(A)

(B)

(C)

(D)

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].
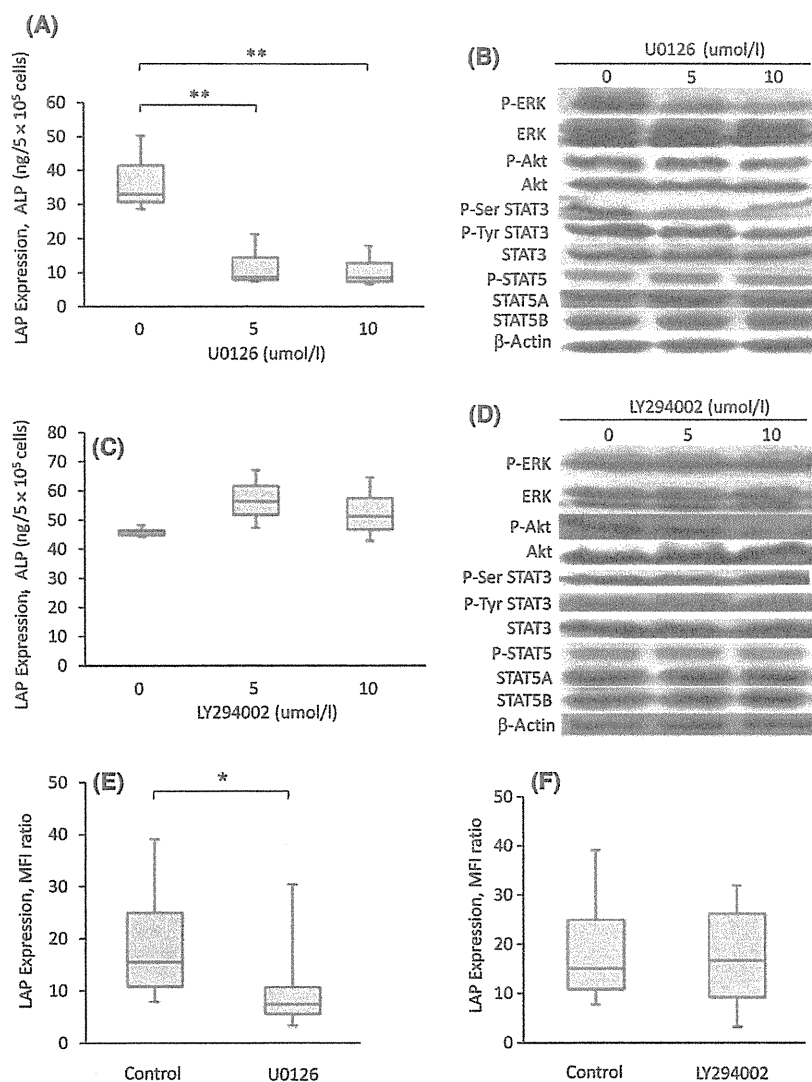
Fig 5. The effects of U0126 (MEK1/2 inhibitor) and LY294002 (PI3K inhibitor) on LAP expression in NB4-derived neutrophils. *Jak2* V617F-transduced NB4 cells were cultured with ATRA (10 μmol/l), G-CSF (10 ng/ml) and U0126 (0–10 μmol/l) (A) or LY294002 (0–10 μmol/l) (C) for 4 d and differentiated into 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
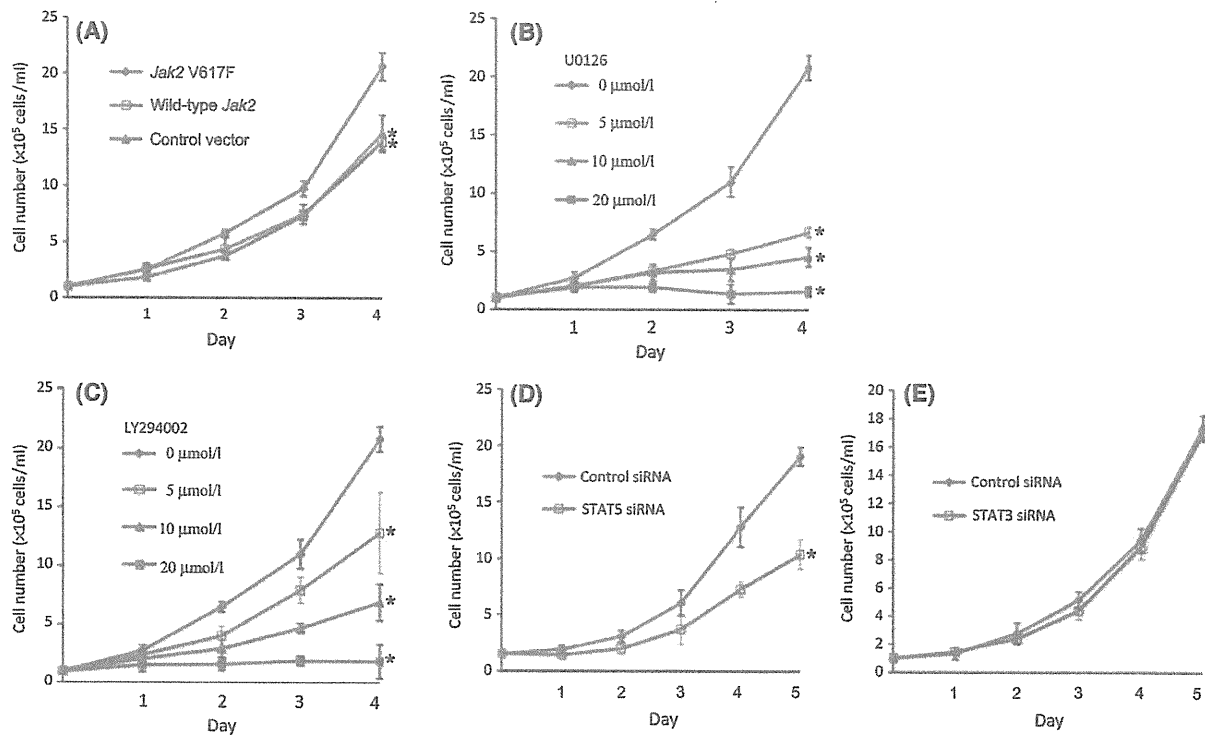
340

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
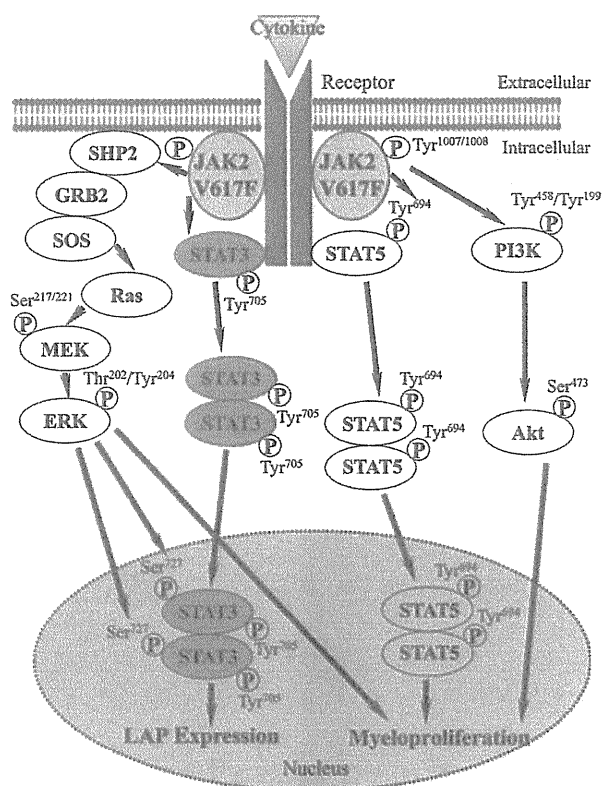
Fig 7. Schematic presentation of JAK2 V617F signalling to activate myeloproliferation and LAP expression. The activated JAK2 kinase induces STAT3/STAT5 tyrosine phosphorylation, and then phosphorylated STATs are dimerized, enter the nucleus, and bind specific regulatory sequences to regulate transcription 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*

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.

104

Functional involvement of Daxx in gp130-mediated cell growth and survival in BaF3 cells

Ryuta Muromoto[1], Makoto Kuroda[1], Sumihito Togi[1], Yuichi Sekine[1], Asuka Nanbo[1], Kazuya Shimoda[2], Kenji Oritani[3] and Tadashi Matsuda[1]

[1] Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, Japan
[2] Department of Internal Medicine II, Faculty of Medicine, University of Miyazaki, Kihara, Kiyotake, Miyazaki, Japan
[3] Department of Hematology and Oncology, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka, Japan

Death domain-associated protein (Daxx) is a multifunctional protein that modulates both cell death and transcription. Several recent studies have indicated that Daxx is a mediator of lymphocyte death and/or growth suppression, although the detailed mechanism is unclear. Previously, we reported that Daxx suppresses IL-6 family cytokine-induced gene expression by interacting with STAT3. STAT3 is important for the growth and survival of lymphocytes; therefore, we here examined the role of Daxx in the gp130/STAT3-dependent cell growth/survival signals. We found that Daxx suppresses the gp130/STAT3-dependent cell growth and that Daxx endogenously interacts with STAT3 and inhibits the DNA-binding activity of STAT3. Moreover, small-interfering RNA-mediated knockdown of Daxx enhanced the expression of STAT3-target genes and accelerated the STAT3-mediated cell cycle progression. In addition, knockdown of Daxx-attenuated lactate dehydrogenase leakage from cells, indicating that Daxx positively regulates cell death during gp130/STAT3-mediated cell proliferation. Notably, Daxx specifically suppressed the levels of Bcl2 mRNA and protein, even in cytokine-unstimulated cells, indicating that Daxx regulates Bcl2 expression independently of activated STAT3. These results suggest that Daxx suppresses gp130-mediated cell growth and survival by two independent mechanisms: inhibition of STAT3-induced transcription and down-regulation of Bcl2 expression.

Key words: Cell death · Daxx · gp130 · IL-6 · STAT3

Introduction

Death domain-associated protein (Daxx) is a multifunctional protein that modulates both cell death and transcription [1]. Daxx is present in most cell types and is mainly located in the nucleus. Because previous studies have demonstrated that Daxx can bear both pro- and anti-cell-death activities depending on the stimulus and the cell

type [1, 2], the precise role of Daxx, in particular its ability to promote or hinder cell death, remains controversial. Nevertheless, in lymphocytes, the following observations indicate that Daxx has pro-cell death and/or growth-suppressing functions. First, Daxx is induced by type I interferon in pro-B cells and is required for interferon-induced apoptosis [3]. Second, in Con A-stimulated splenocytes, Daxx up-regulation and interaction with PML correlate with the induction of B-cell apoptosis [4]. Third, CD40-induced proliferation is profoundly reduced in transgenic B cells over-expressing Daxx [5]. Fourth, analysis of T-cell-specific transgenic mice expressing a dominant negative form of Daxx (Daxx-DN)

Correspondence: Dr. Tadashi Matsuda
e-mail: tmatsuda@pharm.hokudai.ac.jp

has reveled that Daxx-DN protects activated T cells from Fas-induced cell death and that T-lymphocytes expressing Daxx-DN exhibit increased proliferative T-cell responses [6]. These findings have indicated that Daxx is a mediator of lymphocyte death and/or growth suppression. However, the precise mechanism of how Daxx affects cell survival remained unclear.

Daxx interacts with and regulates the transcriptional activities of several DNA-binding transcription factors, including ETS1 [7], PAX5 [8], Glucocorticoid receptor [9], RelA [10], RelB [11], TCF4 [12], SMAD4 [13], C/EBP [14], AIRE [15] and STAT3 [16]. Considering that Daxx binds histone deacetylases [17], DNA methyltransferases and their associated proteins [18–20], and the chromatin-modifying protein α-thalassemia syndrome protein [21, 22], it seems possible that Daxx regulates cellular processes by regulating transcription of specific genes under different conditions. We have previously shown that Daxx suppresses STAT3-mediated transcriptional activity and has a role in regulating IL-6 family cytokine signaling and gene induction in several cancer cell lines [16].

The IL-6 family of cytokines utilizes the membrane glyco-protein gp130 as a critical signal-transducing receptor compo-nent [23]. Dimerization of gp130 activates the JAK family of protein tyrosine kinases, which phosphorylate and activate cytoplasmic STAT3 [24, 25]. Activated STAT3 dimerizes and translocates to the nucleus where it binds to specific DNA response elements and induces expression of STAT3-regulated genes. STAT3 has been known to play critical roles in the regu-lation of various cellular events, malignancies and autoimmune diseases [26–28]. In lymphocyte proliferation, STAT3 activation is responsible for IL-6-dependent T-cell proliferation by preventing apoptosis [29] and STAT3 is also indispensable for IL-27-mediated cell proliferation [30]. Moreover, Chou et al. demonstrated that STAT3 is required for maintaining pro-B-cell survival and for efficient B lymphopoiesis [31].

In the present study, we examined the role of Daxx in the gp130/STAT3-mediated cell proliferation signal. We found that Daxx represses STAT3 activity through the inhibition of DNA-binding, thereby inhibiting cytokine-dependent growth and survival of BaF3 cells. Moreover, Daxx also regulates *Bcl2* gene expression independently of STAT3 regulation. These results suggest that Daxx exerts growth suppression through the modu-lation of transcription.

Results

Daxx suppresses gp130/STAT3-dependent cell proliferation

We used an IL-3-dependent mouse pro-B-cell line, BaF3-derived BaF-G133, as a model for gp130/STAT3-mediated cell prolifera-tion. It has been reported that, in the absence of IL-3, G-CSF-treatment of BaF-G133 cells induces dimerization of GCSFR/gp130 chimeric receptors causing activation of STAT3 and STAT3-dependent cell growth [32].

To examine the role of Daxx in gp130/STAT3-mediated cell growth, we introduced siRNA against Daxx (siDaxx) into BaF-G133 cells. Daxx protein levels were significantly reduced by siDaxx, whereas siDaxx had no effect on the levels of STAT3 or β-actin (Fig. 1A). We then investigated whether this reduction of Daxx in BaF-G133 cells could affect the rate of cell proliferation. After transfection of siRNA, cells were washed in IL-3-free medium and incubated for 48 h in the absence or presence of G-CSF at various concentrations. Silencing of endogenous Daxx by siRNA significantly enhanced the proliferation of BaF-G133 cells at all G-CSF concentrations tested (Fig. 1B). To further elucidate the role of Daxx in gp130/STAT3-mediated cell growth, we established BaF-G133 transfectants over-expressing Daxx
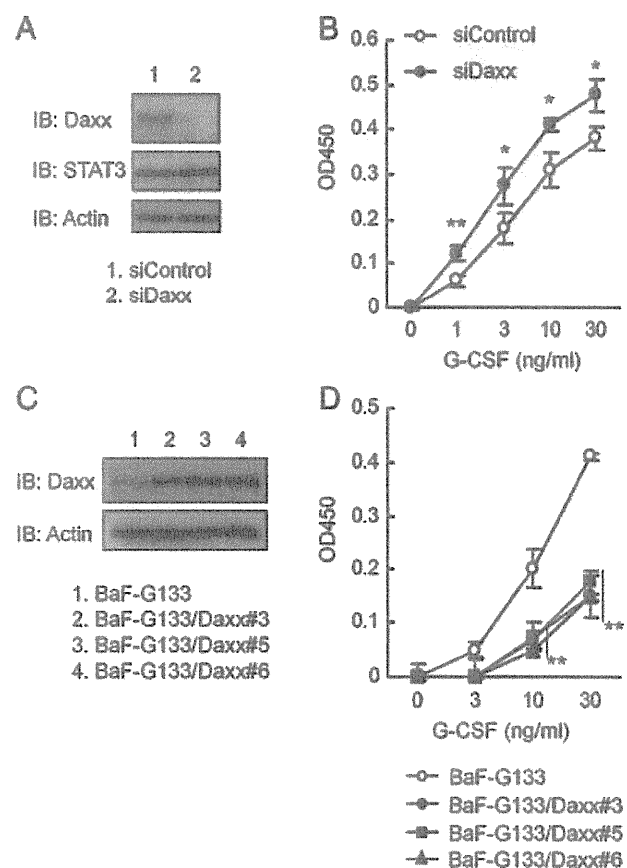

Figure 1. Involvement of Daxx protein in the regulation of gp130/STAT3-dependent cell proliferation. (A) BaF-G133 cells were transfected with Daxx-specific and control siRNA and Daxx knockdown was validated by western blot analysis of Daxx, STAT3 and β-actin. Knockdown of Daxx was comfirmed until 3 days after siRNA transfection. Data are representative of three independent experi-ments. (B) G-CSF-induced cell proliferation of control or Daxx siRNA transfected BaF-G133 cells was determined by the WST8 assay. Data show mean±SD of triplicate samples and representative of three independent experiments. *p<0.05 and **p<0.01 (Student's t-test). (C) Lysates from stable transfectants of BaF-G133 cells over-expressing Daxx were subjected to western blot analysis of Daxx and β-actin. Data are representative of three independent experiments. (D) The WST8 assay was performed on BaF-G133 cells and Daxx transfectants. Data show mean±SD of triplicate samples and representative of three independent experiments. **p<0.01 (Student's t-test).

(Fig. 1C; BaF-G133/Daxx#3, BaF-G133/Daxx#5, BaF-G133/Daxx#6) and performed growth assays in the presence or absence of either G-CSF. Over-expression of Daxx caused a reduction in cell number when cells were stimulated with G-CSF (Fig. 1D). These results indicated that Daxx has the ability to suppress the gp130/STAT3-mediated cell growth.

stained with propidium iodide and then DNA content *per* cell was determined by flow cytometry. As shown in Fig. 2A, BaF-G133 cells transfected with Daxx siRNA exhibited a significant acceleration in gp130-induced cell cycle progression to S and G2/M phases compared with those transfected with control siRNA.

Daxx suppresses cell cycle progression in BaF-G133 cells

To clarify how Daxx regulates cell proliferation, we examined whether gp130-induced cell cycle progression could be affected by knockdown of Daxx. After transfection of siControl or siDaxx, BaF-G133 cells were synchronized in G0/G1 by withdrawing IL-3 and cells were then treated with G-CSF. Cells were fixed and

Daxx increases cell death in BaF-G133 cells

Daxx has been reported to positively or negatively regulate cell death [1, 2]. Thus, we tested whether Daxx is involved in the regulation of cell death in BaF-G133 cells. Leakage of lactate dehydrogenase (LDH) into the medium was used as a marker for membrane breakage and cell death. In cells transfected with control siRNA and cultured without IL-3,
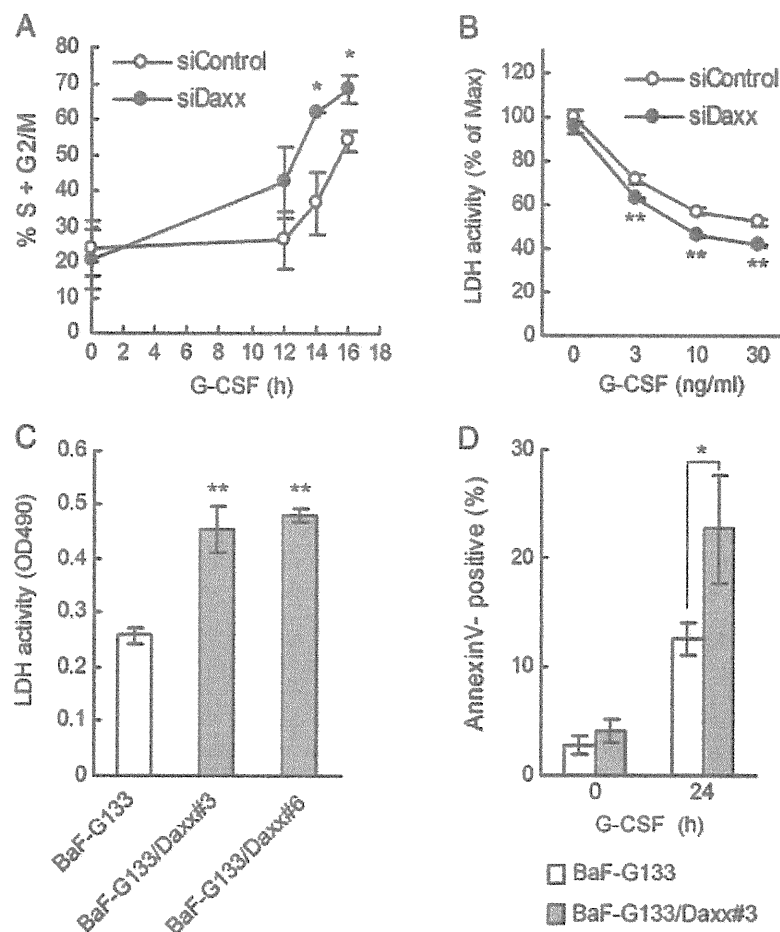

Figure 2. Daxx regulates cell cycle progression and cell death during gp130/STAT3-dependent cell growth. (A) BaF-G133 cells were treated with control siRNA or Daxx siRNA. Cells were IL-3-starved for 12 h and then treated with 30 ng/mL G-CSF for the indicated times. Cells were then fixed and subjected to cell cycle analysis using FACS. The percentages of cells in S plus G2/M phases of the cell cycle are indicated (mean ± SD, $n = 3$). *$p<0.05$ (Student's t-test). (B) Daxx knockdown and control cells were cultured with the indicated concentrations of G-CSF for 48 h and the effect of Daxx knockdown on the G-CSF-induced reduction in cell death of BaF-G133 cells was evaluated by LDH activity in cell culture supernatants. Results are presented as the percentage of the maximum LDH release observed in untreated control cells (mean ± SD, $n = 3$). **$p<0.01$ (Student's t-test). (C) The LDH assay was performed on BaF-G133 cells and Daxx transfectants, cultured with 30 ng/mL of G-CSF for 48 h. Results are presented as OD at 490 nm (Mean ± SD, $n = 3$). **$p<0.01$ (Student's t-test). (D) BaF-G133 and BaF-G133/Daxx#3 cells were treated with 30 ng/mL G-CSF for the indicated times and the percentages of Annexin-V-positive cells were determined by FACS analysis (Mean ± SD, $n = 3$). *$p<0.05$ (Student's t-test).

G-CSF treatment, in a concentration-dependent manner, increased the number of viable cells (as shown in Fig. 1B) with a concomitant reduction of cell death, (Fig. 2B, open circle). Knockdown of endogenous Daxx significantly augmented G-CSF-induced reduction of LDH leakage compared with control cells (Fig. 2B, closed circle).

LDH leakage was increased by about 1.8-fold in Daxx over-expressing transfectants compared with control BaF-G133 cells (Fig. 2C). In addition, BaF-G133/Daxx#3 cells exhibited an increased percentage of Annexin-V-positive cells compared with control BaF-G133 cells (Fig. 2D).

These results indicated that Daxx has the ability to suppress cell cycle progression and to increase cell death, thereby controlling cell proliferation in BaF-G133 cells.

Daxx binds to STAT3 and inhibits its activity

We previously showed that Daxx interacts with STAT3 in transiently transfected 293T cells [16]. STAT3 is essential for gp130-mediated G1 to S phase transition in BaF-G133 cells [33]; therefore, we considered STAT3 to be a potential target of the anti-proliferative function of Daxx. To test whether a Daxx-STAT3 interaction occurs in BaF-G133 cells, we performed co-immunoprecipitation assays (Fig. 3A). An anti-Daxx antibody, but not control rabbit IgG, co-immunoprecipitated endogenous STAT3, regardless of G-CSF stimulation. These data suggest that Daxx endogenously forms a complex with STAT3 in BaF-G133 cells in a constitutive manner.

We next tested whether Daxx affects gp130-mediated phosphorylation of STAT3 and ERK/MAPK. We found that the gp130-mediated upregulation of STAT3 or ERK/MAPK phosphorylation in both BaF-G133/Daxx#3 and/Daxx#6 cells was comparable to that in BaF-G133 cells (data not shown). Therefore, we assumed that Daxx does not participate in the modulation of the activation steps for STAT3 and ERK/MAPK signaling pathways.

We have previously shown that the DNA-binding domain of STAT3 (320–493) interacts with Daxx [16]; therefore, we next examined whether a reduction of Daxx expression affects the DNA-binding activity of STAT3. We introduced siControl or siDaxx into BaF-G133 cells. Cells were washed, starved for 12 h and stimulated with G-CSF for 10 and 30 min. As shown in Fig. 3B, G-CSF-induced STAT3 binding to its consensus oligo-nucleotide was clearly enhanced by siDaxx treatment, suggesting that Daxx has an inhibitory effect on the DNA-binding activity of STAT3. To investigate the effects of Daxx on the DNA-binding activity of STAT3 in the context of chromatin structure, we performed chromatin immunoprecipitation analysis on the STAT3-binding site in the 5′ region of the JunB gene (Fig. 3C). The STAT3-binding site was amplified by PCR in all of the sheared DNA samples (top, indicated as "Input"). In addition, this region was amplified by PCR from the anti-STAT3 immunoprecipitates obtained from G-CSF-stimulated cells (middle, lane 2), but not from control Ig immunoprecipitates (bottom), indicating that STAT3 activated by G-CSF bound to this element. Impor-

tantly, siDaxx transfection enhanced this binding (middle, lane 4). These findings indicate that Daxx also inhibits DNA-binding activity of STAT3 in chromatin.

To further delineate the functional relevance of Daxx in STAT3 regulation, we examined the status of STAT3-target gene expression in BaF-G133 cells. Either siControl- or siDaxx-transfected cells were treated with G-CSF for 30 min, followed by RNA extraction, reverse transcription and RT-PCR analysis using mouse Socs3-specific primers. The Socs3 mRNA levels were normalized against Actb. As shown in Fig. 3D, Socs3 was induced 6-fold with G-CSF treatment in the control cells and Socs3 was induced by almost 13-fold in the siDaxx-transfected cells, suggesting that the siDaxx-transfected cells have enhanced expression of STAT3-target genes. Consistent with these observations, knockdown of Daxx in BaF-G133 cells also resulted in enhanced induction of other known STAT3-target genes, JunB and Pim2 (Fig. 3E).

Daxx is involved in the regulation of Bcl2 mRNA and protein levels

Dimerization of G-CSFR-gp130 chimeric receptors in BaF-G133 cells transduces anti-apoptotic signal through the induction of Bcl2 [32]. Thus, we next examined the effects of Daxx knock-down on the mRNA levels of Bcl2 family genes. After washing with IL-3-free medium, BaF-G133 cells were stimulated with G-CSF and mRNA levels were analyzed by semi-quantitative RT-PCR, as indicated in Fig. 4A. In control siRNA-transfected cells, G-CSF treatment induced the upregulation of Bcl2 mRNA levels, but not of Bcl2l1, which encodes the Bcl-x protein. The mRNA level of pro-apoptotic Bax was unaffected by G-CSF. Notably, knockdown of Daxx increased the basal level of Bcl2 mRNA, indicating that Daxx regulates transcription factors involved in Bcl2 expression other than activated STAT3. Reciprocally, BaF-G133 cells over-expressing Daxx exhibited decreased Bcl2 mRNA levels regardless of G-CSF stimulation (Fig. 4B). In addition, we introduced siDaxx into BaF-G133 to knockdown endogenous Daxx and found an increase in protein levels of Bcl2 in the absence of G-CSF (Fig. 4C). We also prepared cell extracts from BaF-G133 or BaF-G133/Daxx#3 cells and analyzed endogenous protein levels of Bcl2 and Bax by western blotting. As shown in Fig. 4D, reduced expression of Bcl2 was observed in BaF-G133/Daxx#3 cells in G-CSF-treated conditions and also in non-G-CSF-treated conditions. Protein levels of Bax, however, were unaltered by G-CSF stimulation or Daxx expression. Interestingly, we also found that a decrease of Daxx protein preceded the up-regulation of Bcl2 after G-CSF-stimulation. Collectively, these data indicate that Daxx rather specifically regulates Bcl2 expression at the mRNA level under both basement and G-CSF-stimulated conditions.

It has been shown that the cyclic AMP response element (CRE) is a major positive regulatory site in the Bcl2 promoter in B-cells and that mutation of the CRE abolishes the binding of CREB/ATF and CREB-Binding Protein (CBP) transcription factors
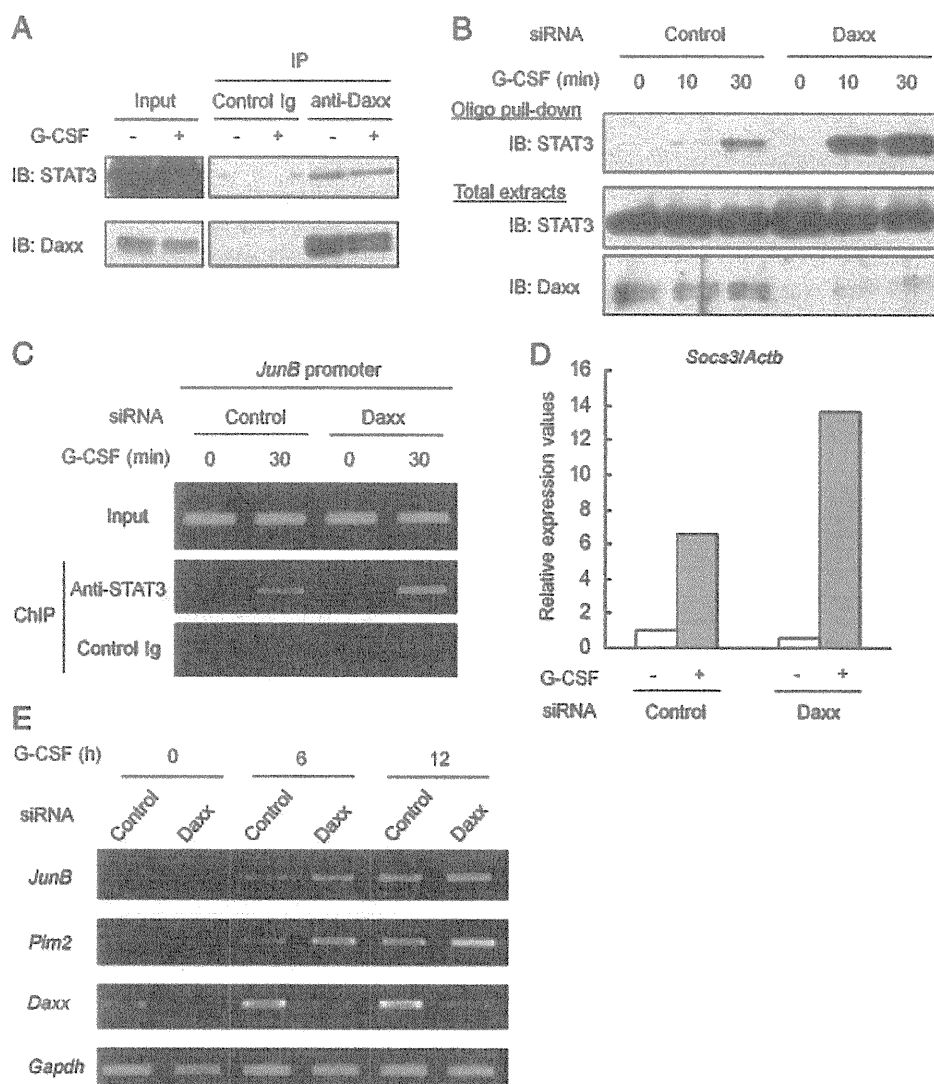
Figure 3. Daxx interacts with STAT3 and inhibits its activity. (A) BaF-G133 cells (5×10^7 cells) were stimulated with G-CSF (30 ng/ml) for 30 min. The cells were lysed, immunoprecipitated with control IgG or anti-Daxx antibody and subjected to western blot analysis of STAT3 and Daxx. An aliquot of total cell extract (input) was also analyzed. (B) BaF-G133 cells (2×10^6 cells) were treated with control siRNA or Daxx siRNA, and cells were stimulated with G-CSF (30 ng/ml) for the indicated periods. Cell extracts were prepared and subjected to pull-down experiments using the immobilized STAT3 consensus oligonucleotide-sepharose conjugate to evaluate DNA binding activity of STAT3. The precipitates and an aliquot of total cell extract were subjected to western blot analysis of STAT3. (C) BaF-G133 cells (4×10^6 cells) were treated with control siRNA or Daxx siRNA and cells were stimulated with G-CSF (30 ng/ml) for the indicated periods. Samples for ChIP were prepared as described in the *Materials and methods* section. STAT3-DNA binding complexes were immunoprecipitated with the anti-STAT3 antibody or with control IgG. The immunoprecipitated DNA was eluted and subjected to PCR. (D) BaF-G133 cells were treated with control or Daxx siRNA and cells were stimulated with G-CSF (30 ng/mL) for 30 min. Total RNA samples isolated from these cells were subjected to quantitative RT-PCR analysis using Socs3 or Actb primers. Data represent the levels of Socs3 mRNA normalized to that of an Actb internal control and are expressed relative to the value of control siRNA-treated samples without G-CSF-stimulation. Results are representative of three independent, duplicate experiments. (E) BaF-G133 cells were treated with control or Daxx siRNA and cells were stimulated with G-CSF (30 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to semi quantitative RT-PCR analysis using JunB, Pim2, Daxx or Gapdh primers. Data in (A), (C) and (E) are representative of three independent experiments; (B) is representative of two independent experiments.

to the *Bcl2* promoter and greatly diminishes the binding of NF-κB factors [34]. Because Daxx can interact with RelA [10] and RelB [11], two members of the NF-κB family of transcription factors, as well as CBP [35], we next examined the binding of these proteins to the region of the *Bcl2* promoter containing the CRE site. The ChIP assays were performed with G-CSF-treated BaF-G133 and BaF-G133/Daxx#3 cells, and an anti-rabbit IgG was used as a nonspecific immunoprecipitation control. The results demonstrated that in BaF-G133 cells, RelA and CBP, but not RelB, binds to the CRE site (Fig. 4E). In addition, a substantial decrease in the binding of RelA and CBP was observed in the BaF-G133/Daxx#3 cells (Fig. 4E). These results indicated that Daxx negatively regulates RelA and CBP binding to the Bcl2 promoter in BaF-G133 cells.

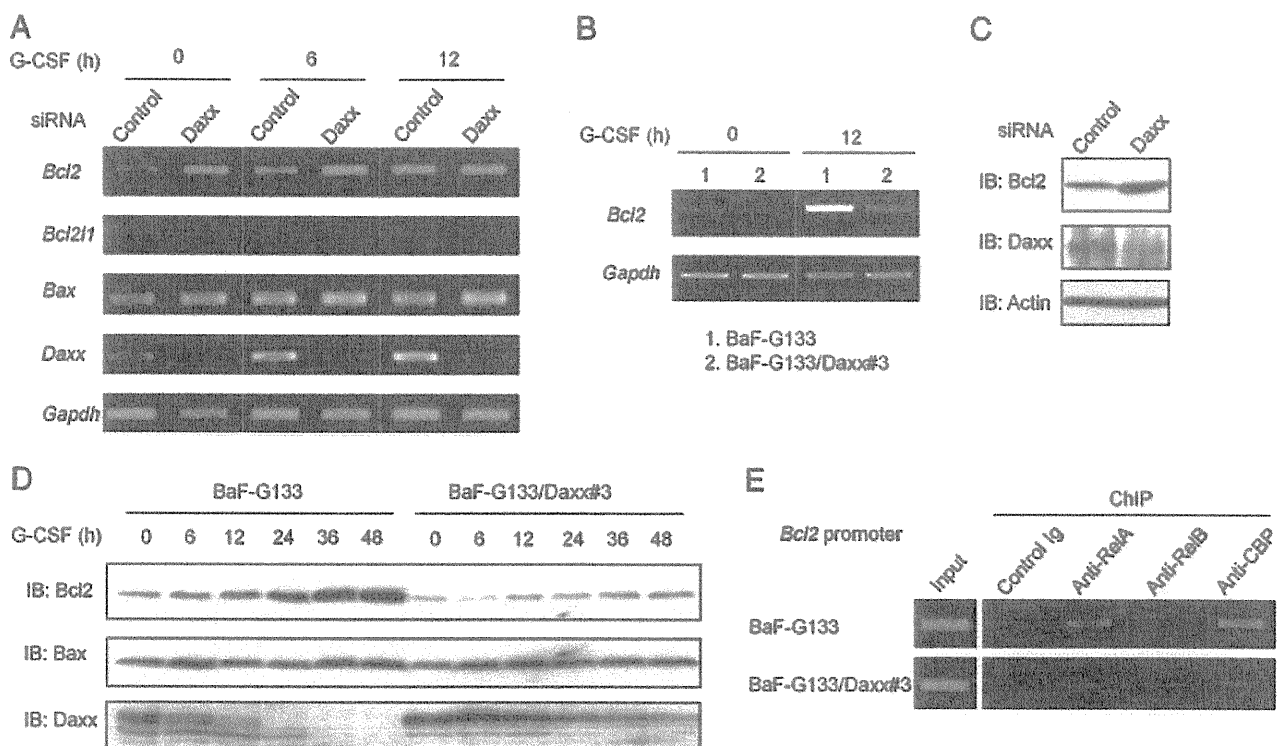**Figure 4.** Daxx suppresses Bcl2 mRNA and protein levels. (A) cDNA samples, prepared as described in Fig. 3E, were subjected to semi quantitative analysis using Bcl2, Bcl2l1 and Bax primers. (B) BaF-G133 or BaF-G133/Daxx#3 cells were treated with G-CSF at 30 ng/mL for 12 h. Total RNA was then extracted and subjected to RT-PCR analysis. (C) BaF-G133 cells were transfected with control siRNA or Daxx siRNA and endogenous Bcl2 protein was detected by western blot analysis. (D) BaF-G133 and BaF-G133/Daxx#3 cells were treated with 30 ng/mL G-CSF for the indicated times. Protein levels of Bcl2, Bax and Daxx in cells were detected by western blot analysis. (E) BaF-G133 or BaF-G133/Daxx#3 cells (4×10^6 cells) were stimulated with G-CSF (30 ng/mL) for 6 h. Samples for ChIP were prepared as described in the Materials and methods section. Transcription factor-DNA binding complexes were immunoprecipitated with indicated antibodies or with control IgG. The immunoprecipitated DNA was analyzed by PCR using primers that amplified a 100-bp product that includes the CRE site in the Bcl2 promoter. Data in this figure are representative of three independent experiments.

Over-expression of Bcl2 represses Daxx-mediated cell death

We then analyzed whether Daxx-mediated cell death of BaF-G133 cells was repressed by over-expression of Bcl2. In G-CSF-treated conditions, over-expression of Bcl2 in BaF-G133/Daxx#3 cells decreased LDH leakage to a level comparable with that observed in control BaF-G133 cells (Fig. 5A, left and 5B). Treatment with HA14-1, a small-molecule Bcl2 inhibitor [36], decreased cell viability in a dose-dependent manner in G-CSF-treated BaF-G133 cells (Fig. 5C), confirming that Bcl2 function contributes to gp130-mediated survival of this cell line. These data indicate that the Daxx-mediated increase in cell death is due, at least in part, to changes in Bcl2 expression in G-CSF-treated BaF-G133 cells.

Discussion

Our present manipulation of Daxx expression has revealed that Daxx negatively regulates gp130-mediated signals. We here proposed and discussed two possible mechanisms concerning to STAT3 and Bcl2. Daxx constitutively interacted with STAT3, resulting in the impaired binding of STAT3 to its consensus DNA sequence in chromatin. In addition, Daxx preferentially down-regulated Bcl2 expression at the mRNA level. These cellular modifications mediated by Daxx are likely to suppress gp130-mediated cell proliferation and survival.

The involvement of STAT3 in IL-6/gp130-mediated growth responses has been indicated by many investigators. For example, the G1 to S phase progression of cell cycle was shown to be a STAT3-dependent process [33]. In BaF-G133 cells, G-CSF-treatment was reported to induce dimerization of G-CSFR/gp130 chimeric receptors causing activation of STAT3 as well as STAT3-dependent cell growth [32]. In lymphocytes, STAT3 activation is reported to be responsible for IL-6-dpendent T-cell proliferation [29] and for normal early B-cell development [31]. With regard to this gp130-mediated STAT3 function, we here showed constitutive interactions between endogenous Daxx and STAT3. Of importance, our data clearly indicated that these interactions gave rise to a decrease in the DNA-binding activity of STAT3 rather than that in the activation steps of STAT3. Our previous report telling that the DNA-binding domain of STAT3 (320–493) was a major domain to interact with Daxx [16] might explain the inhibition of STAT3 transcription by Daxx.
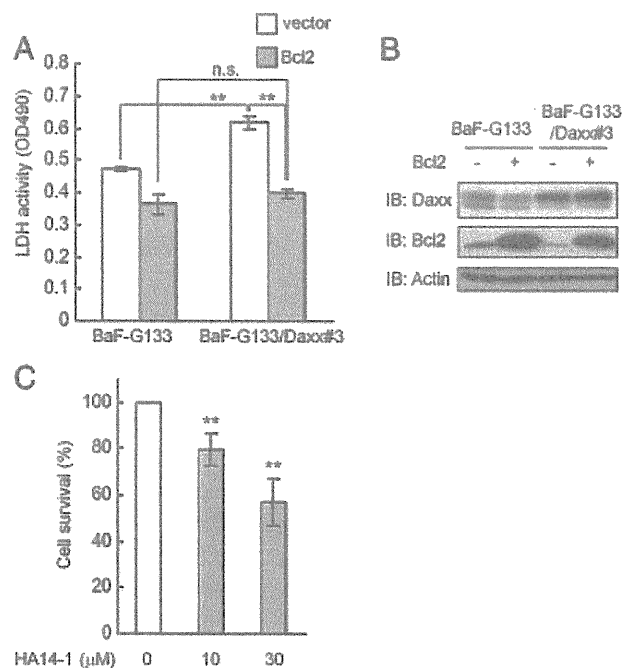
Figure 5. Over-expression of Bcl2 repressed Daxx-mediated cell death. (A) BaF-G133 or BaF-G133/Daxx#3 cells were transfected with empty plasmid or Bcl2 expression plasmid. After treatment with G-CSF (30 ng/mL, left) for 48 h, LDH activities in culture supernatants were measured. Results are presented as OD at 490 nm (mean ± SD, $n = 3$; **$p < 0.01$ and n.s., not significant). (B) Transient over-expression of Bcl2 in BaF-G133 or BaF-G133/Daxx#3 cells was validated by western blot analysis of Daxx, Bcl2 and β-actin. Data are representative of three independent experiments. (C) BaF-133 cells were plated in a 96-well plate in the presence of G-CSF (30 ng/mL) without or with the indicated concentrations of the small-molecule Bcl2 inhibitor, HA14-1. Twenty-four hours after treatment, cell number was determined by the WST8 assay. The value obtained for DMSO-treated (0 μM) cells was set as 100% survival. Data show mean ± SD ($n = 3$). **$p < 0.01$ (Student's t-test).

Although Daxx negatively regulated gp130-mediated *Bcl2* mRNA induction, it also reduced basal level of *Bcl2* expression at the mRNA level. This is likely to suggest that Daxx must have further targets for repression, in addition to activated STAT3. In this regard, we first considered ETS1 to be a candidate, because *Bcl2* is induced by over-expression of ETS1 [7]. However, we could not detect any *Ets1* mRNA expression in BaF-G133 cells (data not shown), indicating that ETS1 is not involved in the Bcl2 expression in those cells. *Bcl2* is also a well-known anti-apoptotic target gene of NF-κB. Daxx can interact with RelA [10], RelB [11] and CBP [35], we therefore assessed the effect of Daxx over-expression on Rel and CBP binding to the *Bcl2* promoter. Our ChIP experiments using the primers amplifying the Bcl2 promoter region containing the CRE clearly indicated that Daxx negatively regulates RelA and CBP in BaF-G133 cells. In addition, our experiments using a Bcl2-specific inhibitor indicated that gp130-mediated Bcl2 expression is indeed involved in the maintenance of cell survival and that Daxx specifically inhibits the *Bcl2* expression. During apoptosis of lymphocytes, an inverse correlation of Daxx and Bcl2 expression levels has been suggested to be important. For example, gene expression profiling of inter-

feron-β-treated progenitor B cells revealed enhanced Daxx expression and nuclear accumulation, with subsequent down-regulation of *Bcl2*, followed by apoptosis [3]. Another report said that certain mantle cell lymphomas have markedly decreased levels of *Daxx* expression and exhibit over-expression of *Bcl2* and *Bcl2l1* [37]. It has also been reported that ectopic over-expression of Daxx down-regulates *Bcl2* in malignant lymphocyte Jurkat T-cells and sensitizes cells to the apoptosis-inducing effect of chemotherapeutic agents [38]. These findings and our results support the idea that Daxx participates in repression, especially that of *Bcl2* gene expression and thereby sensitizes cell death.

As described above, Daxx inhibited gp130-mediated cell growth and survival in BaF-G133 cells. In a case of IL-3 signals, which mainly utilize STAT5 [39–41], manipulation of Daxx expression failed to modify their growth and/or survival (data not shown). This different involvement of Daxx may come from the difference in activated STAT proteins between gp130- and IL-3-mediated signaling. In accordance with this notion, we could not detect the specific interactions between Daxx and STAT5 in our co-immunoprecipitation experiments (data not shown). These observations indicate that Daxx preferentially interacts with and inhibits STAT3 rather than STAT5.

The functions of Daxx are related to its protein level. It has been reported that the peptidyl–prolyl isomerase, Pin1, inhibits Daxx-mediated apoptosis by inducing Daxx ubiquitination and degradation [42], and that the BTB domain-containing speckle-type POZ protein/Cul3 ubiquitin ligase complex inhibits the transcriptional repression function of Daxx by mediating the proteasome-dependent degradation of Daxx [43]. Furthermore, Mdm2 and Hausp regulate Daxx functions by controlling Daxx ubiquitination and stability [44]. Interestingly, we found that gp130-mediated up-regulation of Bcl2 is accompanied by a reduction in the level of endogenous Daxx protein. This reduction of Daxx protein may be regulated by the above factors and may be an important process in the 'de-repression' mechanism for the regulation of both STAT3 activity and Bcl2 expression, although the details of this are still unclear.

G-CSF is a common inducer of the release of hematopoietic progenitor cells (HPC) from the bone marrow into the peripheral blood [45]. One of the molecular mechanisms underlying this action of G-CSF has been implicated to involve the phosphorylation of JAK1/STAT3 pathway [46]. Daxx has been reported to be expressed in HPC [47]. Therefore, it is possible to speculate that Daxx could also influence G-CSF-mediated mobilization of HPC and the further study will be required to clarify this point.

In summary, we suggest that Daxx has two functional roles corresponding to cell death regulation: STAT3 repression and down-regulation of Bcl2. Our results suggest that the ability of Daxx to repress transcription is relevant to its ability to sensitize cell death. In addition, our finding that STAT3 function can be modulated by Daxx will provide insight into the regulation of various cellular events, malignancies and autoimmune diseases in which STAT3 plays critical roles.

Materials and methods

Cell culture

BaF-G133 cells were a kind gift from Dr. Toshio Hirano (Osaka University, Osaka, Japan) and have been described [32]. BaF-G133 cells are derived from an IL-3-dependent mouse pro-B cell line, BaF3, and were maintained in RPMI 1640 medium supplemented with 10% FBS (Trace Biosystems, Sydney, Australia), 10% conditioned medium from WEHI-3B cells as a source of IL-3, 100 U/mL penicillin and 100 µg/mL streptomycin. BaF-G133 cells stably express the chimeric receptor G133, which is composed of the extracellular domain of the G-CSF receptor and the transmembrane and cytoplasmic domains of gp130 (truncation of gp130 occurs 133 amino acids from the transmembrane domain). G133 contains gp130 Tyr767, which is required for STAT3 activation and STAT3 is essential for both gp130-mediated cell survival [32] and gp130-mediated cell-cycle transition [33]. To establish Daxx transfectants, 20 µg of human Daxx expression vector (pCDNA3–FLAG–Daxx) was co-transfected with 2 µg of pMIK–Hyg into BaF-G133 cells by electroporation. Daxx transfectants were selected using 200 µg/mL hygromycin. Independent clones were established using a limiting dilution procedure. Expression levels of Daxx were analyzed by western blotting. Human G-CSF was kindly provided by Chugai Pharmaceutics (Tokyo, Japan).

siRNA Experiments

Mouse Daxx stealth siRNA was purchased from Invitrogen (Carlsbad, CA, USA). The sequence of the mouse Daxx siRNA was 5'-AAGUAGAAGAGACCAUGCCUGCUCC-3'. Stealth siRNA negative control medium GC (Invitrogen) was used as a negative control. BaF-G133 cells were transfected using a Nucleofector (Amaxa Biosystems, Cologne, Germany). Cells were transfected with 200 pmol siRNA in Nucleofector solution V using program X-001. Immediately following transfection medium was added to the BaF-G133 cells, which were then plated in 6-well tissue culture plates and incubated overnight. Cells were collected the following day and analyzed for protein expression by western blotting.

Proliferation assays

Cell proliferation was determined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, cells were plated in a 96-well plate at 1×10^4 cells/well and left untreated or were treated with G-CSF at concentrations indicated in figures. Ten microliters of WST-8 mixture was then added to each well, and the plates were incubated at 37°C in 5% CO_2 for 4 h. The absorbance of each well was then measured on a microplate reader at 450 nm. All assays were performed in triplicate and repeated at least three times. To investigate the role of Bcl2 in G-CSF-induced cell growth, a small-molecule Bcl2 inhibitor, HA14-1 (Calbiochem, San Diego, CA, USA) was used.

Immunoprecipitation, STAT3 DNA-binding assay and western blotting

The immunoprecipitation and western blotting assays were performed as described previously [48]. The DNA-binding activity of STAT3 in cell extracts was measured using an immobilized STAT3 consensus oligonucleotide–Sepharose conjugate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described previously [49]. The immunoprecipitates or consensus oligo-binding proteins from cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes (PerkinElmer, Boston, MA, USA). The membranes were then immunoblotted with the different primary antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore, Bedford, MA, USA). Antibodies used in this study were: anti-Daxx (sc-7152), anti-STAT3 (sc-482) and anti-Bcl2 (sc-7382) from Santa Cruz Biotechnology, anti-Bax (#2772) from Cell Signaling technology (Beverly, MA, USA) and anti-Actin (A1978) from Sigma.

Cell cycle analysis

BaF-G133 (2×10^5) cells were IL-3-starved for 12 h and then treated with 30 ng/mL G-CSF. Cells were then washed once with ice-cold PBS, suspended with 100 µL of PBS and then fixed by the addition of 900 µL of ethanol. Cells were incubated at −20°C for 20 min, pelleted, resuspended with 300 µL of staining buffer (1 mg/mL RNase, 20 µg/mL propidium iodide, 0.01% NP-40 in PBS) and incubated at 37°C for 20 min. The DNA content of nuclei was analyzed using an FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA, USA).

LDH release assay

Cytotoxicity was determined by measuring the release of LDH using an LDH Cytotoxicity Detection Kit (Takara, Otsu, Japan) according to the manufacturer's instructions. The reaction was initiated by mixing 50 µL of cell-free supernatant with a potassium phosphate buffer containing NADH and sodium pyruvate to a final volume of 100 µL in a 96-well plate. The absorbance of the sample was read at 490 nm. Data were normalized to the activity of LDH released from control cells (100%) and are expressed as a percentage of the control value.

Table 1. RT-PCR primers used in this study

Target	Forward primer (5'–3')	Reverse primer (5'–3')
Bax	TGCAGAGGATGATTGCTGAC	GATCAGCTCGGGCACTTTAG
Bcl2	GTCGCTACCGTCGTCACTTC	ACAGCCAGGAGAAATCAAAC
Bcl2l1	TAGGACTGAGGCCCCAGAAG	CAGTCATGCCCGTCAGGAAC
Daxx	CCCATGGCCACCGATGACAGCAT	AGGGTTAGGGCCCGACGCCTCACT
Gapdh	CAGTAGAGGCAGGGATGATGTTC	GAAATCCCATCACCATCTTCCAGG
JunB	CAGGCAGCTACTTTTCGGGTC	AAGGGTGGTGCATGTGGGAGG
Pim2	AGCACCTCCTCCATGTTGAC	ATGGCCACCTGACGTCTATC

Chromatin immunoprecipitation

Cells were treated with 1% formaldehyde for 20 min to crosslink proteins to DNA. Formaldehyde was neutralized for 5 min by the addition of glycine to 125 mM. Cells were washed twice with cold PBS. Cells were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0) and incubated at 4°C for 10 min. The suspension was then sonicated with five 10 s pulses and then clarified by centrifugation for 10 min in a microcentrifuge. The supernatant was diluted 10-fold with dilution buffer (1% Triton X-100, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl at pH 8.0) to yield the solubilized chromatin solution. The resulting material was used for immunoprecipitation with anti-STAT3 (sc-482), anti-RelA (sc-372), anti-RelB (sc-226), anti-CBP (sc-369) from Santa Cruz Biotechnology or with rabbit IgG as a negative control. After overnight incubation with antibody, DNA-protein complexes were collected by the addition of 10 µL of salmon sperm DNA-protein A agarose (Upstate Biotechnology, Lake Placid, NY, USA) and incubated at 4°C with rotation for 2 h. Following immunoprecipitation, beads were washed in RIPA buffer (1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl at pH 8.0), followed by RIPA/500 mM NaCl, LiCl buffer (250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl at pH 8.0), and finally, TE buffer. The immunoprecipitated complexes were eluted in a buffer containing 10 mM Tris pH 8.0, 300 mM NaCl, 5 mM EDTA, 0.5% SDS at 65°C for 6 h. The samples were then treated with proteinase K for 1 h, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. Immunoprecipitates were analyzed by PCR. PCR primers were designed to amplify −454/−186 of the JunB promoter, as described previously [50] and to amplify −1560/−1461 of the Bcl2 promoter region containing the CRE as follows: the forward primer was 5'-GCTCAGAGGAGGGCTTTCTT-3' and the reverse primer was 5'-GGCCCGGCCTCTTACTTC-3'. PCR products were electrophoresed on agarose gels and visualized by EtBr staining.

RNA extraction and RT-PCR analysis

Total RNA was extracted from BaF-G133 cells using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). First-strand cDNA was synthesized from 1 µg of total RNA with Revertra Ace reverse transcriptase (TOYOBO, Osaka, Japan) using random nonamers. The cDNA was the template for PCR using Gene Taq polymerase (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions. PCR products were electrophoresed on agarose gels and visualized by EtBr staining. The primer pairs used in this study were listed in Table 1. Quantitative real-time PCR analysis of Socs3 mRNA transcripts was carried out using the assay-on-demand™ gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system.

Statistical analysis

Statistical evaluation of differences between populations was determined using Student's unpaired, two-tailed t test. Results shown are the means and standard deviations.

References

1 Salomoni, P. and Khelifi, A. F., Daxx: death or survival protein? Trends Cell Biol. 2006. 16: 97–104.

2 Michaelson, J. S., The Daxx enigma. Apoptosis 2000. 5: 217–220.

3 Gongora, R., Stephan, R. P., Zhang, Z. and Cooper, M. D., An essential role for Daxx in the inhibition of B lymphopoiesis by type I interferons. Immunity 2001. 14: 727–737.

4 Zhong, S., Salomoni, P., Ronchetti, S., Guo, A., Ruggero, D. and Pandolfi, P. P., Promyelocytic leukemia protein (PML) and Daxx participate in a novel nuclear pathway for apoptosis. *J. Exp. Med.* 2000. **191**: 631–640.

5 Salomoni, P., Guernah, I. and Pandolfi, P. P., The PML-nuclear body associated protein Daxx regulates the cellular response to CD40. *Cell Death Differ.* 2006. **13**: 672–675.

6 Leal-Sanchez, J., Couzinet, A., Rossin, A., Abdel-Sater, F., Chakrabandhu, K., Luci, C., Anjuere, F. et al., Requirement for Daxx in mature T-cell proliferation and activation. *Cell Death Differ.* 2007. **14**: 795–806.

7 Li, R., Pei, H., Watson, D. K. and Papas, T. S., EAP1/Daxx interacts with ETS1 and represses transcriptional activation of ETS1 target genes. *Oncogene* 2000. **19**: 745–753.

8 Emelyanov, A. V., Kovac, C. R., Sepulveda, M. A. and Birshtein, B. K., The interaction of Pax5 (BSAP) with Daxx can result in transcriptional activation in B cells. *J. Biol. Chem.* 2002. **277**: 11156–11164.

9 Lin, D. Y., Lai, M. Z., Ann, D. K. and Shih, H. M., Promyelocytic leukemia protein (PML) functions as a glucocorticoid receptor co-activator by sequestering Daxx to the PML oncogenic domains (PODs) to enhance its transactivation potential. *J. Biol. Chem.* 2003. **278**: 15958–15965.

10 Park, J., Lee, J. H., La, M., Jang, M. J., Chae, G. W., Kim, S. B., Tak, H. et al., Inhibition of NF-kappaB acetylation and its transcriptional activity by Daxx. *J. Mol. Biol.* 2007. **368**: 388–397.

11 Croxton, R., Puto, L. A., de Belle, I., Thomas, M., Torii, S., Hanaii, F., Cuddy, M. et al., Daxx represses expression of a subset of antiapoptotic genes regulated by nuclear factor-kappaB. *Cancer Res.* 2006. **66**: 9026–9035.

12 Tzeng, S. L., Cheng, Y. W., Li, C. H., Lin, Y. S., Hsu, H. C. and Kang, J. J., Physiological and functional interactions between Tcf4 and Daxx in colon cancer cells. *J. Biol. Chem.* 2006. **281**: 15405–15411.

13 Chang, C. C., Lin, D. Y., Fang, H. I., Chen, R. H. and Shih, H. M., Daxx mediates the small ubiquitin-like modifier-dependent transcriptional repression of Smad4. *J. Biol. Chem.* 2005. **280**: 10164–10173.

14 Wethkamp, N. and Klempnauer, K. H., Daxx is a transcriptional repressor of CCAAT/enhancer-binding protein beta. *J. Biol. Chem.* 2009. **284**: 28783–28794.

15 Meloni, A., Fiorillo, E., Corda, D., Incani, F., Serra, M. L., Contini, A., Cao, A. et al., DAXX is a new AIRE-interacting protein. *J. Biol. Chem.* 2010. **285**: 13012–13021.

16 Muromoto, R., Nakao, K., Watanabe, T., Sato, N., Sekine, Y., Sugiyama, K., Oritani, K. et al., Physical and functional interactions between Daxx and STAT3. *Oncogene* 2006. **25**: 2131–2136.

17 Hollenbach, A. D., McPherson, C. J., Mientjes, E. J., Iyengar, R. and Grosveld, G., Daxx and histone deacetylase II associate with chromatin through an interaction with core histones and the chromatin-associated protein Dek. *J. Cell Sci.* 2002. **115**: 3319–3330.

18 Michaelson, J. S., Bader, D., Kuo, F., Kozak, C. and Leder, P., Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. *Genes Dev.* 1999. **13**: 1918–1923.

19 Puto, L. A. and Reed, J. C., Daxx represses RelB target promoters via DNA methyltransferase recruitment and DNA hypermethylation. *Genes Dev.* 2008. **22**: 998–1010.

20 Muromoto, R., Sugiyama, K., Takachi, A., Imoto, S., Sato, N., Yamamoto, T., Oritani, K. et al., Physical and functional interactions between Daxx and DNA methyltransferase 1-associated protein, DMAP1. *J. Immunol.* 2004. **172**: 2985–2993.

21 Xue, Y., Gibbons, R., Yan, Z., Yang, D., McDowell, T. L., Sechi, S., Qin, J. et al., The ATRX syndrome protein forms a chromatin-remodeling complex with Daxx and localizes in promyelocytic leukemia nuclear bodies. *Proc. Natl. Acad. Sci. USA* 2003. **100**: 10635–10640.

22 Tang, J., Wu, S., Liu, H., Stratt, R., Barak, O. G., Shiekhattar, R., Picketts, D. J. et al., A novel transcription regulatory complex containing death domain-associated protein and the ATR-X syndrome protein. *J. Biol. Chem.* 2004. **279**: 20369–20377.

23 Hirano, T., Nakajima, K. and Hibi, M., Signaling mechanisms through gp130: a model of the cytokine system. *Cytokine Growth Factor Rev.* 1997. **8**: 241–252.

24 Darnell, J. E., Jr., STATs and gene regulation. *Science* 1997. **277**: 1630–1635.

25 Taga, T. and Kishimoto, T., Gp130 and the interleukin-6 family of cytokines. *Annu. Rev. Immunol.* 1997. **15**: 797–819.

26 Hankey, P. A., Regulation of hematopoietic cell development and function by Stat3. *Front. Biosci.* 2009. **14**: 5273–5290.

27 Yu, H., Pardoll, D. and Jove, R., STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat. Rev. Cancer* 2009. **9**: 798–809.

28 Minegishi, Y., Hyper-IgE syndrome. *Curr. Opin. Immunol.* 2009. **21**: 487–492.

29 Takeda, K., Kaisho, T., Yoshida, N., Takeda, J., Kishimoto, T. and Akira, S., Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. *J. Immunol* 1998. **161**: 4652–4660.

30 Owaki, T., Asakawa, M., Morishima, N., Mizoguchi, I., Fukai, F., Takeda, K., Mizuguchi, J. et al., STAT3 is indispensable to IL-27-mediated cell proliferation but not to IL-27-induced Th1 differentiation and suppression of proinflammatory cytokine production. *J. Immunol.* 2008. **180**: 2903–2911.

31 Chou, W. C., Levy, D. E. and Lee, C. K., STAT3 positively regulates an early step in B-cell development. *Blood* 2006. **108**: 3005–3011.

32 Fukada, T., Hibi, M., Yamanaka, Y., Takahashi-Tezuka, M., Fujitani, Y., Yamaguchi, T., Nakajima, K. et al., Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. *Immunity* 1996. **5**: 449–460.

33 Fukada, T., Ohtani, T., Yoshida, Y., Shirogane, T., Nishida, K., Nakajima, K., Hibi, M. et al., STAT3 orchestrates contradictory signals in cytokine-induced G1 to S cell-cycle transition. *EMBO J.* 1998. **17**: 6670–6677.

34 Xiang, H., Wang, J. and Boxer, L. M., Role of the cyclic AMP response element in the bcl-2 promoter in the regulation of endogenous Bcl-2 expression and apoptosis in murine B cells. *Mol. Cell Biol.* 2006. **26**: 8599–8606.

35 Kuo, H. Y., Chang, C. C., Jeng, J. C., Hu, H. M., Lin, D. Y., Maul, G. G., Kwok, R. P. et al., SUMO modification negatively modulates the transcriptional activity of CREB-binding protein via the recruitment of Daxx. *Proc. Natl. Acad. Sci. USA* 2005. **102**: 16973–16978.

36 Wang, J. L., Liu, D., Zhang, Z. J., Shan, S., Han, X., Srinivasula, S. M., Croce, C. M. et al., Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. *Proc. Natl. Acad. Sci. USA* 2000. **97**: 7124–7129.

37 Hofmann, W. K., de Vos, S., Tsukasaki, K., Wachsman, W., Pinkus, G. S., Said, J. W. and Koeffler, H. P., Altered apoptosis pathways in mantle cell lymphoma detected by oligonucleotide microarray. *Blood* 2001. **98**: 787–794.

38 Boehrer, S., Nowak, D., Kukoc-Zivojnov, N., Hochmuth, S., Kim, S. Z., Hoelzer, D., Mitrou, P. S. et al., Expression of Daxx sensitizes Jurkat T-cells to the apoptosis-inducing effect of chemotherapeutic agents. *Pharmacol. Res.* 2005. **51**: 367–374.

39 Mui, A. L., Wakao, H., Kinoshita, T., Kitamura, T. and Miyajima, A., Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. *EMBO J.* 1996. **15**: 2425–2433.

40 Dumon, S., Santos, S. C., Debierre-Grockiego, F., Gouilleux-Gruart, V., Cocault, L., Boucheron, C., Mollat, P. et al., IL-3 dependent regulation of

Bcl-xL gene expression by STAT5 in a bone marrow derived cell line. *Oncogene* 1999. **18**: 4191–4199.

41 Rosa Santos, S. C., Dumon, S., Mayeux, P., Gisselbrecht, S. and Gouilleux, F., Cooperation between STAT5 and phosphatidylinositol 3-kinase in the IL-3-dependent survival of a bone marrow derived cell line. *Oncogene* 2000. **19**: 1164–1172.

42 Ryo, A., Hirai, A., Nishi, M., Liou, Y. C., Perrem, K., Lin, S. C., Hirano, H. *et al.*, A suppressive role of the prolyl isomerase Pin1 in cellular apoptosis mediated by the death-associated protein Daxx. *J. Biol. Chem.* 2007. **282**: 36671–36681.

43 Kwon, J. E., La, M., Oh, K. H., Oh, Y. M., Kim, G. R., Seol, J. H., Baek, S. H. *et al.*, BTB domain-containing speckle-type POZ protein (SPOP) serves as an adaptor of Daxx for ubiquitination by Cul3-based ubiquitin ligase. *J. Biol. Chem.* 2006. **281**: 12664–12672.

44 Tang, J., Qu, L., Pang, M. and Yang, X., Daxx is reciprocally regulated by Mdm2 and Hausp. *Biochem. Biophys. Res. Commun.* 2010. **393**: 542–545.

45 Cashen, A. F., Lazarus, H. M. and Devine, S. M., Mobilizing stem cells from normal donors: is it possible to improve upon G-CSF? *Bone Marrow Transplantation* 2007. **39**: 577–588.

46 Zhang, Y., Cheng, G., Yang, K., Fan, R., Xu, Z., Chen, L., Li, Q. *et al.*, A novel function of granulocyte colony-stimulating factor in mobilization of human hematopoietic progenitor cells. *Immunol. Cell Biol.* 2009. **87**: 428–432.

47 Saffert, R. T., Penkert, R. R. and Kalejta, R. F., Cellular and viral control over the initial events of human cytomegalovirus experimental latency in CD34+cells. *J. Virol.* 2010. **84**: 5594–5604.

48 Matsuda, T., Yamamoto, T., Muraguchi, A. and Saatcioglu, F., Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3. *J. Biol. Chem.* 2001. **276**: 42908–42914.

49 Muromoto, R., Ikeda, O., Okabe, K., Togi, S., Kamitani, S., Fujimuro, M., Harada, S. *et al.*, Epstein-Barr virus-derived EBNA2 regulates STAT3 activation. *Biochem. Biophys. Res. Commun.* 2009. **378**: 439–443.

50 Ezoe, S., Matsumura, I., Gale, K., Satoh, Y., Ishikawa, J., Mizuki, M., Takahashi, S. *et al.*, GATA transcription factors inhibit cytokine-dependent growth and survival of a hematopoietic cell line through the inhibition of STAT3 activity. *J. Biol. Chem.* 2005. **280**: 13163–13170.

Full correspondence: Dr. Tadashi Matsuda, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, 060-0812, Japan
Fax: +81-11-706-4990
e-mail: tmatsuda@pharm.hokudai.ac.jp

Microbiology and Immunology

MINI-REVIEW

The role of B cells in regulating the magnitude of immune response

Seiho Nagafuchi

Department of Medical Science and Technology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan

ABSTRACT

Recently, accumulating evidence has suggested that B cell depletion therapy with rituximab is effective not only in autoantibody-associated, but also in T cell-mediated, autoimmune diseases. It is likely that B cells play an important role in regulating the extent of immune response in both physiological and pathological conditions. When a severe infection occurs, pathogens spread throughout the bloodstream. B cells in the blood capture the pathogens, via their specific antigen receptors (surface immunoglobulins), then present the specific antigen to T cells in the spleen, thus increasing the degree of T-cell immune responses to systemic infection. Similarly, in the exacerbation stage of autoimmunity, a large amount of autoantigens may be released into the blood and be captured by autoantigen specific B cells, and this may be followed by presentation of the antigen to CD4 positive autoreactive T cells resulting in extensive activation and proliferation of autoreactive T cells. Thus, it has been suggested that B-cell depletion therapy for autoimmune diseases is most useful for the "vicious cycle" phase of autoreactive immune response. The recognition of this paradigm for the role of B cells in regulating the magnitude of immune response will help to facilitate both basic and clinical research on the regulation of immune responses.

Key words autoimmune disease, B cell, immunoregulation, rituximab.

Accumulating evidence has suggested that B cell-depletion therapy with rituximab is effective in a wide range of autoimmune diseases. These include not only autoantibody-associated diseases such as hemolytic anemia, autoimmune thrombocytopenic purpura, pemphigus, ANCA-associated vasculitis, systemic lupus erythematosus in the refractory phase and rheumatoid arthritis (1–3), but also the preservation of beta cell function in patients with early onset of type 1 diabetes (4), which is representative of T cell-dependent autoimmune diseases (5). All these observations taken together suggest that B cells play a significant role in regulating autoreactive T-cell responses as well as autoantibody production. However, the exact role of B

cells in the regulation of immune responses *in vivo* remains elusive (6–8).

In terms of its physiological significance, the immune system is constructed to resist pathogenic infections according to the species of pathogen and the severity of infection. It is known that B cells not only produce antibody but also work as antigen presenting cells, and excrete cytokines (8, 9).

Regarding the role of B cells in the immune response against infection, it should be noted that B cells circulate in the bloodstream to meet pathogens via their antigen specific receptors; these are membrane-type surface immunoglobulins. On the other hand, the so-called

487

Local Infection
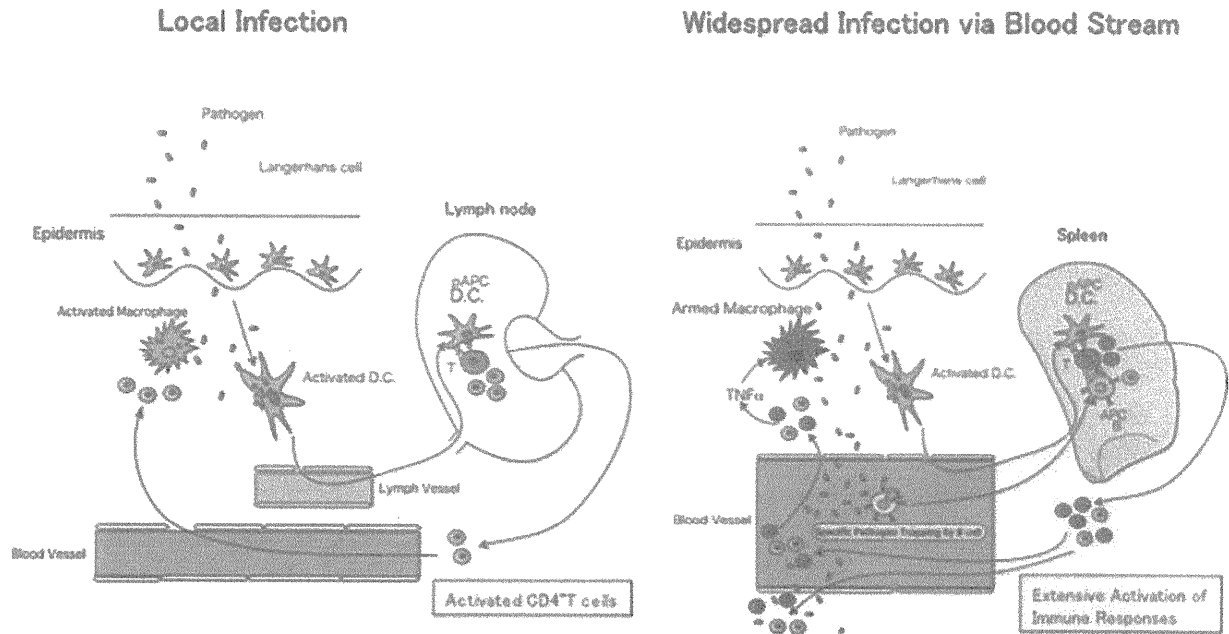

Widespread Infection via Blood Stream

Fig. 1. A paradigm for the role of B cells in regulating the magnitude of T-cell immune response against widespread pathogenic infection. When a local dermal infection occurs, it can be overcome by taking up the pathogen with pAPC such as Langerhans cells at the site of infection. Langerhans cells migrate to regional lymph nodes via the afferent lymphatics, to activate reactive CD4+T cells. The activation of specific immune responses directed against the pathogen will then in turn control the local infection (left panel). In contrast, when a more severe infection develops, pathogens spread throughout the bloodstream. B cells capture the pathogens in the blood via their specific antigen receptors (surface immunoglobulins), then present the specific antigen to T cells in the spleen, thus increasing the extent of T-cell immune responses against systemic infection (right panel). DC, dendritic cells

professional pAPC, such as the Langerhans cells in the skin, but not the body, function at the entry site of infection. As an example, when local dermal infection occurs, the professional antigen presenting Langerhans cells trap the pathogen. This is followed by migration to regional lymph nodes via afferent lymphatics, and presentation of specific antigen to CD4+ T cells, resulting in activation of pathogen-reactive T cells. The reactive T cells in turn move to the site of local infection and work to control it via their specific functions, inducing local inflammatory immune responses and leading to the resolution of local skin infection (Fig. 1, left panel) (10). In contrast, when infection spreads throughout the bloodstream, B cells specifically capture the pathogen in the blood, and this is followed by activation of B cells through B cell receptor signaling pathways associated with increased expression of MHC class II antigens and co-stimulatory signals such as CD80/86 molecules (11). The antigen-stimulated B cells then travel to the spleen and present specific antigens of the pathogen to T cells. This is followed by extensive activation of CD4+ T cells, possibly associated with enlargement of the spleen, thereby contributing to the overall extent of immune re-

sponse (12) (Fig. 1, right panel). Thus, enhanced immune responses work to control severe systemic infection.

Similarly, B cells may enhance exacerbation of autoimmunity as well as influence control of widespread infection. Intact cells are injured by many agents, including environmental factors such as viruses and toxins, autoreactive T cells, and inflammatory chemokines, cytokines, and chemical mediators (8). These damaged cells release autoantigens into the blood. This is followed by capture of autoantigens by autoreactive B cells, which then present autoantigens to autoreactive T cells, mainly in the spleen, thus increasing the extent of specific T-cell mediated autoimmunity (Fig. 2), which is associated with pathogenic T cell receptor clonotype spreading (13), as well as enhancing production of autoantibodies by B cells. B cells may also produce pathogenic cytokines (8, 9) (not shown). The resultant extensive proliferation and activation of autoreactive T cells produces profound T cell mediated autoreactivity, resulting in enhanced deterioration of target cells. This, together with the increased production of autoantibodies, creates a vicious cycle of exacerbated autoreactivity and target cell injury at the site of autoimmunity (Fig. 2).
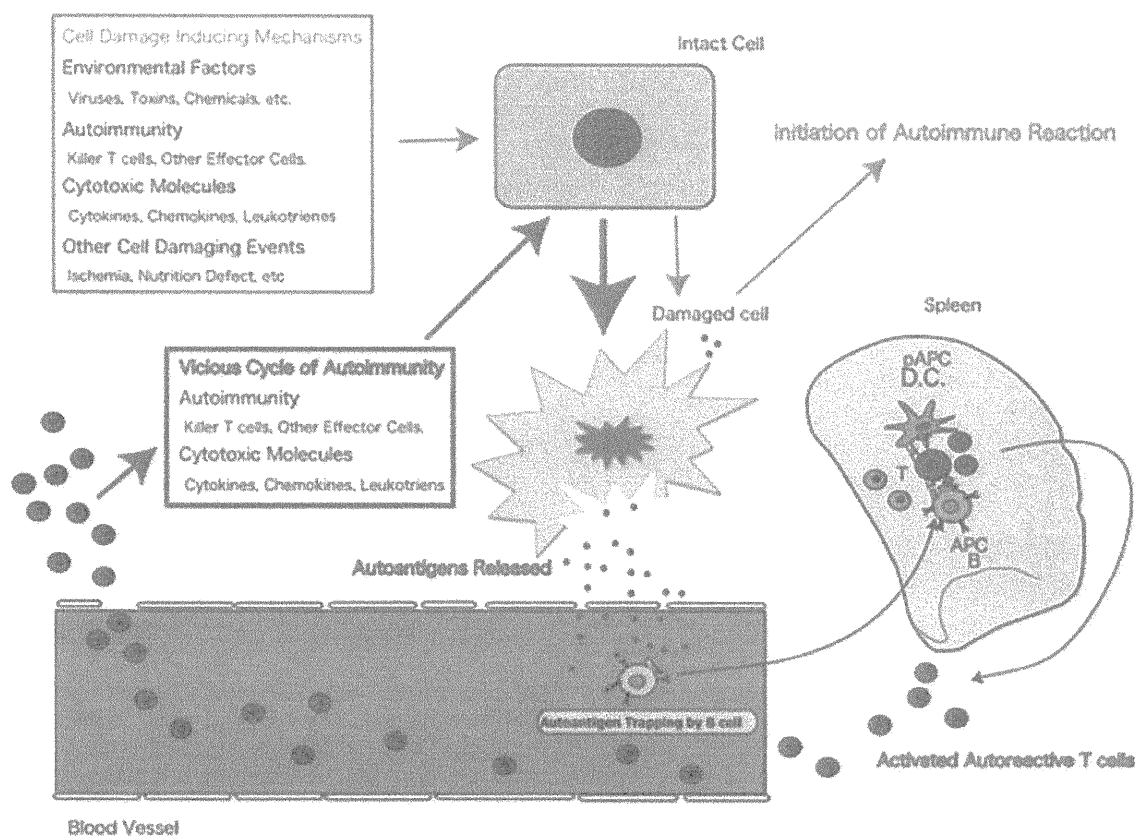
Fig. 2. A paradigm for the role of B cells in the exacerbation stage of autoimmunity. Intact cells may be damaged by various agents. These include environmental factors such as viruses, toxins, and chemicals; autoimmunity due to autoreactive cytotoxic T cells, and other effecter cells as NK cells and macrophages; cytokines, chemokines and leukotrienes as mediators; and other cell damaging events such as ischemia and nutritional defects. These triggering factors may damage the cells, and lead to the induction of an autoimmune reaction. In the exacerbation stage of autoimmunity, a large amount of autoantigens may be released into the blood and be captured by autoantigen-specific B cells. This may be followed by presentation of the antigen to CD4 positive autoreactive T cells. Thus, extensive proliferation of autoreactive T cells occurs in the spleen (figure), as well as stimulation of B cells, which leads to enhanced production of autoantibodies (not shown in the figure). The vicious cycle may proceed to an out-of-control state of autoimmune reaction.

The cycling can be blocked by elimination of autoreactive B cells. As described above, the effectiveness of rituximab has consistently been reported in many autoimmune diseases (1–4, 6–8). However, it should be emphasized that, even in the absence of development of clinical diabetes in B cell-depleted NOD mice (14–16), the presence of minimal but distinct insulitis has been observed, suggesting that B-cell depletion can not entirely prevent the development of autoimmunity.

During B-cell depletion therapy, clinical safeguards should include careful monitoring for adverse reactions including allergy, neutropenia and infections (17–19). Indeed, in the absence of B cells, innate immunity dependent on residual pAPC such as dendritic cells, and minimal but distinct T cell reactivity, continue to protect against local infection, with fewer associated infections (10, 12). Moreover, after elimination of B cells, antibodies which protect against infection may be supplied by administering human immunoglobulins, thus reducing the risk of occurrence of infectious complications (20).

Thus, treatment with B-cell depletion therapy should be capable of stopping the vicious cycle of the out-of-control stage of autoimmunity. However, achieving complete recovery by elimination of autoreactive T cells may be difficult. To accomplish long-lasting resolution of autoimmune diseases, another effective therapy to regulate T cell dependent autoimmunity is required in addition to B-cell depletion therapy.

In conclusion, B cells play an important role in resistance against widespread infection by increasing the extent