifungal treatment. (1→3)-β-D-glucan was

negative in all patients showing false-positive GM test. With a median follow-up of 10 months (range: 1–19 months), these patients did not develop fungal infection without treatment. On the other hand, false-negative GM results were obtained in four patients (3.2 %). In this study, the sensitivity, specificity, PPV, and NPV of the GM ELISA test were 63.6% (7/11), 87.6% (99/113), 33.3% (7/21), and 96.1% (99/103), respectively.

Surprisingly, 11 out of the 14 patients showing the false-positive results had diagnosis of multiple myeloma. The false-positivity of GM antigenemia was significantly higher in myeloma patients (11/22, 50%) than those with other hematological malignancies (3/102, 2.9%) (P < 0.001). Moreover, in myeloma patients false-positive results were exclusively detected in those with IgG subtype. Thus, rate of false-positivity was extremely high in patients with IgG myeloma (11/14, 78.6%). We could not find any difference in the characteristics of IgG myeloma patients with or without GM false-positivity, including serum levels of IgG (5083 ± 2077 mg/dl versus 4713 ± 3729 mg/dl). In addition, the GM test remained to be false-positive even after normalization of IgG levels by chemotherapy in eight of the 11 patients. We also evaluated GM antigenemia in frozen serum samples collected before chemotherapy in three myeloma patients who showed false-positivity after chemotherapy to rule out the possibility that administration of myeloma-specific chemotherapy is associated with the false-positivity, and confirmed GM positivity before chemotherapy in these samples.

In a univariate logistic regression analysis, IgG myeloma and low-risk category were strongly associated with false-positive GM antigenemia. Sex, type of treatment, antibiotics, corticosteroid usage, and serum levels of immunoglobulins were also significant or marginally associated with false-positivity. Multivariate analysis confirmed diagnosis of IgG myeloma as the only independent risk factor for false-positivity (odds ratio, 59.41; 95% confidence interval, 8.19–431.0; P < 0.001) (Supporting Information, Table 2). In patients with other diseases, the GM assay showed a high sensitivity (7/11, 63.6%), specificity (96/99, 97.0%), PPV (7/10, 70%), and NPV (96/100, 96%). In contrast, for patients with IgG myeloma, specificity and PPV of the assay were very low (3/14, 21.4%, and 0/11, 0%), whereas NPV was 100% even in this cohort.

A recent meta-analysis addressing the accuracy of a GM assay for diagnosing IA confirmed the clinical usefulness of this test with a sensitivity of 71% and a specificity of 89% [16]. Although our study demonstrated similar sensitivity (7/11:64%) and specificity (103/117:88%) of the GM test, PPV (33%) was lower, compared with previous studies that demonstrate 40–60% PPV [7,17–19]. This difference is due to an unexpectedly high incidence of GM antigen false-positivity (11.3%) in our study. It should be noted, however, that screening of GM antigen was performed less frequently in this study compared with previous studies, where GM antigenemia was evaluated two to three times per week [1,2,20], and such a frequent monitoring is ideal to assure the optimal PPV and NPV.

Diagnosis of multiple myeloma is a major risk factor for GM false-positivity. In particular, the false-positivity was exclusively observed in patients with IgG myeloma and was not observed in patients with other types of plasma cell disorders. These observations should be confirmed in a larger study because some studies previously reported the usefulness of GM antigen assay as a diagnostic tool for IA among patients with hematological malignancies including IgG myeloma [21,22], and only small numbers of patients with plasma cell disorders other than IgG myeloma were included in this study. Low-risk category of developing IA was a risk for false-positive results in a univariate, but not multivariate analysis. IgG myeloma remained a strong risk for false-positivity even after the compensation by the risk categorization.

Mechanisms of high frequency of GM false-positivity in myeloma patients remain to be investigated. (1→3)-β-D-glucan, which is released from the fungal cell wall, is also widely used to support diagnosis of fungal infections

449

TABLE I. Characteristics of 21 Patients with Positive GM Test

Case	Age/Sex	Disease	Treatment	GM (C.O.I)	Radiological/Clinical	Diagnosis	Times/duration of false-positivity	Antibiotics[a]	Antifungal agents[a]	Steroids	Neutrophil (×10⁹/L)s	IgG (mg/dL)	IgA (mg/dL)	IgM (mg/dL)	Risk of IA
1	49/F	MDS/AML	CTx	1.1	nodules with halo	probable IA	—	CFPM	FLCZ	none	0.01	1419	227	50	High
2	63/M	MDS/AML	CTx	1.2	nodules	probable IA	—	MEPM, AMK	FLCZ	none	0	1253	238	82	High
3	50/F	AML	CTx	1.2	nodules	probable IA	—	MEPM, AMK	ITCZ	none	0	1301	293	235	High
4	57/F	MDS	allo-SCT	2.3	nodules	probable IA	—	CAZ, VCM	VRCZ	none	0.009	1198	256	120	High
5	63/M	ML	CTx	1.3	consolidation with pleural pain	probable IA	—	none	MCFG	DEXA	2.034	689	126	92	Low
6	57/F	ML	CTx	1.5	nodules	probable IA	—	CFPM	FLCZ	PSL	0.081	675	117	67	Low
7	59/F	ML	CTx	2.2	consolidation with pleural pain	probable IA	—	CZOP	MCFG	PSL	0.036	719	95	20	Low
8	70/M	ML	CTx	0.8	pneumoconiosis	F-P	7/7 months	none	FLCZ	PSL	5.096	1870	315	60	High
9	52/M	ML	allo-SCT	2.5	bacterial pneumonia	F-P	2/1 month	CZOP, CPFX	ITCZ	mPSL	0.806	562	49	19	High
10	54/M	ML	CTx	0.9	negative	F-P	5/3 months	CFPM	FLCZ	none	0.395	691	46	10	Low
11	63/F	IgGκ MM	CTx	0.8	negative	F-P	2/1 month	none	ITCZ	DEXA	1.497	4770	25	27	Low
12	56/F	IgGκ MM	none	1.2	negative	F-P	3/2 months	CAZ	FLCZ	none	3.449	7196	5	38	Low
13	69/M	IgGκ MM	CTx	0.5	negative	F-P	4/4 months	none	MCFG	DEXA	0.72	3944	13	<10	Low
14	69/M	IgGκ MM	CTx	1.2	negative	F-P	6/3 months	none	FLCZ	DEXA	1.777	2385	6	28	Low
15	64/M	IgGκ MM	CTx	1.6	old inflammatory change	F-P	5/2 months	none	MCFG	PSL	1.919	3269	30	<10	Low
16	51/F	IgGκ MM	CTx	2.1	IP	F-P	23/15 months	none	ITCZ	PSL	3.791	8006	99	30	Low
17	64/M	IgGκ MM	CTx	1.6	negative	F-P	13/8 months	none	ITCZ	DEXA	0.899	3950	6	<10	Low
18	75/M	IgGκ MM	CTx	0.6	negative	F-P	2/1 month	MEPM	FLCZ	DEXA	0.823	8986	<5	<10	High
19	49/F	IgGκ MM	CTx	1.1	negative	F-P	8/3 months	none	MCFG	DEXA	0.724	4563	37	15	Low
20	74/F	IgGλ MM	CTx	0.7	negative	F-P	6/2 months	none	MCFG	none	1.451	3924	25	18	Low
21	56/M	IgGλ MM	CTx	1.1	negative	F-P	10/3 months	none	ITCZ	DEXA	1.605	4917	9	10	Low

MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; ML, malignant lymphoma; MM, multiple myeloma; CTx, chemotherapy; allo-SCT, allogeneic stem cell transplantation; C.O.I, cut-off index; IP, interstitial pneumonia; F-P, false-positive; CFPM, cefepime; MEPM, meropenem; AMK, amikacin; CAZ, ceftazidime; VCM, vancomycin; CZOP, cefozopran; PIPC/TAZ, piperacillin/tazobactam; CPFX, ciprofloxacin; PSL, prednisolone; mPSL, methylprednisolone; DEXA, dexamethasone. [b]Administrated at initial positive sampling.

and adopted as one of the microbiological criterion for probable IA in the revised EORTC/MSG definition [5]. A previous study reported that high levels of immunoglobulins interfere with the measurement of $(1\rightarrow3)$-β-D-glucan by causing precipitation of insolubilized proteins and increase the nonspecific optical density levels of reaction fluid [23], although $(1\rightarrow3)$-β-D-glucan was negative in patients showing false-positive GM test in this study. This phenomenon has not been reported in the GM assay. However, serum levels of IgG were not directly associated with the false-positivity; IgG levels did not differ between IgG myeloma patients with and without false-positivity. In addition, the GM test remained to be false-positive even after normalization of IgG levels by chemotherapy in eight of the 11 patients.

Causative role of PIPC/TAZ, amoxicillin/clavulanic acid, and amoxicillin in GM false-positivity has been well documented [10–12,15]; therefore, collection of samples before infusion of these antibiotics and the use of a relatively higher cut-off level (>0.7) are recommended in patients receiving these agents [12]. In this study, no patients with GM false-positivity received these antibiotics at the time of sampling. It has been hypothesized that dietary contamination by GM causes GM false-positivity by the translocation of dietary GM into the systemic circulation through the disrupted intestinal mucosal barrier, especially in patients with gastrointestinal GVHD after allogeneic HSCT [6,7,9]. In our cohort, 1(3.6%) of the 28 patients who underwent allogeneic HSCT showed false-positivity. This patient with lymphoma had acute GVHD involving in the skin and intestine. A previous study demonstrated that false-positive results were preferentially observed in patients with febrile neutropenic sepsis [24], although subsequent study was unable to replicate this result [20]. A recent study revealed that serum GM antigen levels was significantly higher in severely neutropenic patients ($< 0.1 \times 10^9$/L) than in the other patients [25]. However, we did not find such an association in this study.

In conclusion, the incidence of false-positive GM antigenemia was high in patients with IgG myeloma. Although the results should be confirmed in a prospective study including larger numbers of patients, positive results of GM antigenemia may be interpreted with caution, and intimate survey including CT scan or other microbiological markers will be recommended in myeloma patients.

[1]Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka Japan; [2]Center for Cellular and Molecular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka Japan; [3]Medicine and Bioregulatory Science, Kyushu University Graduate School of Medical Sciences, Fukuoka Japan

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Takanori Teshima, Center for Cellular and Molecular Medicine, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
E-mail: tteshima@cancer.med.kyushu-u.ac.jp

References

1. Herbrecht R, Letscher-Bru V, Oprea C, et al. Aspergillus galactomannan detection in the diagnosis of invasive aspergillosis in cancer patients. J Clin Oncol 2002;20:1898–1906.
2. Maertens J, Verhaegen J, Lagrou K, et al. Screening for circulating galactomannan as a noninvasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: A prospective validation. Blood 2001;97:1604–1610.
3. Wheat LJ. Rapid diagnosis of invasive aspergillosis by antigen detection. Transpl Infect Dis 2003;5:158–166.
4. Ascioglu S, Rex JH, De Pauw B, et al. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: An international consensus. Clin Infect Dis 2002;34:7–14.
5. De Pauw B, Walsh TJ, Donnelly JP, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 2008;46:1813–1821.
6. Hamaki T, Kami M, Kanda Y, et al. False-positive results of Aspergillus enzyme-linked immunosorbent assay in a patient with chronic graft-versus-host disease after allogeneic bone marrow transplantation. Bone Marrow Transplant 2001;28:633–634.

49

7. Asano-Mori Y, Kanda Y, Oshima K, et al. False-positive Aspergillus galactomannan antigenaemia after haematopoietic stem cell transplantation. J Antimicrob Chemother 2008;61:411–416.
8. Ansorg R, Van Den Boom R, Rath PM. Detection of Aspergillus galactomannan antigen in foods and antibiotics. Mycoses 1997;40:353–357.
9. Murashige N, Kami M, Kishi Y, et al. False-positive results of Aspergillus enzyme-linked immunosorbent assays for a patient with gastrointestinal graft-versus-host disease taking a nutrient containing soybean protein. Clin Infect Dis 2005;40:333–334.
10. Sulahian A, Touratier S, Ribaud P. False positive test for Aspergillus antigenemia related to concomitant administration of piperacillin and tazobactam. N Engl J Med 2003;349:2366–2367.
11. Viscoli C, Machetti M, Cappellano P, et al. False-positive galactomannan platelia Aspergillus test results for patients receiving piperacillin-tazobactam. Clin Infect Dis 2004;38:913–916.
12. Orlopp K, Von Lilienfeld-Toal M, Marklein G, et al. False positivity of the Aspergillus galactomannan Platelia ELISA because of piperacillin/tazobactam treatment: Does it represent a clinical problem? J Antimicrob Chemother 2008;62:1109–1112.
13. Blade J, San Miguel JF, Fontanillas M, et al. Increased conventional chemotherapy does not improve survival in multiple myeloma: Long-term results of two PETHEMA trials including 914 patients. Hematol J 2001;2:272–278.
14. Augustson BM, Begum G, Dunn JA, et al. Early mortality after diagnosis of multiple myeloma: Analysis of patients entered onto the United kingdom Medical Research Council trials between 1980 and 2002—Medical Research Council Adult Leukaemia Working Party. J Clin Oncol 2005;23:9219–9226.
15. Mattei D, Rapezzi D, Mordini N, et al. False-positive Aspergillus galactomannan enzyme-linked immunosorbent assay results in vivo during amoxicillin-clavulanic acid treatment. J Clin Microbiol 2004;42:5362–5363.

16. Pfeiffer CD, Fine JP, Safdar N. Diagnosis of invasive aspergillosis using a galactomannan assay: A meta-analysis. Clin Infect Dis 2006;42:1417–1427.
17. Kawazu M, Kanda Y, Nannya Y, et al. Prospective comparison of the diagnostic potential of real-time PCR, double-sandwich enzyme-linked immunosorbent assay for galactomannan, and a (1-->3)-beta-D-glucan test in weekly screening for invasive aspergillosis in patients with hematological disorders. J Clin Microbiol 2004;42:2733–2741.
18. Alhambra A, Cuetara MS, Ortiz MC, et al. False positive galactomannan results in adult hematological patients treated with piperacillin-tazobactam. Rev Iberoam Micol 2007;24:106–112.
19. Lai CC, Hsu HL, Lee LN, Hsueh PR. Assessment of Platelia Aspergillus enzyme immunoassay for the diagnosis of invasive aspergillosis. J Microbiol Immunol Infect 2007;40:148–153.
20. Maertens J, Verhaegen J, Demuynck H, et al. Autopsy-controlled prospective evaluation of serial screening for circulating galactomannan by a sandwich enzyme-linked immunosorbent assay for hematological patients at risk for invasive Aspergillosis. J Clin Microbiol 1999;37:3223–3228.
21. Miceli MH, Grazziutti ML, Woods G, et al. Strong correlation between serum aspergillus galactomannan index and outcome of aspergillosis in patients with hematological cancer: Clinical and research implications. Clin Infect Dis 2008;46:1412–1422.
22. Woods G, Miceli MH, Grazziutti ML, et al. Serum Aspergillus galactomannan antigen values strongly correlate with outcome of invasive aspergillosis: A study of 56 patients with hematologic cancer. Cancer 2007;110:830–834.
23. Yoshida K. [Recent advances of serodiagnosis for systemic fungal infections]. Nippon Ishinkin Gakkai Zasshi 2006;47:135–142.
24. Swanink CM, Meis JF, Rijs AJ, et al. Specificity of a sandwich enzyme-linked immunosorbent assay for detecting Aspergillus galactomannan. J Clin Microbiol 1997;35:257–260.
25. Cordonnier C, Botterel F, Ben Amor R, et al. Correlation between galactomannan antigen levels in serum and neutrophil counts in haematological patients with invasive aspergillosis. Clin Microbiol Infect 2009;15:81–86.

Mixed phenotype acute leukemia with t(11;19)(q23;p13.3)/MLL-MLLT1(ENL), B/T-lymphoid type: A first case report

Mojdeh Naghashpour, [1]* Jeffrey Lancet, [2] Lynn Moscinski, [1] and Ling Zhang[1]

The majority of cases of acute leukemia belong to a specific lineage origin, either lymphoid or myeloid, and therefore are classified as acute lymphoblastic leukemia (ALL) or acute myelogenous leukemia (AML), based on morphologic features and cytochemical and immunophenotypic profile of the blast cells. A minority of acute leukemias however, show no clear evidence of differentiation along a single lineage. These are now classified under acute leukemias of ambiguous lineage by the most recent WHO classification and account for <4% of all cases of acute leukemia [1]. They include leukemias with no lineage specific antigens (acute undifferentiated leukemias) and those with blasts that express antigens of more than one lineage to such degree that it is not possible to assign the leukemia to any one particular lineage with certainty (mixed phenotype acute leukemias). The latter can either be leukemias with two distinct populations of blasts, each expressing antigens of a different lineage (historically referred to as "bilineal" leukemias) or a single blast population expressing antigens of multiple lineages (historically referred to as "biphenotypic" acute leukemias) [2]. Acute leukemias of ambiguous lineage may harbor a variety of genetic lesions. Those with t(9;22)(q34;q11) or translocations associated with mixed lineage leukemias (MLL) gene, i.e., t(11;V)(q23;V), occur frequently enough and are associated with distinct features, that are considered as separate entities according to the recent WHO classification. Co-expression of myeloid and B-lymphoid antigens is most common in mixed phenotype acute leukemia (MPAL), followed by co-expression of myeloid and T-lymphoid antigens, accounting for 66–70% and 23–24% of MLLs, respectively. Co-expression of B- and T-lineage associated antigens or antigens of all three lineages is exceedingly rare, accounting for <5% of MLLs [3,4]. The requirements for assigning more than one lineage to a single blast population has been established by current WHO classification [1].

Chromosomal rearrangements of mixed lineage leukemias (MLL) gene on chromosomal segment 11q23 occur in a subset of leukemias with poor prognosis and are seen in pediatric, adult and therapy-related acute leukemias [5].

MLL translocations can be found in de novo AML and lymphoid lineages (ALL), secondary myelodysplastic syndrome, as well as mixed phenotype acute leukemias [6–8]. Among them, mixed phenotypic acute leukemia has a high incidence of chromosomal abnormalities involving MLL gene [2,3]. Wild-type MLL is a transcriptional regulatory factor involved in the maintenance of clustered homeobox (Hox) gene expression, particularly during hematopoietic development [9]. MLL translocations generate an in-frame fusion protein, in which the N-terminal portion of the MLL protein is fused to the C-terminal region of a partner protein, resulting in a chimeric protein with oncogenic properties [10]. Leukemias that harbor MLL translocations are a unique biologic subgroup that co-express myeloid and lymphoid associated genes and an overall gene expression profile that characterize those of the precursor cells [11,12]. To date, more than 51 partner genes have been characterized at the molecular level and an additional 35 genetic loci have been identified by cytogenetic analysis [13]. The most frequent translocations, t(4;11)(q21;23) involving MLLT2 (AF4), t(9;11)(p22;23) involving MLLT3 (AF9), and t(11;19)(q23;p13.3) involving MLLT1 (ENL) account for 80% of investigated leukemia samples [13]. The characteristic of the fusion partner seems to determine a bias for generation of myeloid versus lymphoid or mixed lineage type leukemia. While MLL-MLLT3 and MLL-MLLT2 are predominantly associated with a myeloid or a lymphoid/mixed lineage phenotype, MLL-MLLT1 occurs equally likely in both leukemia subclasses [14]. To our knowledge however, B/T-lymphoid MLL harboring t(11;19)(q23;p13.3) has not been reported.

Case Report

A previously healthy 37-year-old male, presented to a local primary care physician with flu-like symptoms. A complete blood count (CBC) was within normal limits, with a normal differential count, at initial visit. He was treated conservatively without relief of his symptoms. A CBC, repeated 1 month later revealed a white blood cell count 10.6×10^9/L, including 3% circulating blasts, hemoglobin 8.6 g/dL, and a platelet count of 179×10^9/L. His absolute neutrophil count was 0.4×10^9/L. With an uncertain diagnosis, the patient was referred to our institution for further consulta-

Alloantigen expression on non-hematopoietic cells reduces graft-versus-leukemia effects in mice

Shoji Asakura,[1] Daigo Hashimoto,[1,2] Shuichiro Takashima,[3] Haruko Sugiyama,[1] Yoshinobu Maeda,[1] Koichi Akashi,[2,3] Mitsune Tanimoto,[1] and Takanori Teshima[2]

[1]Biopathological Science, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan. [2]Center for Cellular and Molecular Medicine and [3]Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Science, Fukuoka, Japan.

Allogeneic hematopoietic stem cell transplantation (HSCT) is used effectively to treat a number of hematological malignancies. Its beneficial effects rely on donor-derived T cell–targeted leukemic cells, the so-called graft-versus-leukemia (GVL) effect. Induction of GVL is usually associated with concomitant development of graft-versus-host disease (GVHD), a major complication of allogeneic HSCT. The T cells that mediate GVL and GVHD are activated by alloantigen presented on host antigen-presenting cells of hematopoietic origin, and it is not well understood how alloantigen expression on non-hematopoietic cells affects GVL activity. Here we show, in mouse models of MHC-matched, minor histocompatibility antigen–mismatched bone marrow transplantation, that alloantigen expression on host epithelium drives donor T cells into apoptosis and dysfunction during GVHD, resulting in a loss of GVL activity. During GVHD, programmed death-1 (PD-1) and PD ligand-1 (PD-L1), molecules implicated in inducing T cell exhaustion, were upregulated on activated T cells and the target tissue, respectively, suggesting that the T cell defects driven by host epithelial alloantigen expression might be mediated by the PD-1/PD-L1 pathway. Consistent with this, blockade of PD-1/PD-L1 interactions partially restored T cell effector functions and improved GVL. These results elucidate a previously unrecognized significance of alloantigen expression on non-hematopoietic cells in GVL and suggest that separation of GVL from GVHD for more effective HSCT may be possible in human patients.

Introduction

Donor immunity in allogeneic hematopoietic stem cell transplantation (HSCT) harnesses beneficial graft-versus-leukemia (GVL) effects; therefore, allogeneic HSCT represents a very potent form of immunotherapy for hematological malignancies (1, 2). Induction of GVL is usually associated with the development of graft-versus-host disease (GVHD), which is a major complication after allogeneic HSCT. T cell depletion of the donor inocula prevents GVHD and leads to a loss of the GVL effect (3–5). Both GVL and GVHD are mediated by donor T cells, which recognize alloantigens presented on host APCs (6, 7). Donor CTLs and inflammatory cytokines are major effectors of GVHD, whereas CTLs are primarily responsible for GVL (8, 9). In patients with advanced-stage leukemia and lymphoma, relapse is still a major cause of mortality after allogeneic HSCT even after the development of severe GVHD. Thus, improvements in our understanding of the pathophysiology of GVHD and GVL are urgently needed to develop more effective therapies for malignant diseases.

Alloantigens are expressed on the three major components in HSCT recipients in the context of GVHD and GVL: hematopoietically derived APCs, GVHD target epithelium, and leukemia cells. Several studies have shown that host APCs are crucial for the induction of both GVHD and GVL (6, 7, 9–11). Alloantigen expression on epithelium is also critical for the induction of GVHD in MHC-matched, minor histocompatibility antigen–mismatched (mHA-mismatched) models of bone marrow transplantation (BMT) (10), but GVHD can occur in the absence of alloantigen expression on

epithelium in MHC-mismatched models of BMT (9). However, the effect of alloantigen expression on non-hematopoietic cells such as the epithelium in GVL is not well defined. In this study, we addressed this important issue in mHA-mismatched models of BMT.

Results

Alloantigen expression on host non-hematopoietic cells augments acute GVHD but reduces GVL effects. We generated BM chimeric mice that express alloantigens on APCs, which are essential for the induction of both GVHD and GVL (6, 7, 12). BM chimeras were created by reconstituting lethally irradiated C3H.Sw (C3: H-2^b) mice with 5×10^6 T cell–depleted (TCD) BM cells isolated from C57BL/6 (B6, H-2^b) mice that differ from C3 mice at multiple mHAs ([B6→C3] chimeras). Control chimeras, [B6→B6], were identically created. Four months later, donor repopulation of hematopoiesis was confirmed by flow cytometry as shown previously (6, 9, 12). Thus, [B6→C3] chimeric mice expressed B6-derived mHAs on hematopoietically derived APCs but not on non-hematopoietic target cells. In contrast, [B6→B6] mice expressed B6-derived mHAs on both APCs and target epithelium. These chimeras were used as BMT recipients; they were reirradiated and injected with 5×10^6 TCD BM cells alone or with various doses of CD8$^+$ T cells from C3 donors. After BMT, GVHD mortality was higher in [B6→B6] mice than in [B6→C3] mice (Figure 1A). Clinical GVHD scores (13) in surviving animals were also higher in [B6→B6] mice than in [B6→C3] mice (Figure 1B). Mortality and morbidity from GVHD in [B6→C3] mice were almost equivalent to those in [B6→B6] mice given a 1-log lower T cell dose. This finding confirmed the previous observation of a lack of alloantigen expression on host epithelium significantly reducing GVHD across mHA disparity (10). We
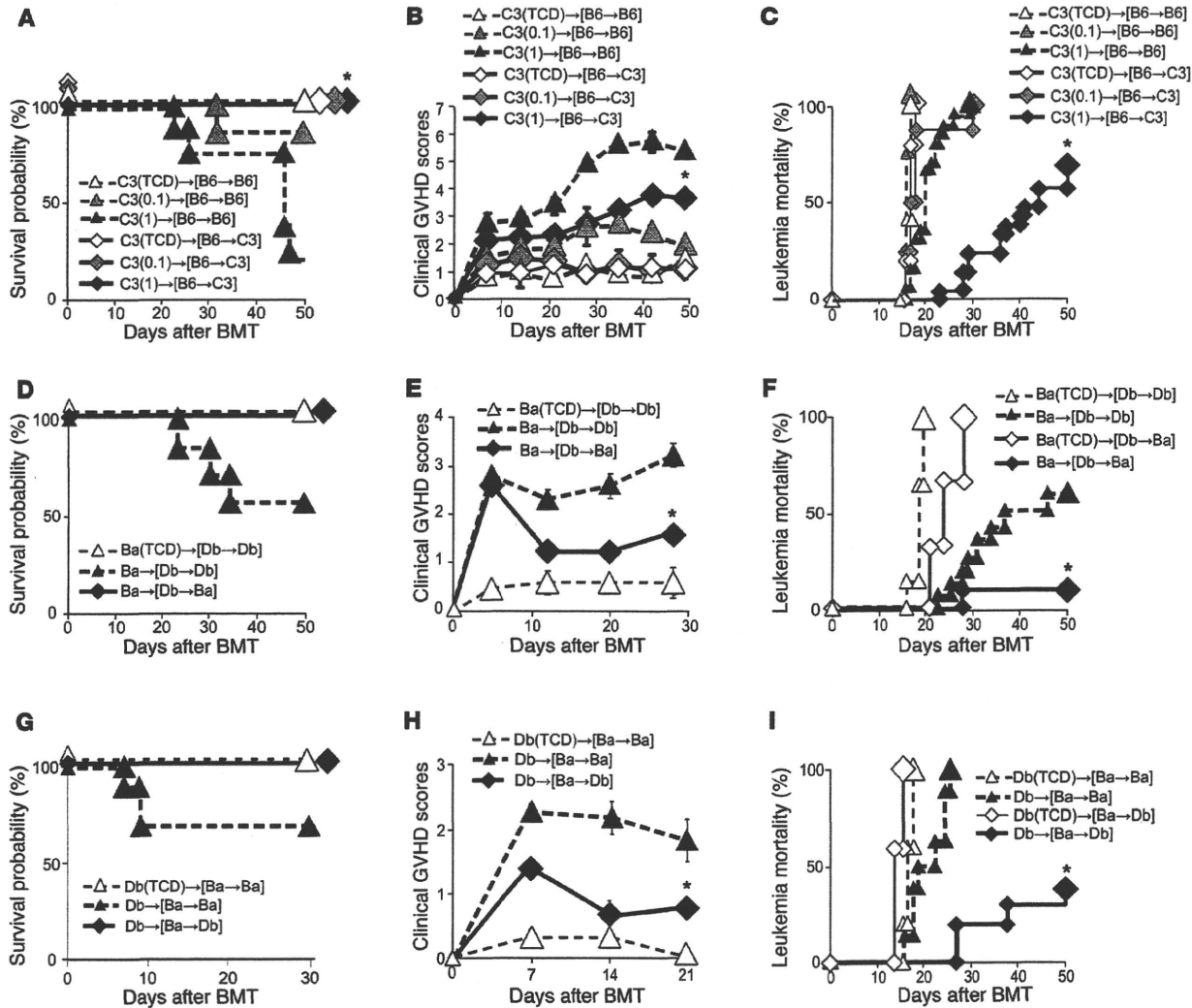
Figure 1
Alloantigen expression on host non-hematopoietic cells augments acute GVHD but reduces GVL effects. (**A–C**) [B6→C3] (diamonds) and [B6→B6] chimeras (triangles) were created by reconstituting lethally irradiated C3 and B6 mice with 5×10^6 TCD BM cells from B6 mice. Four months later, the chimeras were reirradiated and injected with 5×10^6 TCD BM cells alone (open symbols) or with 1×10^6 (black symbols) or 0.1×10^6 (gray symbols) CD8+ T cells from C3 donors (as indicated in parentheses × 10^6). Survival (**A**) and clinical GVHD scores (**B**) after BMT ($n = 3$–8/group). (**C**) Leukemia mortality after BMT in chimeras injected with EL4 cells ($n = 5$–21/group). Data from 3 similar experiments were combined. (**D–F**) [Db→Ba] (diamonds) and [Db→Db] (triangles) chimeras were reirradiated and injected with TCD BM alone (open symbols) or with 2×10^6 T cells from Ba donors (filled symbols). Survival (**D**) and clinical GVHD scores (**E**) after BMT from a representative experiment of 2 similar experiments ($n = 4$–7/group). (**F**) Leukemia mortality after BMT in mice injected with P815 cells. Data from 2 similar experiments were combined ($n = 6$–18/group). (**G–I**) [Ba→Db] (diamonds) and [Ba→Ba] (triangles) chimeras were similarly transplanted with 5×10^6 TCD BM cells alone (open symbols) or with 2×10^6 T cells from Db donors (filled symbols). Survival (**G**) and clinical scores (**H**) after BMT ($n = 3$–10/group). (**I**) Leukemia mortality after BMT in chimeras injected with A20 cells ($n = 5$–10/group). Data from 2 similar experiments were combined. Clinical scores are shown as the mean ± SEM. *$P < 0.05$ compared with allogeneic controls.

then tested the effect of alloantigen expression on GVHD target epithelium on GVL effects. These chimeric mice were transplanted as described above together with 2,500 B6-derived EL4 cells as a model of residual leukemia after BMT. As expected, 100% of both types of chimeric mice that received TCD BM cells died from leukemia by day +20 after BMT (Figure 1C), whereas leukemia-free survival was significantly prolonged in mice that received donor T cells, demonstrating a significant GVL effect. However, this GVL

effect was not potent in [B6→B6] mice, and all mice subsequently died from leukemia. Surprisingly, leukemia mortality was significantly lower in [B6→C3] mice that did not express alloantigens on their non-hematopoietic cells (62% vs. 100%; $P < 0.05$). GVL effects in [B6→B6] mice appeared to be almost equivalent to those in [B6→C3] mice given a 1-log lower T cell dose.

We further confirmed these observations in a different strain combination: BALB/c (Ba, H-2^d) and DBA/2 (Db, H-2^d) mice that
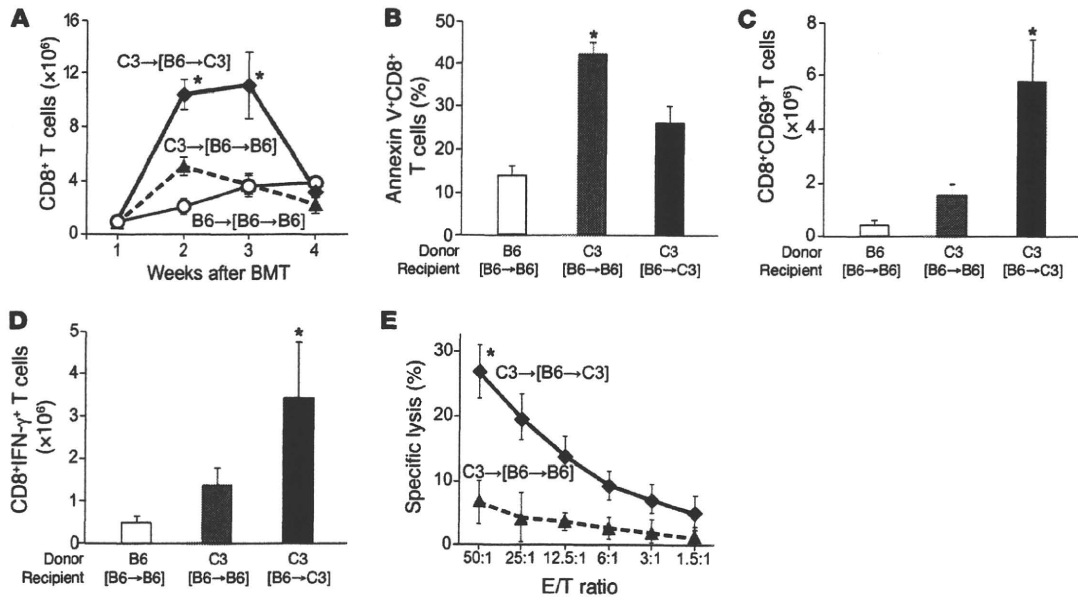
Figure 2
Alloantigen expression on host non-hematopoietic cells enhances the apoptosis and dysfunction of alloreactive T cells. [B6→C3] (diamonds and black bars) and [B6→B6] (triangles and gray bars) chimeras were transplanted as indicated in the legend for Figure 1. Syngeneic controls were [B6→B6] recipients of B6.Ly5.1 (CD45.1⁺) cells (open circles and white bars). (**A**) Numbers of donor CD8⁺ T cells in spleens. (**B**) Frequencies of annexin V⁺ donor CD8⁺ T cells. (**C**) Numbers of annexin V⁻ donor CD69⁺CD8⁺ T cells. (**D**) Numbers of annexin V⁻ IFN-γ–producing donor CD8⁺ T cells. (**E**) CTL activity against EL4. (**B–E**) Analysis was performed 14 days after BMT (n = 3–8/group). Representative data from 1 of the experiments are shown as the mean ± SD. *P < 0.05 compared with allogeneic controls.

differed at multiple mHAs from each other. [Db→Ba] and control [Db→Db] chimeras were lethally reirradiated and injected with 5 × 10⁶ TCD BM cells alone or with 2 × 10⁶ Ba T cells. Mortality (Figure 1D, P = 0.08) and morbidity from GVHD (Figure 1E, P < 0.05) were higher in [Db→Db] mice than in [Db→Ba] mice. When cells were transplanted together with 2,000 Db-derived P815 cells, leukemia mortality was significantly lower in [Db→Ba] mice than in [Db→Db] mice (10% vs. 60%; P < 0.05) (Figure 1F).

Similar results were obtained when [Ba→Db] and control [Ba→Ba] chimeras were transplanted with 5 × 10⁶ TCD BM cells with or without 2 × 10⁶ Db T cells. In [Ba→Db] recipients, in which non-hematopoietic cells do not express alloantigens, mortality (Figure 1G, P = 0.08) and morbidity of GVHD (Figure 1H, P < 0.05) were lower, but GVL effects against Ba-derived A20 lymphoma cells were significantly more potent as compared with [Ba→Ba] controls (leukemia mortality: 30% vs. 100%; P < 0.05) (Figure 1I). Taken together, these results demonstrate that GVHD is decreased but GVL activity is enhanced in the absence of alloantigen expression on non-hematopoietic cells.

Alloantigen expression on non-hematopoietic cells enhances apoptosis and dysfunction of alloreactive T cells. GVHD and GVL in the C3 and B6 strain combination is dependent on donor CD8⁺ T cells (12, 14). To elucidate the mechanisms responsible for the enhancement of the GVL effect in [B6→C3] chimeric mice, which lack alloantigen expression on non-hematopoietic cells, the kinetics of donor CD8⁺ T cell expansion and activation were evaluated after BMT. Expansion of donor CD8⁺ T cells identified as CD5.1⁺CD8⁺ cells peaked on day +14 in the spleens of allogeneic [B6→B6] recipients and decreased thereafter (Figure 2A), as previously shown in this model (15). CD8 expansion was significantly greater in [B6→C3]

mice than in [B6→B6] mice on days +14 and +21. We next assessed donor T cell apoptosis as a determinant of the kinetics of T cell expansion. Frequencies of annexin V⁺ apoptotic donor CD8⁺ T cells were significantly greater in the spleen of [B6→B6] mice as compared with that of [B6→C3] mice on day +14 (Figure 2B). Notably, surviving donor CD8⁺ T cells were significantly less activated in [B6→B6] mice than in [B6→C3] mice when evaluated based on the expression of CD69 (Figure 2C) and intracellular IFN-γ (Figure 2D) on annexin V⁻ donor CD8⁺ T cells. We next evaluated CTL activity in donor T cells isolated from the spleen on day +14 after BMT. CTL activity against EL4 targets was significantly reduced in the splenocytes of [B6→B6] mice as compared with [B6→C3] mice (Figure 2E). These results suggest that alloantigen expression on non-hematopoietic cells induces apoptosis and dysfunction of alloreactive T cells.

Absence of alloantigen expression on host non-hematopoietic cells restores GVL effects. Self-recognition in the periphery facilitates the reactivity of mature T cells to foreign antigens (16). Therefore, it is possible that the expression of syngeneic MHC molecules and not the absence of alloantigens on non-hematopoietic cells may be responsible for the enhancement of the GVL effect in [B6→C3] chimeras. This possibility was tested in B6-background β₂m⁻/⁻ mice. [B6→β₂m⁻/⁻] chimeras lacking functional MHC class I molecules on non-hematopoietic cells did not develop GVHD after transplantation with CD8⁺ T cells from C3 donors, as shown previously (17) (Figure 3A). In these mice, however, leukemia mortality was significantly delayed even in the absence of GVHD as compared with [B6→B6] recipients (Figure 3B, P < 0.05). The expansion and CTL activity of donor CD8⁺ T cells was significantly greater in [B6→β₂m⁻/⁻] recipients than in [B6→B6] recipients (Figure 3, C and D).
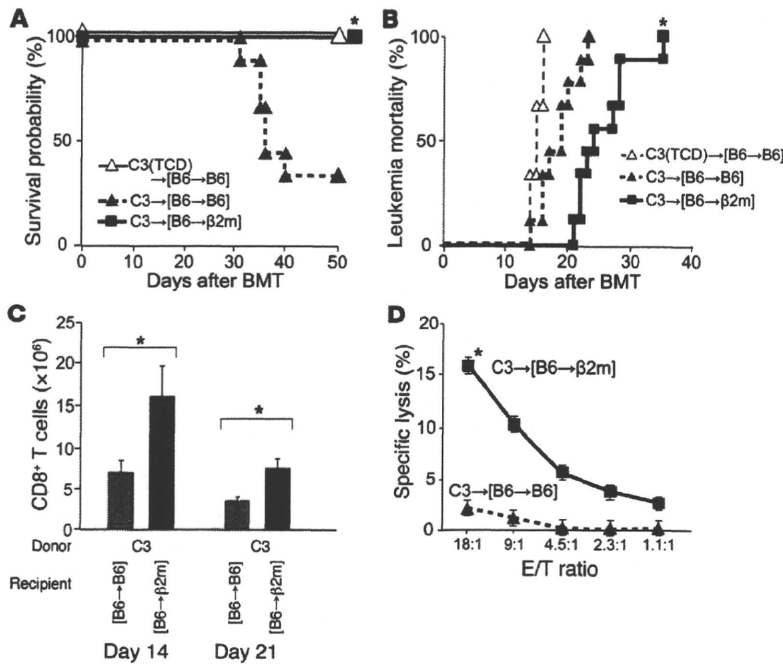
These results confirm that alloantigen expression on host epithelium induces apoptosis and dysfunction of alloreactive T cells, which results in impaired GVL effects.

Alloantigen expression on host non-hematopoietic cells stimulates programmed death-1 and its ligand pathway. Programmed death–1 (PD-1) is a negative regulator of activated T cells and regulates T cell exhaustion during chronic infections (18–20). PD-1 interacts with at least 2 ligands: PD ligand–1 (PD-L1) and PD-L2 (21). In particular, the PD-1/PD-L1 pathway has been proposed as one of the most important mechanisms of T cell exhaustion and tolerance induction against infectious agents and tumors (19, 22–25). We therefore hypothesized that the PD-1/PD-L1 pathway plays a role in the loss of GVL effects in [B6→B6] mice. To test this hypothesis, we examined PD-1 expression on donor CD8⁺ T cells in lymph nodes on day +14 and +21 after BMT. It was significantly upregulated in allogeneic [B6→B6] recipients as compared with syngeneic controls but was low in [B6→C3] mice (Figure 4, A and B). We also investigated the expression of another inhibitory receptor, CTLA-4, on donor CD8⁺ T cells. Although the expression of cytoplasmic CTLA-4 was slightly upregulated in allogeneic animals as compared with syngeneic animals, its level did not differ between [B6→B6] and [B6→C3] mice (5.5% ± 1.0% vs. 4.5% ± 0.2%, respectively; P = 0.50).

We next examined PD-L1 expression in the liver by real-time PCR after BMT. PD-L1 expression was markedly upregulated in the liver of allogeneic controls as compared with syngeneic controls (Figure 4C). In allogeneic [B6→C3] mice, it was slightly upregulated on day +14 but not on day +21. Immunohistochemical analysis confirmed upregulated expression of PD-L1 in the liver of [B6→B6] mice, as previously reported (Figure 4D) (21, 26). These results showed that alloantigen expression on GVHD target epithelium is associated with upregulation of the PD-1/PD-L1 interactions between donor T cells and GVHD target tissue.

Blockade of the interaction between PD-1 and PD-L1 enhances GVL activity. We next examined whether blocking the PD-1/PD-L1 pathway could enhance GVL activity. [B6→C3] and [B6→B6]

chimeras were reirradiated and injected with TCD BM cells and CD8⁺ T cells from C3 donors. Mice were i.p. injected with 500 μg of anti–PD-L1 mAb on day 0 and then with 200 μg on days +3, +6, +9, +12, +15, and +18 after BMT. In [B6→B6] recipients, injection of anti–PD-L1 mAbs significantly restored T cell functions on day +14, as assessed by CD69 expression (Figure 5A), IFN-γ production (Figure 5B), and CTL activity (Figure 5C). In [B6→C3] mice, it marginally upregulated CD69 expression, IFN-γ production, and CTL activity, although differences were not statistically significant (Figure 5, A, B, and D). As a consequence, anti–PD-L1 mAb administration significantly increased the severity of GVHD in [B6→B6] mice (Figure 5E) but not in [B6→C3] mice (Figure 5F). PD-L1 blockade also significantly augmented GVL activity in [B6→B6] recipients injected with EL4 cells on day 0 (Figure 5G, P < 0.05). It also delayed leukemia death in [B6→C3] mice, although the difference was not statistically significant (Figure 5H, P = 0.38). In controls, PD-L1 blockade did not affect leukemia mortality in TCD-BMT recipients (Figure 5H) or [B6→B6] recipients of syngeneic B6 CD8⁺ T cells (data not shown).

Discussion

Alloantigens are expressed in three major sites in HSCT recipients: APCs, GVHD target epithelium, and leukemia cells. Alloantigen expression on APCs is essential for the induction of GVHD (6), and an optimal GVL response occurs when alloantigens are expressed on both host APCs and tumor cells (7). Alloantigen expression on the epithelium is also critical for the induction of GVHD across mHA disparities (10), but GVHD can occur in the absence of alloantigen expression on epithelium in MHC-mismatched BMT (9). In this study, we addressed the effect of alloantigen expression on target epithelium in GVL using chimeric mouse models of GVHD and GVL across mHA disparities. Our models mimic clinical BMT in patients not in remission, since most of the mice relapsed after allogeneic BMT. This high tumor burden enabled us to compare the magnitude of GVL activity in our models, and we made sur-