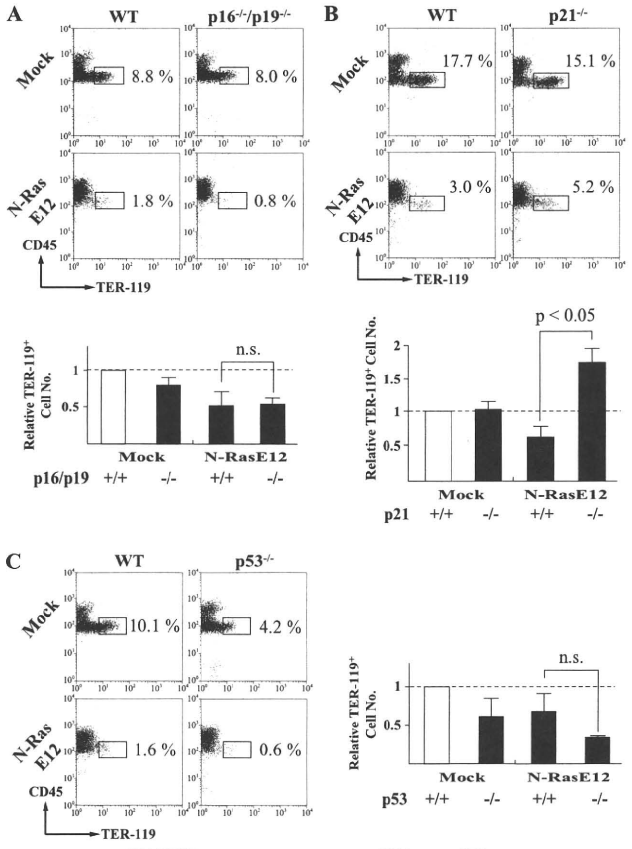

FIGURE 6. **p21$^{CIP1/WAF1}$ but not p53 or p16^{INK4a}/p19^{ARF} mediates oncogenic Ras-induced suppression of erythropoiesis.** *A–C*, LSK cells were isolated from BM of the indicated mice. After retrovirus infection, GFP$^+$ cells were sorted and cocultured with MS-5 in the presence of rmSCF and rhEPO. The expression of CD45 and TER-119 was analyzed after 5 days. Bar graphs represent the relative TER-119$^+$ cell numbers normalized to mock-transduced WT cells (*dashed lines*). *n.s.*, not significant.

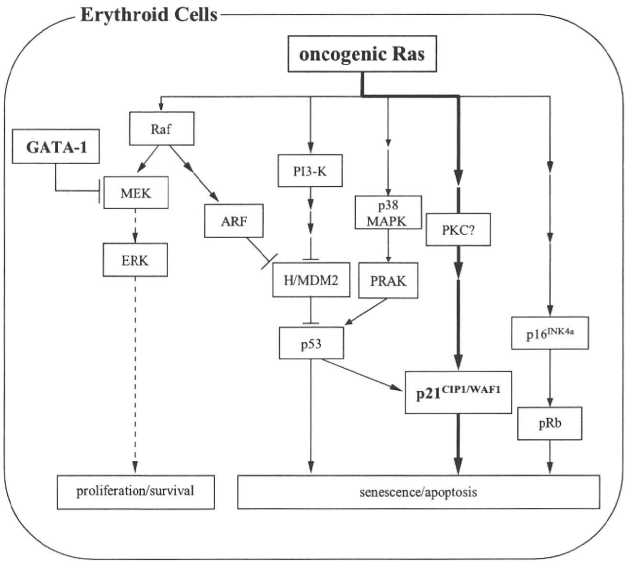

FIGURE 7. **A proposed model for oncogenic Ras-induced suppression of erythropoiesis.** Oncogenic Ras simultaneously activates several downstream molecules including Raf, PI3-K, and p38 MAPK. The Raf/MEK/ERK pathway mainly transduces proliferation and survival signals, while the remaining pathways commonly induce growth arrest (senescence) through cell cycle regulatory molecules such as p16^{INK4a}, p19^{ARF}, p21$^{CIP1/WAF1}$, and p53. So, oncogenic Ras is supposed to induce proliferation or senescence dependently on the balance between these two signals. In this study, we found that GATA-1 inhibits mitogenic signal from Ras through its interaction with MEK1 in erythroid cells, which resulted in their growth inhibition due to the dominance of senescence-inducing signals. In addition, we found that p21$^{CIP1/WAF1}$ is a crucial regulator of oncogenic Ras-induced senescence of erythroid cells.

as Jun N-terminal kinase (JNK) and protein kinase C (PKC) in CML cells (49). Alternatively, it is also possible that the interaction between GATA-1 and MEK might be inhibited in megakaryocytes due to the presence of some nuclear protein(s) specific for this lineage. However, further studies are required to clarify how megakaryocytes develop and platelets are effectively produced in CML patients.

Among various signaling molecules downstream of Ras, the Raf/MEK/ERK pathway mainly promotes cell growth and prevents apoptosis of hematopoietic cells (14). On the other hand, oncogenic stimuli including constitutively activated Ras, also cause growth inhibition (senescence) that acts as a fail-safe mechanism against malignant transformation (15, 16, 21). Although the mechanism of Ras-induced senescence is not fully understood, recent findings have unveiled several MEK/ERK-independent pathways (19). These pathways regulate the function of two main tumor-suppressor molecules, p53 and retinoblastoma protein (pRb) (50). Downstream of oncogenic Ras, p38-regulated/activated protein kinase (PRAK), a substrate of p38 mitogen-activated protein kinase (p38 MAPK), activates p53 by direct phosphorylation (20). Ras/Raf stabilizes p53 independently of MEK through the up-regulation of p19^{ARF} (21). The PI3-K pathway also stabilizes p53 through the inhibition of H/MDM2 (19). So, we speculated that N-RasE12 might induce growth arrest in erythroid cells even if MEK activities are blocked by GATA-1.

Ras-induced senescence is executed by CDK inhibitors such as p16^{INK4a} and p21$^{CIP1/WAF1}$, and a tumor-suppressor, p19^{ARF}, which consequently activate both p53 and pRb pathways. Among these molecules, we here found that p21$^{CIP1/WAF1}$ is a major player of Ras-induced suppression of erythropoiesis (may well be called nearly equal to senescence). Although p21$^{CIP1/WAF1}$ is a transcriptional target of p53 (51), p53 deficiency did not cancel Ras-induced suppression of erythropoiesis. So, p53-independent expression of p21$^{CIP1/WAF1}$ was supposed to be important for Ras-induced suppression of erythropoiesis. Because Darley *et al.* (18) previously showed that oncogenic N-Ras conferred developmental abnormalities on human erythroid cells through the activation of PKC, one of the reported activators of p21$^{CIP1/WAF1}$ (52), PKC may be a candidate molecule involved in Ras-induced expression of p21$^{CIP1/WAF1}$ and consequent suppression of erythropoiesis.

Mutation and/or deletion of the p53 gene and the INK4a/ARF locus are frequently observed in CML blast phase (1), but to our knowledge, there is no report demonstrating the inactivation of the p21$^{CIP1/WAF1}$ gene. So, our findings that p21$^{CIP1/WAF1}$ but not p53 or p16^{INK4a}/p19^{ARF} is the major regulator of Ras-induced suppression of erythropoiesis are again consistent with the clinical features that anemia is continued and erythroid transformation is a rare event in blast-phase

CML (7, 8). Furthermore, because loss-of-function mutations of the p21$^{CIP1/WAF1}$ gene are rare in most of the hematologic malignancies, anemia observed in these diseases might be attributable to p21$^{CIP1/WAF1}$.

In conclusion, we here show that BCR-ABL but not JAK2 V617F inhibits erythropoiesis through the Ras signal. We also identified p21$^{CIP1/WAF1}$ as a central regulator of Ras-induced suppression of erythropoiesis. Ras transmits both growth promoting and inhibitory signals, and then induces proliferation or senescence dependently on their balance. In erythroid but not in myeloid progenitors, the growth promoting signal is inhibited at the level of MEK by GATA-1, which would lead to the relative dominance of the growth inhibitory signal mediated by p21$^{CIP1/WAF1}$ (Fig. 7). These mechanisms would explain why oncogenic Ras simultaneously reveals conflicting effects according to the cell lineage, *i.e.* growth promotion in myeloid cells and growth inhibition in erythroid cells. This model may be also useful to understand the mechanism of anemia caused by other oncogenic TKs.

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bjh research paper

Procoagulant properties of microparticles released from red blood cells in paroxysmal nocturnal haemoglobinuria

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Summary

Thrombosis in paroxysmal nocturnal haemoglobinuria (PNH) has been suggested to be due to several pathophysiological states: a suppressed fibrinolytic system, increased leucocyte-derived tissue factor, complement (C′)-mediated damage to platelets and endothelia, or increased platelet- and endothelium-derived microparticles (MPs). Because haemolytic attack is often accompanied by thrombosis in PNH, we studied the role of C′-induced release of MPs in the thrombogenesis of PNH. C′ activation induced procoagulant alteration in PNH red blood cells (RBC), when assessed by thrombin generation in the presence of C′-activated PNH RBC, which was abolished by their subsequent treatment with annexin V. Significant amounts of procoagulant MPs, measured by phosphatidylserine-binding prothrombinase activity, were released from PNH RBC in association with the formation of C5b-9, but not significantly before C5b-8. Generation of procoagulant, annexin V-binding, MPs from C′-activated RBC was studied also by flow cytometry. While phorbol 12-myristate 13-acetate, an activator of protein kinase C (PKC), induced the release of MPs from normal RBC as well as PNH RBC, C′-induced release of MPs from PNH RBC was Ca^{2+}-independent and not associated with the activation of PKC, calpain or caspase. Procoagulant properties of MPs released from PNH RBC could contribute to the thrombogenesis of PNH.

Keywords: PNH, microparticle, thrombosis, RBC, PKC.

Paroxysmal nocturnal haemoglobinuria (PNH) is a rare acquired stem cell disorder caused by a somatic mutation of *PIGA*, diverse clinical manifestations of which include intravascular haemolysis, bone marrow failure and thrombosis (Parker *et al*, 2005). The most important factor related to the poor prognosis of PNH is the complication of visceral thrombosis, cerebrovascular thromboembolism and pulmonary embolism (Ziakas *et al*, 2008). Several factors involved in thrombogenesis in PNH have been proposed: (i) chronic haemolysis, (ii) impaired fibrinolytic system, (iii) microparticles (MPs) released from injured platelets and vascular endothelia (Ploug *et al*, 1992; Wiedmer *et al*, 1993; Ninomiya *et al*, 1999; Simak *et al*, 2004). Complement (C′) sensitivity of PNH red blood cells (RBC) is due to a deficiency in the expression of glycosyl-phosphatidylinositol (GPI)-anchored membrane proteins with C′-regulatory activity, CD55 and CD59, on PNH-affected RBC (Wilcox *et al*, 1991; Parker *et al*, 2005; Brodsky, 2008). The clinical evidence that eculizumab (Soliris, Alexion Pharmaceuticals, Chelshire, CT, USA), a humanized monoclonal anti-C5 antibody therapy, reduced the risk of clinical thromboembolism in addition to its reducing effect on intravascular haemolysis strongly suggests a major role of C′-induced haemolysis in thrombogenesis in PNH (Hillmen *et al*, 2004, 2007).

In the human RBC membrane, phospholipids are organized asymmetrically: phosphatidylserine (PS), phosphatidylethanolamine (PE) and probably phosphatidylinositol (PI) are located mainly in the inner monolayer while phosphatidylcholine (PC) and sphingomyelin (SM) are essentially in the outer monolayer (Devaux, 1991). An ATP-requiring mechanism responsible for the specific translocation of aminophospholipids (PS and PE), aminophospholipid translocase, has been demonstrated (Seigneuret & Devaux, 1984). An increase of the intracellular Ca^{2+} concentration in RBC is known to activate the scrambling of membrane phospholipids (Zhou *et al*, 1997; Bucki *et al*, 1998). Phospholipid scrambling plays a stimulatory role in MP

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generation (Kamp *et al*, 2001), although little is known about the signalling that links cell injury to Ca^{2+} entry and subsequent activation of the phospholipid scramblase, leading to PS exposure. Phospholipid scrambling and MP generation have been shown, although closely regulated, to proceed by independent pathways (Bucki *et al*, 1998).

Human RBC contain protein kinase C (PKC), which mediates the phosphorylation of cytoskeletal proteins, such as band 4.1, 4.9 and, probably, adducin (Danilov & Cohen, 1989). It has been reported that PKC is involved in human RBC Ca^{2+} entry (Andrews *et al*, 2002) and subsequent PS exposure on RBC (de Jong *et al*, 2002). In the case of platelets, calpain (Bachelot-Loza *et al*, 2006) or caspase (Shcherbina & Remold-O'Donnell, 1999) is suggested to be involved in the mechanisms inducing PS exposure and/or MP release.

In this study, we investigated the release of procoagulant MPs from PNH RBC by C′ activation. Ca^{2+} dependency and the role of PKC, calpain or caspase in the C′-induced release of procoagulant MPs from PNH RBC were also examined.

Materials and methods

Materials

A ZYMUPHEN-MP Activity enzyme-linked immunosorbent assay kit (HYPHEN BioMed, Neuville sur Oise, France) was used to measure MP procoagulant activity. Thrombin (IIa)-specific chromogenic substrate, Tos–Gry–Pro–Arg–ANBA–IPA (Sysmex, Kobe, Japan), was used for the enzymatic assay of IIa generation. Annexin V derived from human placenta was from Sigma Chemical Co. (St Louis, MO, USA). Goat antisera to human C5 (anti-C5) and human C9 (anti-C9) were from Cappel (Aurora, OH, USA). Phorbol 12-myristate 13-acetate (PMA) was from Sigma. Calphostin C, a cell-permeable, highly specific PKC inhibitor, and RO-31-8220, a PKC inhibitor, were from Merck (Darmstadt, Germany). Calpain inhibitors, ALLN, E64d, PD150606, PD145305 and calpastatin peptide, were from Merck. A broad caspase inhibitor, z–Val–Ala–DL–Asp–fluoromethylketone (z–VAD–fmk) was from Sigma. PYCOERYTHRIN-labelled anti-human CD59 mouse IgG2a [p272(H19)] (BD Pharmingen, San Jose, CA, USA) and fluorescein isothiocyante (FITC)-labelled annexin V (Sigma) were used for immunostaining. Phycoerythrin-labelled mouse IgG_2 (DakoCytomation, Kyoto, Japan) was used as an isotype-matched control antibody. Staining medium for flow cytometry (FCM) was phosphate-buffered saline, pH 7·2 (Nissui Pharmacology, Tokyo, Japan), containing 0·1% bovine serum albumin (BSA), 0·1% NaN_3. FACSort (BD Bioscience, San Jose, CA, USA) was used for FCM and the data were analysed with CELL-QUEST (BD Bioscience, San Jose, CA, USA).

PNH patients and normal donors

Two patients with classical PNH and three normal individuals were used for the study. PNH Patients 1 and 2 had 44·8% and 92·2% CD59-negative (PNH-III) RBC, respectively. Freshly drawn blood with anticoagulant sodium citrate from normal individuals and PNH patients was used. RBC, washed and resuspended in 50 mmol/l Tris-buffered saline, pH 7·4 (TBS), were then treated by C′-activation in sucrose buffer as described below. C′-inactivated (56°C, 30 min) normal human serum (ABO-matched) and serum preincubated with anti-C5 or anti-C9 were also used in some experiments. Freshly drawn and separated normal human plasma was used as a source of coagulation factors for IIa-generation assays. Written informed consent was obtained from the patients and normal donors. This study was approved by the ethics committee of the University of Tsukuba.

C′ activation of RBC in sucrose buffer

Sucrose buffer was prepared as 270 mmol/l sucrose dissolved in 5 mmol/l phosphate buffer, pH 6·5. C′ activation of RBC was performed by incubation of 50 μl RBC (2×10^9/ml in TBS) with 50 μl freshly isolated ABO-matched serum (or inactivated serum) in 850 μl sucrose buffer (37°C, 30 min). For some experiments, serum was pretreated with anti-C5 or anti-C9 (4°C, 30 min). After C′-activation of RBC, the supernatants were isolated by centrifugation (2300 ***g***, 5 min) and then used for procoagulant MPs activity assay or IIa-generation assay, as described below. C′-activated RBC were resuspended in TBS and then added to the IIa-generation assay, as described below.

IIa-generation assay

IIa generation in the plasma was assayed enzymatically with Tos–Gry–Pro–Arg–ANBA–IPA as a IIa-specific chromogenic substrate on a 96-well microplate (Nunc, Rosklide, Denmark): 50 μl of the samples (RBC suspensions or supernatants of RBC after C′ activation) were added to wells containing 10 μl 4 mmol/l IIa-substrate, 20 μl 250 mmol/l $CaCl_2$ and 50 μl Tyrode buffer (135 mmol/l NaCl, 10 mmol/l HEPES, 5 mmol/l glucose, 2·9 mmol/l KCl, 1 mmol/l $MgCl_2$, 12 mmol/l $NaHCO_3$, 0·34 mmol/l Na_2HPO_4, 0·3% BSA); and the assay was started by the addition of 50 μl normal human plasma, four-times diluted in TBS, to the wells. The optical density at 405 nm (OD_{405}) of the microplate wells were read at 23°C over time (0–120 min). For the IIa-generation assay in the presence of RBC, IIa-generation data were obtained by subtracting the data (OD_{405}) of the reference wells containing RBC plus plasma without IIa substrate and $CaCl_2$ on the same microplate. The rate of IIa generation was calculated from the change of OD_{405} using calibration curves generated with standard bovine α-thrombin (0, 15, 30, 60 and 120 u/l) (Fig 1A).

MP release from RBC by C′ activation

MPs generated from C′-activated RBC in sucrose buffer were measured using a ZYMUPHEN MP-Activity Assay kit. Briefly,

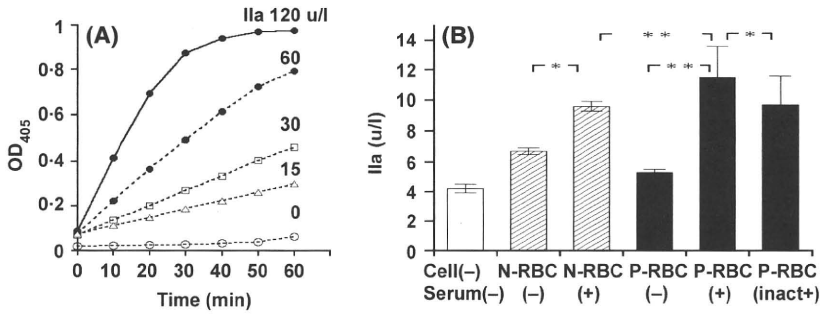

Fig 1. Procoagulant properties of the supernatants of C′-activated RBC in sucrose buffer. Panel A: Enzymatic assay for IIa was employed for the measurement of IIa generation in diluted plasma. Calibration curve generated by serially diluted bovine α IIa shows the linear relationship between time (0–60 min) and absorbance in a range of IIa (0–60 u/l) added to wells containing IIa-specific chromogenic substrate. Panel B: The supernatants of C′-activated PNH-RBC (P-RBC) in sucrose buffer showed enhanced procoagulant properties more than those of normal RBC (N-RBC) similarly treated or PNH-RBC treated with inactivated serum. Only the supernatant of C′-activated PNH-RBC showed apparent haemolysis in this experiment. Bars indicate the mean ± SD of triplicate assays. This figure shows data for Patient 1. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

the samples (PS-containing supernatants) were incubated with Annexin V-streptavidin-coated microplates and allowed to bind to the plates; then IIa generated from prothrombin by the action of Xa–Va (prothrombinase) added to the wells was enzymatically measured. PS-exposed MPs accelerate IIa generation. Assays were performed exactly according to the manufacturer's instructions. Results are expressed as PS concentration by calculation from the calibration data of standard PS material included in the assay kit.

PKC activation and inhibition of RBC for IIa-generation assay

PKC activation of RBC was induced by treatment of RBC, normal and PNH, with PMA (0·1 μmol/l, 23°C, 30 min) in HEPES-buffered saline, 145 mmol/l NaCl, pH 7·4, containing 2 mmol/l $CaCl_2$. PKC inhibition of RBC was induced by preincubation of RBC with Calphostin C (1·67 μmol/l, 37°C, 30 min).

FCM

PS-exposed MPs or PS-exposed RBC upon C′-activation were analysed using an Annexin V-FITC Apoptosis Detection kit (Sigma). Aliquots (50 μl) of samples (RBC suspensions after C′ activation in sucrose buffer) were added to test tubes containing 1× binding buffer (10 mmol/l HEPES/NaOH, pH 7·5, containing 140 mmol/l NaCl and 2·5 mmol/l $CaCl_2$), and then 1 μl Annexin V-FITC conjugate was added. After a 10-min incubation at 23°C, samples were analysed by FCM. For some experiments, RBC were pretreated (30 min, 23°C) with PKC inhibitors (10 μmol/l Calphostin C or 100 nmol/l Ro-31-8220), calpain inhibitors or a caspase inhibitor, and then treated by C′ activation. To clarify the effects of Ca^{2+} on MP generation, 2 mmol/l chelating agents, either ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′-*N*′-tetra-acetic acid (EGTA) or 2 mmol/l ethylenediaminetetraacetic acid (EDTA), were added to the C′-activation condition in the sucrose buffer containing 1 mmol/l $MgCl_2$.

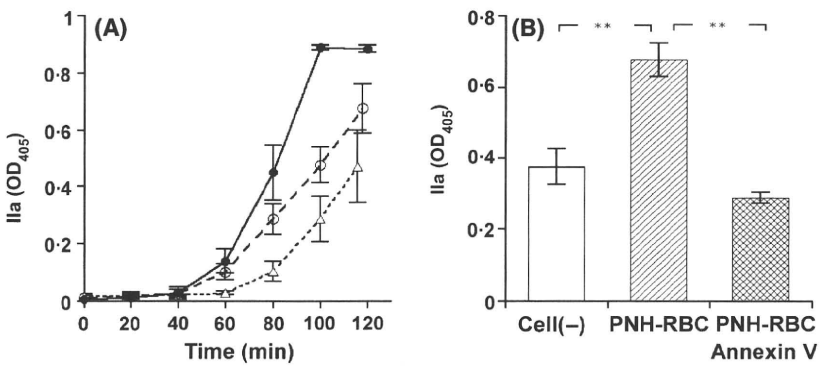

Fig 2. Procoagulant alteration of PNH-RBC by C′ activation. Panel A: IIa generation (OD_{405}) in diluted plasma was measured enzymatically using IIa-specific chromogenic substrate. The presence of PNH-RBC (black circle) that were C′-activated in sucrose buffer, accelerated the coagulation process in diluted plasma in comparison with the presence of normal RBC (open circle), which were treated the same, or under cell-free conditions (open triangle). Panel B: When IIa generation in diluted plasma at 100 min was compared, the enhanced IIa generation in the presence of C′-activated PNH-RBC was diminished by their subsequent treatment with annexin V (10 μg/ml, 23°C, 30 min). Horizontal bars indicate the mean ± SD of triplicate assays. This figure shows data for Patient 2. $**P < 0{\cdot}01$.

Data analysis and statistics

Data were analysed using graphical data analysis software packages, Microsoft Excel (Seattle, WA, USA) and KALEIDA GRAPH (Synergy Software, Reading, PA, USA). Statistical significance between two groups was examined by the unpaired *t*-test. Statistical significance was accepted for $P < 0{\cdot}05$.

Results

Procoagulant alteration of PNH RBC by C′ activation

IIa generation in the diluted plasma by the addition of Ca^{2+} was measured in the presence of RBC. In the presence of PNH RBC that were C′ activated in sucrose buffer, IIa generation was enhanced in comparison with normal RBC treated similarly or under cell-free conditions (Fig 2A). Such procoagulant alteration of PNH RBC was reversed by the subsequent treatment of PNH RBC with Annexin V, which binds to the PS exposed on RBC (Fig 2B).

Release of procoagulant MPs from C′-activated PNH RBC

The IIa-generation assay used in the present study, showed a linear correlation between time (0–60 min) and OD_{405} of IIa at 0–60 u/l, added to the wells (Fig 1A). The procoagulant properties of the supernatants of RBC (normal and PNH) treated by C′ activation in sucrose buffer were examined by acceleration of the IIa-generation process in the diluted plasma. The supernatants of PNH RBC, which were treated by C′ activation, showed enhanced procoagulant properties in comparison with those of normal RBC similarly treated or PNH RBC treated with inactivated serum (Fig 1B). Both supernatants of normal RBC treated with serum and PNH-RBC treated with inactivated serum in sucrose buffer showed enhanced procoagulant properties, suggesting that some components included in the serum may accelerate coagulation.

To measure procoagulant MPs in the supernatants, we also employed a ZYUMUPHEN-MP Activity kit, which is based upon PS-exposed MPs captured in Annexin V-coated wells accelerating the action of prothrombinase added to the wells. C′ activation induced the release of significant amounts of MPs from PNH RBC but not from normal RBC; interruption of terminal C′ activation with anti-C5 or anti-C9 inhibited MP release from PNH RBC (Fig 3).

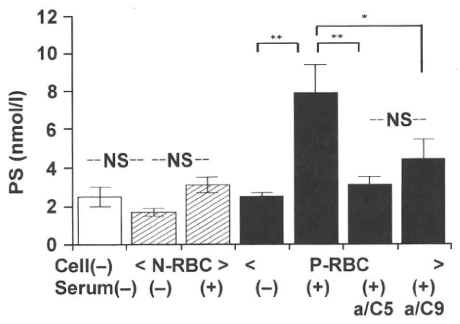

Fig 3. PS-exposed (procoagulant) MP release from RBC by C′-activation. Annexin V binding, i.e. PS-exposed MPs released from RBC by C′ activation was measured using a procoagulant MP Activity Assay kit. C′ activation induced significant amounts of PS-exposed MPs released from PNH-RBC (P-RBC), but not from normal RBC (N-RBC). Interruption of C′ activation with anti-C5 (a/C5) or anti-C9 (a/C9) inhibited the release of MPs from PNH-RBC. This figure shows data for Patient 2. Bars indicate the mean ± SD of triplicate assays. NS, not significant. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

FCM for MP release and PS exposure on RBC

FCM detects MPs as low forward scatter (FSC-low) and Annexin V-binding regions. While no significant MPs were generated from normal RBC by C′ activation (Fig 4Ab), significant amounts of MPs were released from PNH-RBC by C′ activation (26·4% in Fig 4Ae). FCM also detected a significant population of PS-exposing RBC (3·3%) that bound to Annexin V (Fig 4Ae). PNH-RBC were composed of CD59-negative and -positive RBC (a and b, respectively, lower panel in Fig 4B). As expected, only CD59-negative (PNH-affected, i.e. C′-sensitive) RBC released MPs by C′ activation (Fig 4B).

PMA- and C′-induced release of procoagulant MPs from RBC

Procoagulant MPs were released from RBC (both normal and PNH) by treatment with PMA, a PKC activator (Fig 5A,B; ●). This release was inhibited by the pretreatment of RBC with Calphostin C (Fig 5A,B; ■). By contrast, the release of procoagulant MPs from C′-activated PNH RBC was not influenced by the pretreatment of RBC with Calphostin C (Fig 5C).

Effects of PKC inhibitors on C′-induced MP release from PNH RBC

A previous report indicated that PKC activation is involved in PS exposure on RBC induced by calcium and ionophore (de Jong *et al*, 2002), although this was unclear in MP generation from RBC. Our study revealed that PKC activation of RBC (normal RBC as well as PNH-RBC) with PMA enhanced the procoagulant properties of the supernatant of RBC that were inhibited by the pretreatment of RBC with Calphostin C, a blocker of the PMA binding site (Fig 5A,B). As MP generation and PS exposure on RBC are considered to occur in the same phase, we studied the role of PKC in MP generation from PNH-RBC by C′ activation. Procoagulant properties of the supernatants of C′-activated PNH-RBC were not influenced by the pretreatment of PNH-RBC with Calphostin C (Fig 5C). To confirm this phenotypically, the release of Annexin V-binding MPs from PNH-RBC by C′ activation was examined by FCM. Neither Calphostin C nor

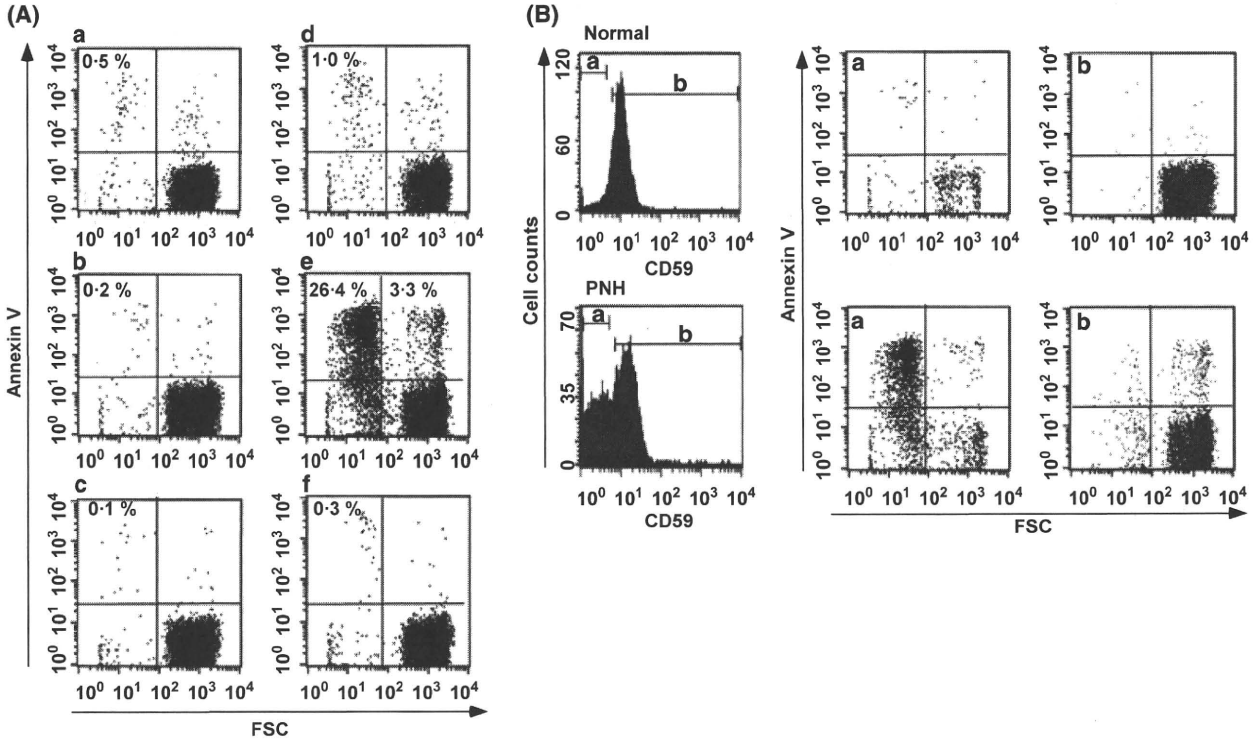

Fig 4. FCM for procoagulant MP release and PS-exposed RBC by C' activation. Panel A: Normal RBC (a,b,c) and PNH-RBC (d,e,f) were incubated with serum (b,e), inactivated serum (c,f), or normal saline (a,d) in sucrose buffer. MPs (FSC-low and Annexin V-high) were detected only when PNH-RBC were treated with serum in sucrose buffer (26·4% in Fig 4Ae). It should be noted that a significant population of PS-exposed RBC was also detected in PNH-RBC treated by C' activation (3·3% in Fig 4Ae). Figures in each panel denote the percentages of the regions with MP or PS-exposed RBC, detected as FSC-low, Annexin V-high or FSC-high, Annexin V-high, respectively. Panel B: PNH phenotype of RBC inducing MP release was analysed by two-colour FCM (FITC-Annexin-V and phycoerythrin-anti-CD59). MPs were shown to be derived from CD59-negative (PNH-affected) RBC (a fraction in lower panel B). CD59-positive, PNH-unaffected RBC were resistant to both MP release and haemolysis upon C' activation (b fraction in lower panel B). This figure shows data for Patient 1.

Ro-31-8220, both PKC inhibitors, affected MP release from C′-activated PNH-RBC in comparison with the controls, distilled water (DDW) or dimethyl sulfoxide (DMSO), respectively (Fig 6).

Involvement of calpain or caspase and Ca^{2+} dependency in C′-induced MP release from PNH-RBC

In the case of activated platelets, it has been reported that PS exposure is regulated by various molecules, such as calpain (calcium-dependent thiol proteinase) or caspase (apoptosis-related protein), although this has not been studied well in RBC. We have studied the role of these molecules in MP generation from PNH-RBC by C′ activation using several inhibitors: calpain inhibitors (ALL, E64d, PD150606, PD145305, calpastatin peptide) and a caspase inhibitor [Z–Val–Ala–DL–Asp–fluoromethylketone (z–VAD–fmk)]. These inhibitors did not affect MP release from PNH-RBC (Fig 7A).

In addition, we examined the requirement of Ca^{2+} for MP release from C′-activated PNH-RBC. In sucrose buffer containing 1 mmol/l Mg^{2+}, EGTA did not affect either haemolysis or MP generation from PNH-RBC (Fig 7B). This explains why EDTA (2 mmol/l) blocked both C′-mediated haemolysis and MP release, because EDTA inhibits the assembly of C′ membrane attack complex (MAC) on RBC (Fig 7B).

Discussion

Increased incidence of thrombosis has been reported in haemolytic anaemias, particularly anaemias with intravascular haemolytic features, which include sickle cell disease (SCD), thalassaemia and PNH (Cappellini *et al*, 2000; Ataga & Orringer, 2003; Ziakas *et al*, 2008). Pathophysiological mechanisms contributing to coagulation abnormalities in haemolytic anaemias could be classified three ways: (i) RBC membrane alteration and MPs, (ii) RBC/endothelium interaction, (iii) nitric oxide (NO) deficiency (Cappellini, 2007). Although diverse PNH abnormalities in multi-lineages of blood cells, deficient in the expression of GPI-anchored membrane proteins, may contribute to thrombogenesis, the effectiveness of eculizumab (anti-C5) on the reduction of thrombotic incidence as well as the reduction of clinical

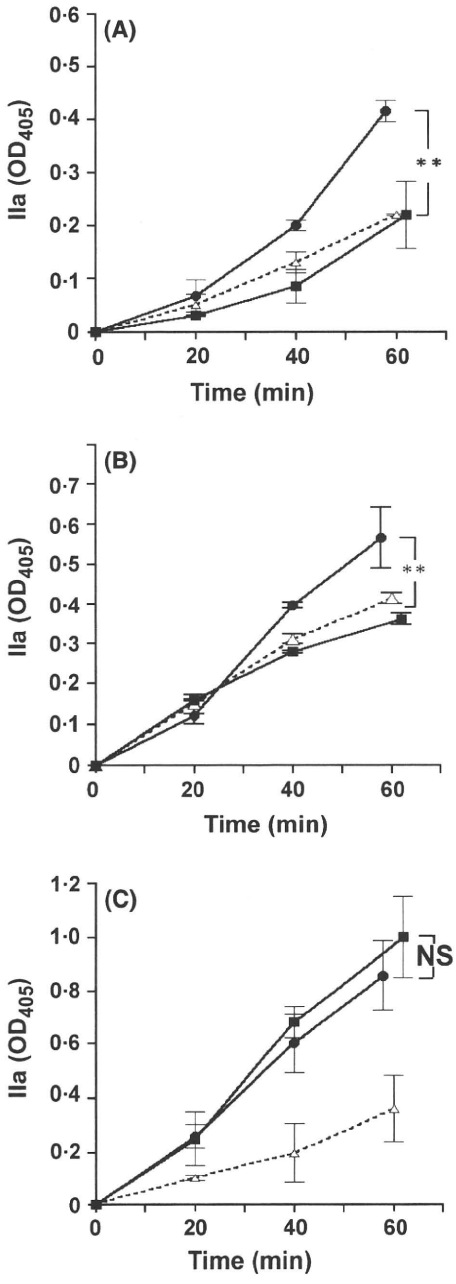

Fig 5. PMA- and C'-induced procoagulant MPs release from RBC. Panel A,B: PMA-induced procoagulant MP release from RBC. The supernatant of normal RBC (A) and PNH-RBC (B), which were treated with PMA (0·1 μmol/l, 30 min), showed enhanced procoagulant properties (black circle) in comparison with those of untreated RBC (open triangle). The supernatant of RBC, pretreated RBC with Calphostin C (1·67 μmol/l, 30 min) followed by treatment with PMA, showed no enhancement of procoagulant properties (black square). There were no differences in PMA-induced MP release between normal and PNH-RBC. This figure shows data for a normal donor (A) and for Patient No.1 (B). Panel C: Effects of Calphostin C on MP release from C'-activated PNH-RBC. Both supernatants of PNH-RBC pretreated with (black square) or without (black circle) Calphostin C followed by C'-activation showed enhanced procoagulant properties. Data of untreated RBC are shown as open triangles. Bars indicate the mean ± SD of triplicate assays. NS, not significant. $^{**}P < 0{\cdot}01$. This figure shows data for Patient 2.

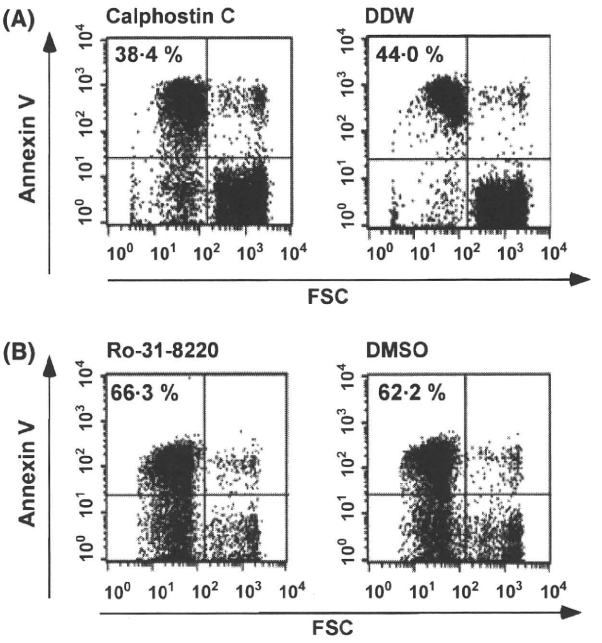

Fig 6. PKC activation is not involved in MP release from C'-activated PNH-RBC. PNH-RBC were pretreated with PKC inhibitors, Calphostin C or Ro-31-8220, followed by C' activation. MP release was analysed by FCM. Panel A: Pretreatment of PNH-RBC (Patient 1) with Calphostin C (10 μmol/l, 23°C, 30 min) did not inhibit MP release from C'-activated PNH-RBC in comparison with the control (double-distilled water, DDW). Panel B: Pretreatment of PNH-RBC (Patient 2) with Ro-31-8220 (100 nmol/l, 23°C, 30 min) did not inhibit MP release from C'-activated PNH-RBC in comparison with the control (dimethyl sulfoxide, DMSO). Figures in each panel denote the percentages of regions of MPs (FSC-low, Annexin V-high). Panels (A) and (B) show data for Patients 1 and 2, respectively.

haemolysis (Hillmen *et al*, 2004, 2007) indicate the importance of C'-mediated mechanisms, haemolysis in particular, for thrombogenesis in PNH. As has been well documented for platelets, cell membrane-derived MPs provide the catalytic surface necessary for the assembly of procoagulant enzyme complexes, prothrombinase and tenase. In PNH, the increased levels of circulating procoagulant MPs, derived from haemolysed RBC or activated platelets, could be responsible for the prothrombotic status (Hugel *et al*, 1999; Simak *et al*, 2004), as well as in sepsis, heparin-induced thrombocytopenia, thrombotic thrombocytopenic purpura and SCD (Piccin *et al*, 2007). While elevated levels of circulating MPs could contribute to the thrombophilia in PNH, their precise correlation with the thrombotic events in PNH remains to be clarified. Because NO plays an important role in the maintenance of normal platelet functions through the downregulation of platelet aggregation and adhesion, NO reduction due to intravascular haemolysis also contributes to thrombogenesis in PNH to some extent (Cappellini, 2007). In addition, the effects of eculizumab on the clinical markers of coagulation and fibrinolysis indicate that endothelial cell activation also plays some role(s) in thrombogenesis in PNH (Helley *et al*, 2009).

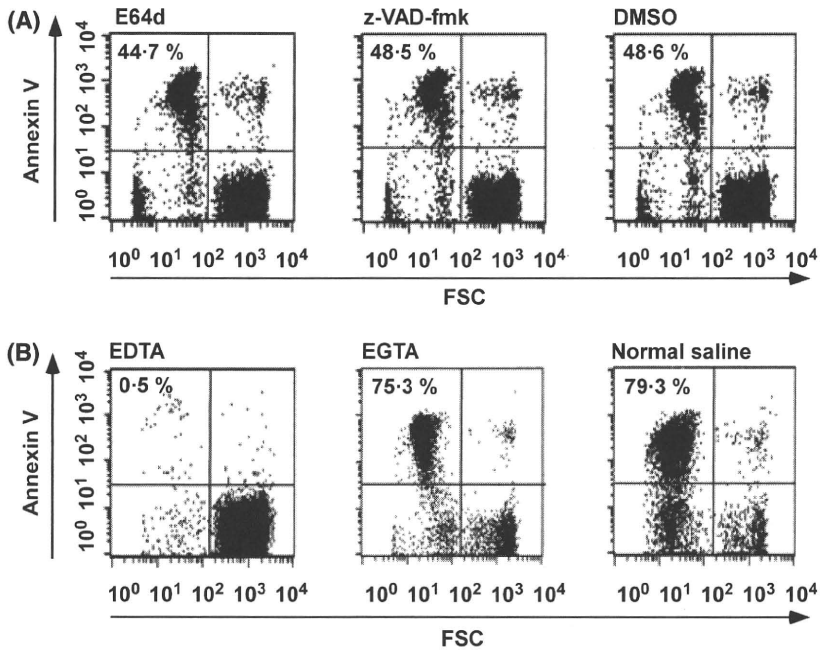

Fig 7. Panel (A): Effects of calpain and caspase inhibitor on C'-induced MPs release from PNH-RBC. Pretreatment of PNH-RBC (Patient 1) with E64d (40 μmol/l, 23°C, 15 min), a calpain inhibitor, or with z-VAD-fmk (100 μmol/l, 23°C, 15 min), a broad caspase inhibitor, did not influence MP release from PNH-RBC by C' activation. DMSO indicates the control. Calpain inhibitors other than E64d showed the same results. Panel (B): Effects of chelating agents on C'-induced MP release from PNH-RBC. C' activation of PNH-RBC (Patient 2) was induced in the presence of EDTA (2 mmol/l), EGTA (2 mmol/l), or normal saline (control) in sucrose buffer containing 1 mmol/l $MgCl_2$. EDTA inhibited both haemolysis and MP release. EGTA and haemolysis did not affect MP release from PNH-RBC. Figures in each panel denote the percentages of regions of MPs (FSC-low, Annexin V-high).

Mammalian cells have an asymmetric transbilayer distribution of phospholipids, in which most PS is located in the inner monolayer. This asymmetry is maintained by the activity of a lipid-specific transporter that regulates the distribution of PS between bilayer leaflets, a process that is Ca^{2+}-dependent (Sims & Wiedmer, 2001). It has been well established that PS exposure is regulated by activation of calcium-dependent phospholipid scramblase activity in concert with inactivation of the aminophospholipid translocase. MP release, generally accompanied by the loss of transversal phospholipid asymmetry leading to PS exposure, occurs during cell ageing and in disease states (Allan *et al*, 1982) or in *in vitro* conditions as the increase of intracellular Ca^{2+} induced by an ionophore (Allan & Thomas, 1981).

PS exposure and/or procoagulant MP generation in RBC can be induced by various mechanisms, such as intracellular calcium increase (Dekkers *et al*, 1998), PKC activation (de Jong *et al*, 2002) and ATP depletion (Beleznay *et al*, 1997); C5b-9 deposition on cell membranes is one such mechanism, in RBC as well as platelets (Wiedmer *et al*, 1993; Ninomiya *et al*, 1999). Our study demonstrated that the accumulation of C′ MAC on RBC induces procoagulant MP release, which was shown both functionally by IIa generation assay and phenotypically by FCM. The data that the treatment of serum (source of C′) with anti-C9 significantly diminished MP generation from PNH-RBC upon C′ activation indicate that PS exposure and MP release are not induced significantly before the step of C5b-8 MAC formation. While PNH-RBC, deficient in the expression of GPI-anchored proteins, including CD55 and CD59, have been shown to be impaired in MP release in response to Ca^{2+} ionophore stimulation (Whitlow *et al*, 1993), our data showed that PNH-RBC release MPs in response to C5b-9 accumulation in a Ca^{2+}-independent manner; normal RBC expressing GPI-anchored proteins were resistant to PS exposure and MP release in response to C′ activation, as clearly shown in Fig 4. Normal RBC are resistant to C′ activation inducing the assembly of C5b-9 MAC leading to haemolysis and MPs release, because of the normal expression of membrane C′ inhibitors, CD55 and CD59.

It is known that human RBC contain PKC, which mediates the phosphorylation of cytoskeletal proteins (Danilov & Cohen, 1989) and the human Na^+/H^+ antiport (Bourikas *et al*, 2003). PKCα, which translocates to the RBC membrane upon stimulation with PMA, mediates PS exposure following glucose depletion of RBC (Klarl *et al*, 2006). In the case of platelet activation accompanied by MP release in response to calcium ionophore, the involvement of calpain-mediated proteolysis of cytoskeletal proteins was also suggested (Fox *et al*, 1990), but not in the response to C5b-9 (Wiedmer *et al*, 1990). Our data indicated that PKC, calpain and caspase were not involved in MP release from RBC by terminal C′ MAC accumulation. This finding is also consistent with MP release from PNH RBC by C′ activation being Ca^{2+}-independent.

Although the precise mechanism underlying PS exposure and/or procoagulant MP release from PNH-RBC by C′ stimulation is still to be clarified, procoagulant MPs derived from PNH-affected blood cells could contribute to thrombogenesis in PNH, in addition to other pathophysiological mechanisms, particularly due to activated platelets and/or injured endothelial cells. To clarify the precise contribution of elevated MPs derived from PNH-RBC to the thrombogenesis in PNH, a clinical study in a large scale, further studies on the MPs in PNH patients are required.

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Notch2 Signaling Is Required for Potent Antitumor Immunity In Vivo

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CD8$^+$ T cells play a central role in cancer immunosurveillance, and the efficient induction of CTLs against tumor Ags is required for successful immunotherapy for cancer patients. Notch signaling directly regulates the transcription of effector molecules in CTLs. However, it remains unclear whether Notch signaling in CD8$^+$ T cells is required for antitumor CTL responses and whether modulation of Notch signaling can augment antitumor CTL responses. In this study, we demonstrate that signaling by Notch2 but not Notch1 in CD8$^+$ T cells is required for antitumor CTL responses. Notch2$^{flox/flox}$ mice crossed with E8I-cre transgenic (N2F/F-E8I) mice, in which the Notch2 gene is absent only in CD8$^+$ T cells, die earlier than control mice after inoculation with OVA-expressing EG7 thymoma cells. In contrast, Notch1$^{flox/flox}$ mice crossed with E8I-cre transgenic mice inoculated with EG7 cells die comparable to control mice, indicating that Notch2 is crucial for exerting antitumor CTL responses. Injection of anti-Notch2 agonistic Ab or delta-like 1-overexpressing dendritic cells augmented the antitumor response in C57BL/6 mice inoculated with EG7 cells. These findings indicate that Notch2 signaling in CD8$^+$ T cells is required for generating potent antitumor CTLs, thus providing a crucial target for augmenting tumor immune responses. ***The Journal of Immunology*****, 2010, 184: 4673–4678.**

The efficacy of antitumor CTLs critically depends on interaction of the TCR and MHC-presented tumor Ags as well as signals through costimulatory molecules or cytokine receptors (1). T cell costimulatory molecules include both activating and inhibitory receptors, and several attempts have been tried to modulate costimulatory molecule activity to augment antitumor immune responses (1, 2). For instance, blockade of feedback inhibition of costimulation through CD28 by blockade of CTLA-4 with a mAb induces remarkable therapeutic responses in tumor-bearing mice (3). Tumor-associated dendritic cells (DCs) overexpress PD-L1 and PD-1, inhibitory receptors for T cells, and PD-L1 blockade by anti–PD-L1–augmented DC-mediated T cell activation (4). Therefore, modulation of stimulatory or inhibitory receptors has a great impact on augmenting T cell immune responses against tumor cells (1).

Notch signaling controls both development and functional differentiation of T cells (5–7). Our group demonstrated that Notch2 signaling directly regulates granzyme B expression and promotes CTL differentiation (8). Notch-mediated CTL differentiation appears to be independent of Eomesodermin (8). Therefore, CTL differentiation is coregulated by several transcriptional pathways. Furthermore, we found that Notch signaling contributes to DC-mediated NK cell activation, which enhanced the killing activity of NK cells (9). These studies suggest that Notch signaling is crucial for exerting cytotoxic responses in immune cells.

In this report, we investigated whether Notch signaling is required for an efficient antitumor response and, if that is the case, whether modulation of Notch signaling augments antitumor responses. We found that Notch2- but not Notch1-deficient CD8$^+$ T cells fail to expand in response to tumor inoculation. The injection of anti-Notch2 agonistic Ab in mice augmented antitumor immunity. These results indicate that stimulation of Notch2 could provide a new strategy to strengthen CD8$^+$ T cell-mediated antitumor immune responses.

Materials and Methods

Mice

Six- to 8-wk-old C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Notch2$^{flox/flox}$ crossed with E8I-cre transgenic mice (N2F/F-E8I) were previously reported (8, 10). Notch1$^{flox/flox}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and crossed with E8I-cre transgenic mice. OT-I TCR transgenic mice were purchased from Taconic Farms (Germantown, NY). All of the mice were maintained under specific pathogen-free conditions in the animal research center at the University of Tokushima, Japan. All of the animal studies were approved by the animal research committee of the University of Tokushima.

Flow cytometry

PE-conjugated anti-CD8 mAb (eBioscience, San Diego, CA) was used for cell staining. In some experiments, cells were fixed with 4% paraformaldehyde and stained with allophycocyanin-conjugated anti-granzyme B mAb (Caltag Laboratories, Burlingame, CA) in saponin-containing buffer. Fluorescence intensity of $\sim 10^5$ cells was examined using a FACSCalibur or FACSCanto II flow cytometer (BD Biosciences, Mountain View, CA).

Inoculation of tumor cells

EG7 cells are EL4 cells transfected with OVA, which were obtained from American Type Culture Collection (Manassas, VA). Animals were injected s.c. on the flank with 3×10^6 EG7 cells. Cells were washed three times in

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Abbreviations used in this paper: BMDC, bone marrow-derived dendritic cell; cont-DC, control dendritic cell; DC, dendritic cell; DL1, delta-like 1; DL1-DC, dendritic cell overexpressing delta-like 1; Jagged2-DC, dendritic cell transduced with Jagged2.

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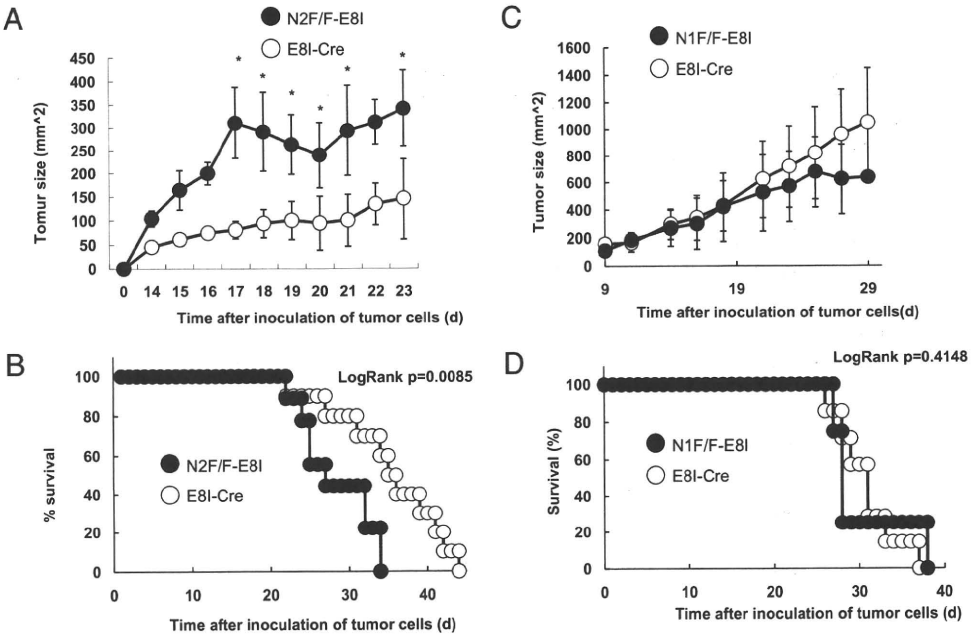

FIGURE 1. Notch2 regulates tumor immune response against EG7. EG7 tumor cells were s.c. inoculated in (*A*, *B*) E8I-Cre (open) and N2F/F-E8I (closed) or (*C*, *D*) E8I-Cre (open) and N1F/F-E8I (closed) mice, and tumor size (*A*, *C*) and mice survival (*B*, *D*) were monitored. Results are shown as mean ± SD. $*p < 0.01$. The experiments are representative of four independent experiments.

endotoxin-free RPMI 1640 medium without FCS and once in endotoxin-free PBS before injection. In some experiments, anti-Notch2 mAb (HMN2-29) (8) or hamster IgG (100 μg/mouse) was injected on days 1, 3, 7, and 10 after tumor inoculation. The anti-Notch2 mAb specifically recognizes Notch2 but not Notch1, Notch3, or Notch4 (Supplemental Fig. 1). Bone marrow-derived DCs (BMDCs) (2×10^6) were injected proximal to the tumor inoculation region 10, 12, and 14 d after tumor inoculation. The BMDCs were infected with delta-like 1 (DL1)- or Jagged2-encoding retrovirus three times as previously reported (8). Then BMDCs were further cultured with irradiated EG7 cells for 2 d before injection, and $CD11c^+$ cells were purified by CD11c magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) before injection into mice. The larger and smaller diameters of the s.c. tumors were measured using calipers at 2-d intervals; these two diameters were multiplied to obtain an estimate of the tumor area. The data are displayed as the mean ± SD of the tumor areas for each group of animals at a given time point.

Vectors and constructs

cDNA for mouse delta-like 1 or Jagged2 was cloned into the retroviral vector pKE004 (11). Retroviruses were generated by transfecting Plat-E cells (12) with vectors using the transfection reagent GeneJuice (Novagen, Darmstadt, Germany). The cDNA for mouse Notch1 or Notch2 was cloned into pcDNA3.1. Each plasmid was transfected into CHO cells by GeneJuice, and a G418-resistant cell line was obtained.

Luciferase assay

CHO cells transfected with Notch1- or Notch2-encoding plasmids were further transfected with a reporter plasmid carrying HES-1 promoter regions (13) and 1.25 ng of the control *Renilla* luciferase reporter plasmid pRL-TK by a Gene Pulser II system (Bio-Rad, Hercules, CA). Anti-Notch2 mAb (10 μg/ml) was added soon after transfection. Twenty-four hours after transfection, luciferase activity was measured by Dual-Luciferase Reporter Assay (Promega, Madison, WI) according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity using pRL-CMV (Promega). All of the experiments were done in triplicate and repeated at least three times.

Cell purification and cell culture

$CD8^+$ T cells were purified from total spleen or lymph node cells by incubating cells with anti-CD8 mAb (53–6.72), followed by positive selection of $CD8^+$ cells with anti-rat IgG MicroBeads (Miltenyi Biotec). Purified $CD8^+$ T cells (5×10^6) were transferred into C57BL/6 mice that had received EG7 cells 12 d previously. Total spleen cells from OT-I TCR transgenic mice were stimulated with OVA peptide (SIINFEKL) (100 pM) in the presence of anti-Notch2 mAb (HMN2-29) or control hamster IgG (10 μg/ml) for 5 d. The resultant $CD8^+$ cells (1×10^6) were transferred into C57BL/6 mice that had received EG7 cells 12 d previously.

In vitro killing assay

Single-cell suspensions of lymph node cells (2×10^6) were cultured with 1×10^6 irradiated spleen cells for 5 d in the presence of OVA peptide (SIINFEKL) (1 μM) and 5 U/ml human recombinant IL-2. Five days after stimulation, live cells were purified with Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) and incubated with target EL4 pulsed with or without OVA peptide and labeled with ^{51}Cr (PerkinElmer, Waltham, MA) at the indicated E:T ratios. After 5 h of incubation, supernatants were harvested, and ^{51}Cr release was measured with a γ scintillation counter (Aloka, Tokyo, Japan). The corrected percentage of lysis was calculated as: corrected % lysis = 100 × (test ^{51}Cr released − spontaneous ^{51}Cr released)/(maximum ^{51}Cr released − spontaneous ^{51}Cr released).

Statistical analysis

All of the data expressed as mean ± SD are representative of at least three different experiments. Comparisons between individual data points were made using Student *t* tests. Differences in survival between experimental groups were analyzed using the Kaplan-Meier approach. The statistical significance of group differences was assessed using the log-rank test. $p < 0.05$ was considered significant.

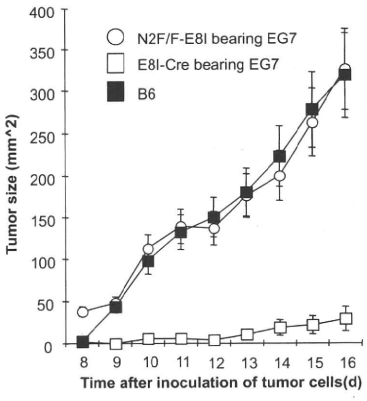

FIGURE 2. Notch2-deficient T cells do not eradicate tumor cells. EG7 tumor cells were s.c. inoculated into E8I or N2F/F-E8I mice. The $CD8^+$ cells from E8I (open square) or N2F/F-E8I mice (open circle) were purified 12 d after inoculation and transferred into C57BL/6 mice that had received EG7 tumor cells 12 d previously. As a control, C57BL/6 mice that did not receive any primed T cells were used (closed square). Results are shown as mean ± SD. The experiments are representative of four independent experiments.

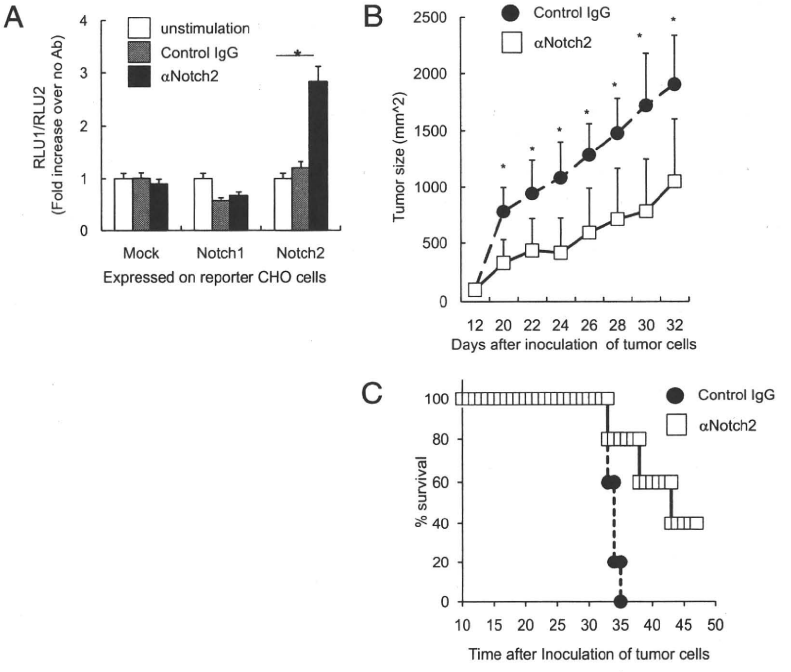

FIGURE 3. Anti-Notch2 agonistic Ab augments antitumor $CD8^+$ T cell responses. *A*, Anti-Notch2 mAb (clone HMN2-29) (black) or control hamster IgG (gray) were incubated with CHO cells transfected with control vector or the cDNA for Notch1 or Notch2. A Notch reporter plasmid was transfected, and luciferase activity was measured and compared with that without an Ab (white). Results are shown as mean ± SD. *B* and *C*, The spleen cells from OT-I TCR transgenic mice were stimulated with OVA peptide (100 pM) in the presence of anti-Notch2 mAb (open square) or control IgG (closed circle) for 5 d in vitro, and purified $CD8^+$ T cells were transferred into C57BL/6 mice that were inoculated s.c. with EG7 tumor cells 12 d before. Then tumor size (*B*) and survival (*C*) of each mouse was monitored. Results are shown as mean ± SD. $*p < 0.01$. The experiments are representative of four independent experiments.

Results

Notch2-deficient mice have lower antitumor activity

We have previously reported that Notch2 has a crucial role in exerting CTL responses (8). To investigate the contribution of Notch2 signaling to $CD8^+$ T cell antitumor immunity, we inoculated EG7 cells (EL4 thymoma cells transfected with the OVA gene) into $Notch2^{flox/flox}$ crossed with E8I-cre transgenic mice (N2F/F-E8I) and control E8I-cre transgenic (E8I) mice. EG7 cells grow gradually in E8I mice, and the growth was much faster in N2F/F-E8I mice (Fig. 1*A*). Accordingly, E8I mice die later than N2F/F-E8I mice after inoculation of EG7 cells (Fig. 1*B*). These data suggest that Notch2 deficiency in $CD8^+$ T cells attenuates EG7 antitumor immune responses.

Because Notch1 is also highly expressed on activated $CD8^+$ T cells (8), we next used $Notch1^{flox/flox}$ crossed with E8I-cre transgenic mice (N1F/F-E8I) inoculated with EG7 cells to determine whether Notch1 deficiency on $CD8^+$ T cells reduces T cell-mediated antitumor immune responses. In contrast to N2F/F-E8I mice, EG7 cells inoculated into N1F/F-E8I mice grow comparable to those in E8I mice (Fig. 1*C*), and N1F/F-E8I mice die with kinetics similar to those seen in E8I mice (Fig. 1*D*). These data indicate that Notch2 on $CD8^+$ T cells contributes to antitumor immunity.

Notch2-deficient mice do not generate antitumor CTLs

We next examined whether tumor Ag-specific killing activity is defective in N2F/F-E8I mice. N2F/F-E8I or E8I $CD8^+$ T cells were harvested from spleen and lymph nodes and enriched from mice 12 d after inoculation of EG7 cells. These cells were then transferred into naive C57BL/6 mice in which EG7 cells had been inoculated 12 d previously. EG7 cells grew poorly in C57BL/6 mice that received T cells from E8I mice, indicating the presence of strong tumor-specific CTLs (Fig. 2). In contrast, EG7 cells grew rapidly in C57BL/6 mice that received T cells from N2F/F-E8I mice, similar to mice in which naive T cells were transferred (Fig. 2). These data indicate that N2F/F-E8I mice are not able to generate enough or high-quality tumor Ag-specific CTLs.

Stimulation of Notch2 by a specific Ab augments antitumor immune responses

We next determined whether overstimulation of Notch2 augments antitumor immune responses. CHO cells were transfected with the genes for mouse *notch1* or *notch2*. These cells were next transfected with Notch reporter plasmids and stimulated by anti-Notch2 mAb. The anti-Notch2 mAb stimulated Notch-mediated

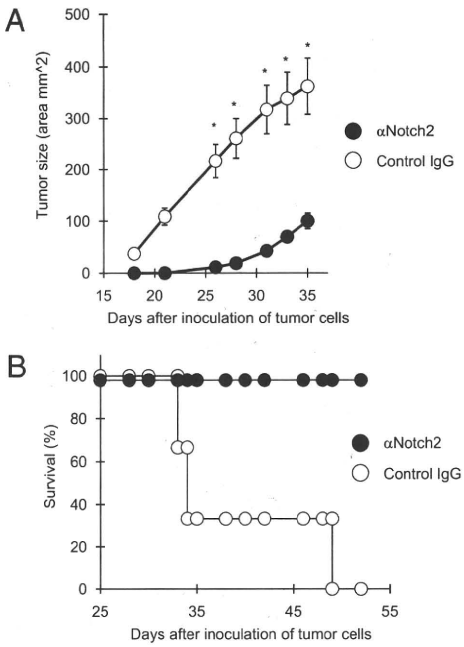

FIGURE 4. Treatment of mice with anti-Notch2 agonistic Ab promotes antitumor immunity in vivo. EG7 tumor cells were s.c. inoculated into C57BL/6 mice that 12 d later received control IgG (open) or anti-Notch2 mAb (closed) 3, 6, 7, and 10 d after tumor inoculation. Tumor size (*A*) and mouse survival (*B*) were monitored every day. Results are shown as mean ± SD. $*p < 0.01$. We used at least five mice in each group, and the experiments are representative of four independent experiments.

signaling only in CHO cells transfected with Notch2 and not Notch1, as evaluated by a HES-1 reporter gene system (Fig. 3*A*). The anti-Notch2 mAb stimulates Notch2 in a concentration-dependent manner, and this stimulatory effect was blocked in the presence of γ secretase inhibitor (Supplemental Fig. 2).

The $CD8^+$ T cells from OT-I TCR transgenic mice were activated with OVA peptide in the presence or absence of anti-Notch2 mAb for 5 d in vitro. Then activated $CD8^+$ T cells were transferred into C57BL/6 mice that had received EG7 cells 12 d previously, and tumor size (Fig. 3*B*) and mouse survival (Fig. 3*C*) were monitored. $CD8^+$ T cells stimulated with anti-Notch2 mAb exhibited greater suppression of tumor growth and enhanced mouse survival compared with those of mice receiving activated control cells (Fig. 3*B*, 3*C*). We did not observe any death at least until day 70 in anti-Notch2 mAb-treated mice that survived at day 50 (data not shown).

Anti-Notch2 mAb promotes the eradication of tumor cells in vivo

We next injected anti-Notch2 mAb or control hamster IgG in C57BL/6 mice that had received EG7 cells 12 d previously. Treatment of tumor-bearing C57BL/6 mice with anti-Notch2 mAb inhibited the growth of EG7 cells compared with that of control IgG (Fig. 4*A*). Similarly, treatment of C57BL/6 mice with anti-Notch2 mAb enhanced mouse survival, and significantly, all mice eventually eradicated the tumor cells (Fig. 4*B*). In addition, we have observed similar antitumor effects of anti-Notch2 mAb when 50 μg Ab in each injection was used (data not shown). These data indicate that stimulation of Notch2 promotes the eradication of tumor cells in vivo.

Delta-like 1-transfected dendritic cells suppress tumor growth

DC-mediated tumor immunotherapy is widely used in clinical settings currently. We have previously reported that DL1 upregulates CTL activity (8). To determine whether DCs overexpressing DL1 (DL1-DCs) are able to suppress tumor growth in vivo, we three times peritumorally injected DL1-DCs or control DCs (cont-DCs) that had been cultured with irradiated EG7 cells in vitro. Tumor cell growth was slower in DL1-DC–injected mice than in cont-DC–injected mice (Fig. 5*A*). The T cells from draining lymph nodes more highly expressed *hes1*, a Notch target gene, in DL1-DC–inoculated mice than those in cont-DC–inoculated mice (Supplemental Fig. 3). The draining lymph node cells from mice inoculated with EG7 and DL1-DCs or cont-DCs were cultured with irradiated EG7 cells in the presence of IL-2 for 5 d. The cytotoxic activity of T cells against EL4 cells pulsed with OVA peptide was evaluated by ^{51}Cr release assay. No apparent lysis of EL4 cells not pulsed with OVA peptide was exerted by T cells from mice that received cont-DCs or DL1-DCs (Fig. 5*B*). Effector cells from mice that received DL1-DCs exhibited much stronger specific lysis of target EL4 cells pulsed with OVA than effector cells from mice that received cont-DCs (Fig. 5*B*). In addition, we could not detect any cytotoxic activity of T cells from DL1-DC–treated mice if T cells are not cultured in vitro in the presence of

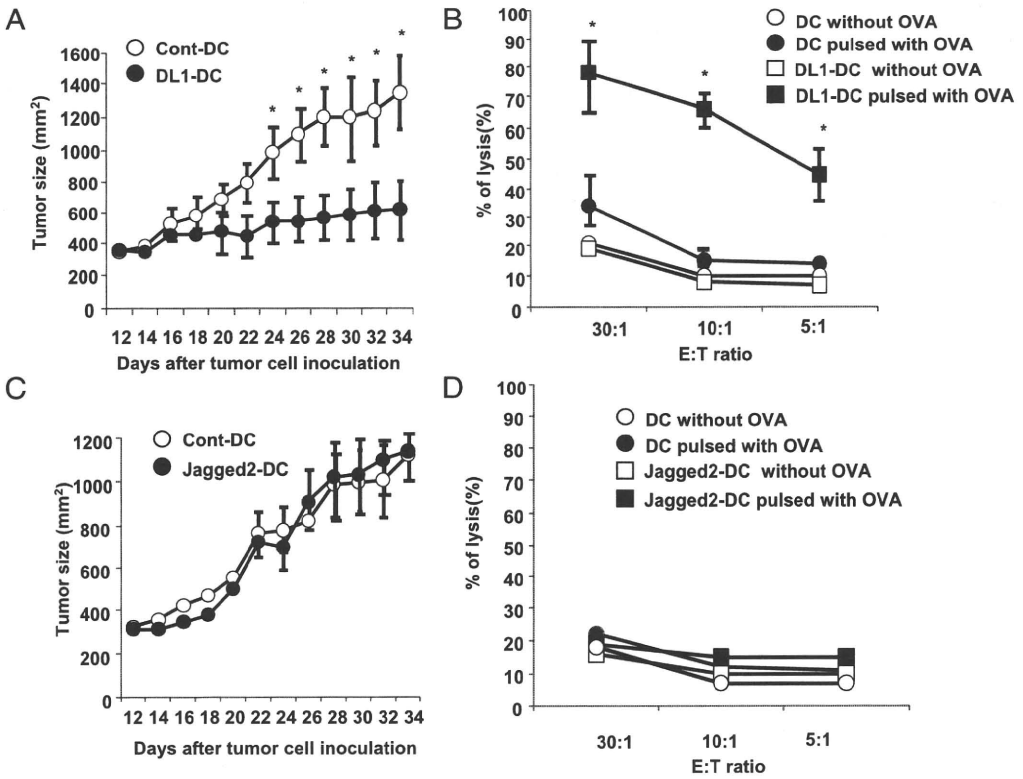

FIGURE 5. Overexpression of delta-like 1 but not Jagged2 on DCs augments antitumor responses by $CD8^+$ T cells. Mouse full-length *dll1* or *Jagged2* was overexpressed in BMDCs (DL1-DCs and Jagged2-DCs) cultured with irradiated EG7 tumor cells for 2 d. (*A*) DL1-DCs (closed) or cont-DCs (open) or (*B*) Jagged2-DCs (closed) or cont-DCs (open) were inoculated into proximal regions of tumors in C57BL/6 mice 10, 12, and 14 d after injecting EG7 cells, and tumor size was monitored. Lymph node cells were recovered 12 d after tumor inoculation and were cultured with irradiated spleen cells in the presence of OVA peptide and recombinant human IL-2 (5 U/ml) for 5 d. Cytolytic activity against EL4 cells pulsed with (closed) or without (open) OVA peptide was evaluated by ^{51}Cr release assay. Killing activity of T cells from mice that received (*C*) DL1-DCs (square), or cont-DCs (circle) or (*D*) Jagged2-DCs (square) or cont-DCs (circle) is shown. Results are shown as mean ± SD. $*p < 0.01$. The experiments are representative of four independent experiments.

OVA peptide (data not shown), probably due to a very low number of Ag-specific T cells. We also tested whether another Notch ligand, Jagged2, has a similar ability to DL1. However, inoculation of DCs transduced with Jagged2 (Jagged2-DCs) did not suppress EG7 growth (Fig. 5*C*), and T cells from Jagged2-DCs did not exhibit cytolytic activity against OVA-pulsed EL4 cells (Fig. 5*D*). The *hes1* expression in T cells from Jagged2-DC–inoculated mice was higher than that from cont-DC–inoculated mice but lower than that from DL1-DC–inoculated mice (Supplemental Fig. 3). Taken together, these data indicate that manipulation of Notch signaling induced by DL1 could be beneficial as a new strategy to augment antitumor CTL activity.

Discussion

Notch signaling controls mature T cell differentiation and activation by directly regulating transcription of effector molecules (7, 8, 14, 15). In particular, our group has recently demonstrated that Notch2 signaling directly controls CTL effector molecules, including granzyme B, by integrating RBP-J and CREB1 (8). In this study, we revealed that Notch2, but not Notch1, signaling in $CD8^+$ T cells is required for efficient induction of antitumor CTLs. Furthermore, treating tumor-bearing mice with anti-Notch2 mAb or DL1-DCs strengthened antitumor CTL responses. These data indicate that Notch2 signaling is required for augmenting antitumor CTL activity and suggest that manipulation of Notch2 signaling might provide a new clinical approach for cancer immunotherapy.

We have recently demonstrated that Notch signaling controls cytotoxic responses in both CTL and NK cells (8, 9). These data suggest that Notch signaling is a crucial signaling pathway required for cytotoxic responses in immune cells. In the studies described in this report, we found that deficiency of Notch2 but not Notch1 decreased the antitumor responses in vivo, although Notch1 is highly expressed on activated $CD8^+$ T cells (8). These data suggest a lower affinity of Notch1 and Notch ligands present in our tumor model relative to those that activate the Notch2 pathway in $CD8^+$ T cells. Notch–Notch ligand interaction is tightly regulated by Notch glycosylation (16), which might be crucial for the distinct Notch receptor utilization controlling CTL responses that we observe. We also found that treatment with anti-Notch2 agonistic Ab in tumor-bearing mice increased antitumor responses. These data strongly suggest that the major target cells for anti-Notch2 mAb in terms of antitumor effects would be $CD8^+$ T cells, although we cannot completely deny the possibility that anti-Notch2 mAb interacts with Notch2 on non-$CD8^+$ T cells, which may indirectly affect Notch2 signaling in $CD8^+$ T cells. Notch2 is widely expressed on many tissues, and thus treatment with anti-Notch2 mAb might have some adverse effects for the host, although we have never seen any macroscopic changes in mice after Ab treatment. Nevertheless, to reduce the possibility of adverse effects of anti-Notch2 mAb, the appropriate route or dose must be considered.

Our previous study showed that DL1 is able to augment CTL responses in vivo (8). We demonstrated in this study that injection of DL1-DCs peritumorally suppresses tumor growth compared with cont-DCs. These data suggest a potential therapeutic strategy to augment antitumor CTLs by injecting DL1-DCs pulsed with tumor-specific Ags. However, we should be cautious with this approach in terms of clinical use because Notch signaling regulates angiogenesis, which nurtures tumor cells (17–19). Those studies revealed that delta-like 4 contributes to angiogenesis. Although the contribution of DL1 to angiogenesis around tumor cells has not been reported, DL1 might also be able to activate Notch receptors that control angiogenesis. Thus, injection of DL1-DCs around the tumor burden may promote angiogenesis for tumor growth, although DL1-DCs would also help to strengthen CTL-mediated killing of tumor cells. The i.v. or s.c. route as a method to transfer DCs has been used in human clinical trials for treating cancer patients. Therefore, it would be important to carefully evaluate whether i.v. or s.c. injection of DL1-DCs affects angiogenesis at the tumor site before applying those methods for clinical use.

In the present work, we have focused on investigating the role of Notch2 signaling in tumor immunity and the effect of anti-Notch2 mAb or DL1-DC treatment on tumor eradication. We show that these treatments enhance survival and decrease the size of the tumor by augmenting CTL activity. The data suggest that stimulation of Notch2 would be a new way to stimulate antitumor immune responses. Combining anti-Notch2 mAb with other therapeutic approaches, such as DC-mediated tumor vaccines, is likely to yield further clinical benefits.

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Disclosures

The authors have no financial conflicts of interest.

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