[29] Y. Tujimoto, Cell death regulation by the Bcl-2 protein family in the mitochondria, J. Cell Physiol. 195 (2003) 158–167.
[30] S.J. Korsmeyer, BCL-2 gene family and the regulation of programmed cell death, Cancer Res. 59 (1999) 1693–1700.
[31] E. Yonish-Rouach, D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, M. Oren, Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6, Nature 352 (1991) 345–347.
[32] S.P. Tuck, L. Crawford, Characterization of the human p53 gene promoter, Mol. Cell. Biol. 9 (1989) 2163–2172.
[33] M. Eguchi, M. Eguchi-Ishimae, A. Tojo, K. Morishita, K. Suzuki, Y. Sato, S. Kudoh, K. Tanaka, M. Setoyama, F. Nagamura, S. Asano, N. Kamada, Fusion of ETV6 to neurotrophin-3 receptor TRKC in acute myeloid leukemia with t(12;15)(p13;q25), Blood 93 (1999) 1355–1363.
[34] T.R. Golub, A. Goga, G.F. Barker, D.E. Afar, J. McLaughlin, S.K. Bohlander, J.D. Rowley, O.N. Witte, D.G. Gilliland, Oligomerization of the ABL tyrosine kinase by the Ets protein TEL in human leukemia, Mol. Cell. Biol. 16 (1996) 4107–4116.
[35] Y. Kuno, A. Abe, N. Emi, M. Iida, T. Yokozawa, M. Towatari, M. Tanimoto, H. Saito, Constitutive kinase activation of the TEL-Syk fusion gene in myelodysplastic syndrome with t(9;12)(q22;p12), Blood 97 (2001) 1050–1055.
[36] S.W. Hiebert, W. Sun, J.N. Davis, T. Golub, S. Shurtleff, A. Buijs, J.R. Downing, G. Grosveld, M.F. Roussell, D.G. Gilliland, N. Lenny, S. Meyers, The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription, Mol. Cell. Biol. 16 (1996) 1349–1355.
[37] S. Fears, M. Gavin, D.E. Zhang, C. Hetherington, Y. Ben-David, J.D. Rowley, G. Nucifora, Functional characterization of ETV6 and ETV6/CBFA2 in the regulation of the MCSFR proximal promoter, Proc. Natl. Acad. Sci. USA 94 (1997) 1949–1954.
[38] H. Gunji, K. Waga, F. Nakamura, K. Maki, K. Sasaki, Y. Nakamura, K. Mitani, TEL/AML1 shows dominant-negative effects over TEL as well as AML1, Biochem. Biophys. Res. Commun. 322 (2004) 623–630.
[39] Y. Iijima, T. Ito, T. Oikawa, M. Eguchi, M. Eguchi-Ishimae, N. Kamada, K. Kishi, S. Asano, Y. Sakaki, Y. Sato, A new ETV6/TEL partner gene, ARG (ABL-related gene or ABL2), identified in an AML-M3 cell line with a t(1;12)(q25;p13) translocation, Blood 95 (2000) 2126–2131.
[40] F. Salomon-Nguyen, V. Della-Valle, M. Mauchauffe, M. Busson-Le Coniat, J. Ghysdael, R. Berger, O.A. Bernard, The t(1;12)(q21;p13) translocation of human acute myeloblastic leukemia results in a TEL-ARNT fusion, Proc. Natl. Acad. Sci. USA 97 (2000) 6757–6762.
[41] F. Nakamura, Y. Nakamura, K. Maki, Y. Sato, K. Mitani, Cloning and characterization of a novel chimeric gene TEL/PTPRR in acute myelogenous leukemia with inv(12)(p13q13), Cancer Res. 65 (2005) 6612–6621.

Development of megakaryoblastic leukaemia in *Runx1-Evi1* knock-in chimaeric mouse

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Chromosomal translocations involving the *Runx1* gene create various chimaeric proteins that are believed to cause human leukaemia. Runx1-ETO and Runx1-Evi1, generated as consequences of t(8;21) and t(3;21), respectively, share molecular structural similarities; they both contain DNA-binding domain of Runx1 and transcriptional repression domains from either ETO or Evi1. Despite such similarity, the subtypes of leukaemia related to each of the chimaeric protein are different; Runx1-ETO typically occurs with acute myelocytic leukaemia of M2 subtype in the French–American–British classification, whereas Runx1-Evi1 is mostly associated with megakaryoblastic leukaemia of M7 subtype or megakaryoblastic crisis in chronic myelocytic leukaemia. Experimental animals that express the fusion proteins in the hematopoietic cells have been created to recapitulate the diseases. Both Runx1-ETO transgenic[1] and conditional knock-in[2] mice do not develop leukaemia by itself and require additional genetic aberrations to transform myeloid progenitors. Here, we report the development of megakaryoblastic leukaemia in *Runx1-Evi1* knock-in chimaeric mouse.

Runx1-Evi1 knock-in chimaeric mice were created by injecting recombinant TT2 ES cells[3] into wild-type blastocyst.[4] We created six of such chimaeric mice, and five of them showed sudden deaths after 7 months of age without any significant finding in post mortem. Interestingly, one of the chimaeric mice that died at 5 months of age showed marked hepatosplenomegaly. Wright–Giemsa staining of stump preparation from the enlarged spleen demonstrated massive infiltration of large dysplastic cells, some of which contained multi-lobulated nuclei with various size of cytoplasm reminiscent of megakaryoblastic leukaemia (Figure 1a). Histology section showed disrupted gross architecture of the spleen, with white and red pulp intermingling (Figure 1c). In the liver, substantial infiltration of leucocytes was observed around the portal vein (Figure 1d). Most of the infiltrating cells consisted of reactive neutrophils, with partial presence of the dysplastic megakaryocytic cells observed in the spleen. Multiple fibrin thrombi were identified in the portal vein, indicating the occurrence of disseminated intravascular coagulation. These findings all

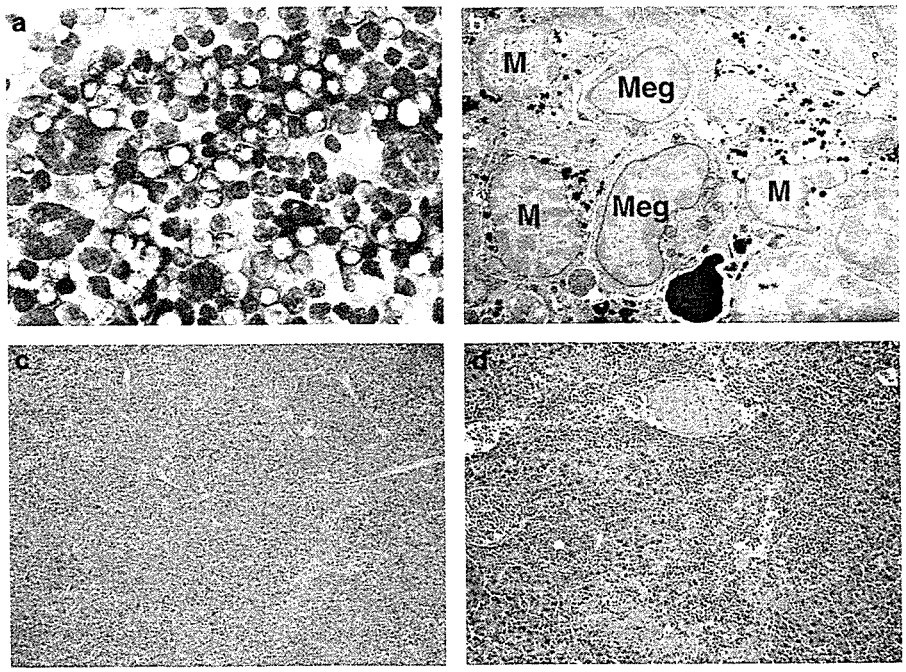

Figure 1 Development of megakaryoblastic leukaemia in *Runx1-Evi1* chimaeric mouse. (**a**) Wright–Giemsa staining of stump preparation from spleen (objective lens (OL), × 40/0.65; original magnification (OM), × 400). (**b**) Electron micrograph of spleen cells (OM, × 3000). M, myeloid cell; Meg, megakaryocytic cell. (**c, d**) Hematoxylin–eosin staining of sections from spleen (**c**) (OL, × 10/0.40; OM, × 100) and liver (**d**) (OL, × 20/0.70; OM, × 200).

indicate an aggressive form of leukaemia. The electron microscopic analysis of the infiltrating cells in the spleen showed 20% of the cells positive for platelet-peroxidase, substantiating megakaryocytic origin of the leukaemic cells (Figure 1b). Taken together, we conclude that this chimaeric mouse developed megakaryoblastic leukaemia.

The key aspect of our observation is that Runx1-Evi1 protein is leukaemogenic *per se*, unlike Runx1-ETO. Such clear difference in the pathophysiological outcome likely arises from the Evi1 portion of Runx1-Evi1 protein. Evi1 is reported to stimulate activator protein 1 activity,[5] repress transforming growth factor-β signaling,[6] and inhibit c-Jun N-terminal kinase function.[7] Such versatile function of Evi1 may underlie stronger oncogenic capacity of Runx1-Evi1 than Runx1-ETO. Another important aspect is that the affected lineage in human is conserved in the experimental animal. Our observation indicates strong causal relationship between the expression of Runx1-Evi1 protein and megakaryoblastic leukaemia. Indeed, the *Runx1-Evi1* chimaeric gene was isolated from a patient developing megakaryoblastic crisis in chronic myelocytic leukaemia that accompanied emergence of t(3;21).[8] It is unknown whether Runx1-Evi1 is preferentially oncogenic in megakaryoblast, or show exclusive maturation block in the megakaryocytic lineage when expressed in early haematopoietic cells. Foetal liver cells from *Runx1-Evi1* knock-in heterozygous embryo can give rise to dysplastic megakaryocytes.[4] Such abnormal progenitors may have persisted in the adult bone marrow and expanded to cause massive infiltration of the megakaryoblasts in the liver and spleen.

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References

1 Yuan Y, Zhou L, Miyamoto T, Iwasaki H, Harakawa N, Hetherington CJ *et al.* AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. *Proc Natl Acad Sci USA* 2001; **98**: 10398–10403.
2 Higuchi M, O'Brien D, Kumaravelu P, Lenny N, Yeoh EJ, Downing JR. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. *Cancer Cell* 2002; **1**: 63–74.
3 Yagi T, Tokunaga T, Furuta Y, Nada S, Yoshida M, Tsukada T *et al.* A novel ES cell line, TT2, with high germline-differentiating potency. *Anal Biochem* 1993; **214**: 70–76.
4 Maki K, Yamagata T, Asai T, Yamazaki I, Oda H, Hirai H *et al.* Dysplastic definitive hematopoiesis in AML1/EVI1 knock-in embryos. *Blood* 2005; **106**: 2147–2155.
5 Tanaka T, Nishida J, Mitani K, Ogawa S, Yazaki Y, Hirai H. Evi-1 raises AP-1 activity and stimulates c-fos promoter transactivation with dependence on the second zinc finger domain. *J Biol Chem* 1994; **269**: 24020–24026.
6 Kurokawa M, Mitani K, Irie K, Matsuyama T, Takahashi T, Chiba S *et al.* The oncoprotein Evi-1 represses TGF-β signalling by inhibiting Smad3. *Nature* 1998; **394**: 92–96.

7 Kurokawa M, Mitani K, Yamagata T, Takahashi T, Izutsu K, Ogawa S *et al.* The evi-1 oncoprotein inhibits c-Jun N-terminal kinase and prevents stress-induced cell death. *EMBO J* 2000; **19**: 2958–2968.

8 Mitani K, Ogawa S, Tanaka T, Miyoshi H, Kurokawa M, Mano H *et al.* Generation of the AML1-EVI-1 fusion gene in the t(3;21)(q26;q22) causes blastic crisis in chronic myelocytic leukemia. *EMBO J* 1994; **13**: 504–510.

The role of the *bax* gene polymorphism G(−248)A in chronic lymphocytic leukemia

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Two recent publications in *Leukemia* have investigated the potential role of the *bax* G(−248)A polymorphism in chronic lymphocytic leukemia (CLL),[1,2] which pertain to our previous study,[3] and that of Saxena *et al.*[4] Our single centre study of 203 patients showed that this polymorphism significantly influenced Bax protein expression, overall survival ($P=0.05$) and in particular survival from first treatment ($P=0.012$). In contrast to the original paper by Saxena *et al.*[4] we showed no difference in allele frequency between CLL samples and those derived from healthy volunteers, nor an increased incidence of the polymorphism in advanced stage disease. Unfortunately, Nuckel *et al.*[2] misquote our work by stating that we showed an association between the polymorphism and advanced stage disease — we did not ($P=0.62$). In all other aspects, Nuckel *et al.*[2] assessed clinical and prognostic parameters that we and Skogsberg *et al.*[1] had previously identified as irrelevant with regards to the *bax* G(−248)A polymorphism. Crucially, they failed to address the pivotal question of the role of the polymorphism on therapeutic response.

Although many of the findings from the study of Skogsberg *et al.*[1] (463 patients) and our own are compatible, there are a number of major differences. (1) They failed to identify any significant overall survival difference (median survival 85 months versus 102 months; $P=0.21$ in 350 patients) or survival from first treatment (median survival 36 months versus 63 months; $P=0.26$ in 98 patients). (2) They could not demonstrate a difference in *bax* RNA expression between the polymorphic groups. These differences between the two studies are worthy of further comment.

Our study was a single centre study in which all the clinical data was complete, whereas in the study of Skogsberg *et al.*[1] (a multicentred international collaboration involving seven centres in three countries), there were some significant omissions of clinical data. Survival from treatment data was presented for only 98 patients when their study contains 154 Binet B/C patients. In addition, with a median follow-up of 60 months, a significant number of the 243 stage A patients would also have required therapy and could therefore have been included too. Despite this, their study showed a 27-month difference in survival from therapy between the different polymorphic groups (36 months versus 63 months; $P=0.26$) that is, a trend towards worse survival. We make explicit in our study that virtually all the patients were entered into the prevailing Medical Research Council CLL study at the time. This means that the indications to commence therapy and disease assessment were standardized according to internationally accepted criteria and indeed the therapies the patients received were not too dissimilar – very difficult to control for in a retrospective, multinational, multi-centre study such as Skogsberg *et al.*[1] More disconcerting is how the 98 patient 'subset' were selected? It is clear from Figure 3b that Skogsberg *et al.*[1] chose to look at 73 GG patients who had died. It is clear that for the GA subgroup they chose dead and alive patients. Why did they not just compare the dead GG subgroup with the dead GA subgroup? – this would have at least stopped any, albeit unintentional bias being introduced. How did the authors choose which of the over 60 live GA subgroup to include in this analysis?

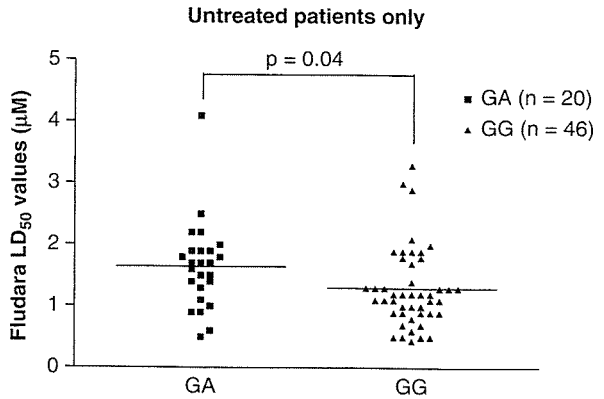

Figure 1 *In vitro* sensitivity (LD_{50}) to fludarabine in 66 previously untreated CLL patients defined by *bax* genotype.

Skogsberg *et al.*[1] stated that they could find no difference in *bax* RNA expression for the different polymorphic groups. However, they only studied 35 patient samples and failed to state how many of these samples were derived from patients who had received prior therapy. This is important as it has been shown that previous treatment exposure is a contributory factor in determining Bax protein expression.[3–5] In our study, we measured Bax protein (122 patients) not RNA and found overlapping, but significantly different Bax expression between the different polymorphic groups. In new data presented here, we show that CLL samples derived from previously untreated patients with the *bax* G(−248)A polymorphism are more resistant to *in vitro* fludarabine (Figure 1). However, the large overlap in fludarabine LD_{50} values between the polymorphic groups indicate that *bax* genotype is not the only determinant of drug sensitivity in CLL cells.

As outlined above, the Skogsberg *et al.*[1] study has several flaws and we look forward to larger prospective studies in CLL and other human malignancies that will hopefully offer definitive evidence of the importance of this single-nucleotide polymorphism in determining treatment response and clinical outcome.

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showed the marked growth suppression of MPC-1$^-$ immature myeloma cells, but not MPC-1$^+$ mature myeloma cells in the MM2 case. In the primary myeloma cells from 20 cases, we summarized that the combination of Dex with baicalein showed consistent growth suppression in MPC-1$^-$ immature myeloma cells from all cases, and MPC-1$^+$ mature myeloma cells were significantly suppressed in seven out of 20 cases.

The ligand-induced activation of PPARβ is reported to suppress the NF-κB activity. Two possible mechanisms for its downregulation of NF-κB activity were reported: one was the upregulation of IkB gene expression[7] and the other was the inhibitory physical interaction between the p65 subunit of NF-κB and PPAR in the nucleus.[8] We examined whether PPARβ interacted with the p65 of NF-κB after IL-1α stimulation by the immunoprecipitation followed by Western blotting. Costimulation of IL-1α with Dex and baicalein strongly enhanced the physical interaction between p65 of NF-κB and PPARβ (data not shown). The binding activity of NF-κB (p65/p50) to the κB site was also suppressed in the treatment with Dex and baicalein. Furthermore, the expression of the NF-κB target genes, such as *IL-6* and *IkBα*, was markedly reduced by the treatment of Dex and baicalein combined with IL-1α stimulation in U266 cells.

With regard to the therapeutic aspect, the cooperative growth suppression of Dex and baicalein in myeloma cells might be useful for myeloma therapy. Combinatory treatment with both may overcome the Dex resistance and the baicalein resistance in primary myeloma cells, as well as myeloma cell lines. It is possible that baicalein combined with Dex can induce the activation of PPARβ and glucocorticoid receptor (GR), which cooperatively suppress the transcriptional activity of NF-κB in the nucleus of myeloma cells. Clinical trials would rapidly evaluate the utility of this combinatory treatment in relapsed and refractory myeloma cases.

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References

1 Dreyer C, Krey G, Keller H, Givel F, Helftenbein G, Wahli W. Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell* 1992; **68**: 879–887.
2 Park BH, Vogelstein B, Kinzler KW. Genetic disruption of PPARδ decreases the tumorigenicity of human colon cancer cell. *Proc Natl Acad Sci USA* 2001; **98**: 2598–2603.
3 Bharti AC, Donato N, Singh S, Aggarwal BB. Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kB and IkBα kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. *Blood* 2003; **101**: 1053–1062.
4 He T, Chan TA, Vogelstein B, Kinzler KW. PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* 1999; **99**: 335–345.
5 Ma Z, Otsuyama K, Liu S, Abroun S, Ishikawa H, Tsuyama N *et al.* Baicalein, a component of *Scutellaria* radix from Huang-Lian-Jie-Du-Tang (HLJDT), leads to suppression of proliferation and induction of apoptosis in human myeloma cells. *Blood* 2004; **105**: 3312–3318.
6 Kawano MM, Huang N, Harada H, Harada Y, Sakai A, Tanaka H *et al.* Identification of immature and mature myeloma cells in the bone marrow of human myelomas. *Blood* 1993; **82**: 564–570.
7 Delerive P, Gervois P, Fruchart JC, Staels B. Induction of IkBα expression as a mechanism contributing to the anti-inflammatory activities of peroxisome proliferators-activated receptorα activators. *J Biol Chem* 2000; **275**: 36703–36707.
8 Planavila A, Laguna JC, Vazquez-Carrera M. Nuclear factor-kB activation leads to down-regulation of fatty acid oxidation during cardiac hypertrophy. *J Biol Chem* 2005; **280**: 17464–17471.

Chronic idiopathic myelofibrosis expressing a novel type of *TEL-PDGFRB* chimaera responded to imatinib mesylate therapy

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Constitutive activation of platelet-derived growth factor receptor (PDGFR) is one of the features of myeloproliferative disease (MPD).[1] PDGFR is a member of type III receptor tyrosine kinase that transmits mitotic signals in cells of mesenchymal origin. Both *PDGFRA* and *PDGFRB* genes, located on chromosomes 4q12 and 5q33 respectively, form fusion gene as a result of chromosome translocation. Representative examples are *TEL-PDGFRB* in t(5;12)(q33;p13)[2] and *FIP1L1-PDGFRA* in del(4)(q12),[3] both of the gene products known to have ligand-independent kinase activity. The former is observed in chronic myelomonocytic leukaemia (CMMoL) as well as other MPDs with eosinophilia, whereas the latter has been reported in hypereosinophilic syndrome (HES).

We report here a case of chronic idiopathic myelofibrosis (CIMF) with t(5;12)(q33;p13), which transformed to acute myelogenous leukaemia (AML). This case is unique in two aspects: (1) this is the first report of CIMF with *TEL-PDGFRB* fusion and (2) a novel type of *TEL-PDGFRB* fusion transcript that retained the internal and ETS domains of TEL was expressed. The leukaemic cells responded well to a series of chemotherapy, which was followed by administration of imatinib mesylate, a kinase inhibitor for both PDGFR and ABL. It gave a partial cytogenetic response but failed to maintain a remission of leukaemia. This case implies the existence of various types of *TEL-PDGFRB* fusion gene with possible impact on the phenotype of the disease.

A 37-year-old male presented with nasal and gingival bleeding in August 2005. He showed marked hepatosplenomegaly at presentation: spleen was palpable 10 cm below the left

costal margin, and liver 2 cm below the right costal margin. Laboratory examination revealed a haemoglobin level of 7.6 g/dl, a white blood cell count of 2.8×10^9/l (13.5% blasts) and a platelet count of 9.0×10^9/l. Six per cent erythroblasts as well as dacryocytes were observed in the peripheral blood. The bone marrow could hardly be aspirated. Histological examination of the biopsied specimen showed marked myelofibrosis accompanied by focal proliferation of blast-like cells and megakaryocytes among reticulin fibres. The blastic cells in the peripheral blood were negative for myeloperoxidase, nonspecific esterase, CD13 and CD41, but positive for CD33 (94.2%), CD34 (78.8%), CD56 (92.1%) and HLA-DR (88.4%). Because neither lymphoid markers nor electron microscopic platelet peroxidase activities were detected in the leukaemic cells, he was diagnosed as AML M0 according to the FAB classification. The preceding history of slight leukocytosis (15.6×10^9/l) at 16 months before the diagnosis and the presence of severe myelofibrosis with massive splenomegaly and tear drop cells at presentation suggested that his leukaemia had been transformed from CIMF. Cytogenetic analysis of the peripheral blood cells showed abnormality of 46, XY, t(5;12)(q33;p13)[2]/46, idem, −17, +r1[17]/47, idem, +8, −17, +r1[1]. The patient was treated with induction course of chemotherapy consisting of cytarabine and idarubicin. After one course of chemotherapy, complete remission was achieved morphologically with improvement of myelofibrosis and recovery of platelet count. Additional two courses of chemotherapy with high-dose cytarabine for consolidation maintained morphological remission. Although cells with normal karyotype transiently appeared in the bone marrow during these courses of chemotherapy, the stem line clone carrying t(5;12) never disappeared and formed 100% of the dividing cells at the end of the consolidation therapy. Imatinib mesylate treatment (400 mg/day) was commenced in November 2005 and continued for 3 months until February 2006 when anaemia and leukocytopenia emerged accompanied by high fever and unconsciousness. Serum calcium and C-reactive protein levels increased up to 13.8 and 14.43 mg/dl, respectively. Bone scintigraphy showed abnormal uptakes in multiple regions such as right shoulder and left femur, suggesting the relapse of AML with multiple blastoma in the bone. Although the bone marrow aspirate became dry again, chromosome analysis revealed re-emergence of normal clone but also the sidelines with the same additional abnormalities as those seen at presentation. Despite the intensive chemotherapy with cytarabine and idarubicin, leukaemic cells reappeared in the peripheral blood. The patient succumbed to multiple organ failure owing to disseminated intravascular coagulation syndrome and died in March 2006 (Figure 1).

Based on the physical and laboratory findings, and the clinical course, this patient was diagnosed as AML transformed from CIMF. No t(5;12)(q33;p13) has been reported in CIMF cases so far. Nested reverse transcription-PCR experiment of the peripheral blood specimen at diagnosis using HemaVision leukaemia typing/subtyping system (DNA Technology A/S, Aarhus, Denmark) showed single *TEL-PDGFRB* fusion product that was larger in size than expected. Sequencing analysis of the product revealed in-frame fusion between exon 7 of *TEL* gene and exon 10 of *PDGFRB* gene (*TEL7-PDGFRB10*; Figure 2). All other common leukaemogenic fusion genes screened by HemaVision including major *BCR-ABL* were not detected. $JAK2^{V617F}$, frequently found in MPDs including CIMF, was not detected in this case.

In all previously reported cases with the t(5;12)(q33;p13) translocation, exon 4 of *TEL* gene was fused in-frame to exon 11 of *PDGFRB* gene (2) (*TEL4-PDGFRB11*). TEL4-PDGFRβ11 consists of the sterile-alpha-motif (SAM) domain of TEL, which fuses to the transmembrane and catalytic domains of PDGFRβ. The SAM domain of TEL is known to act as the interface for

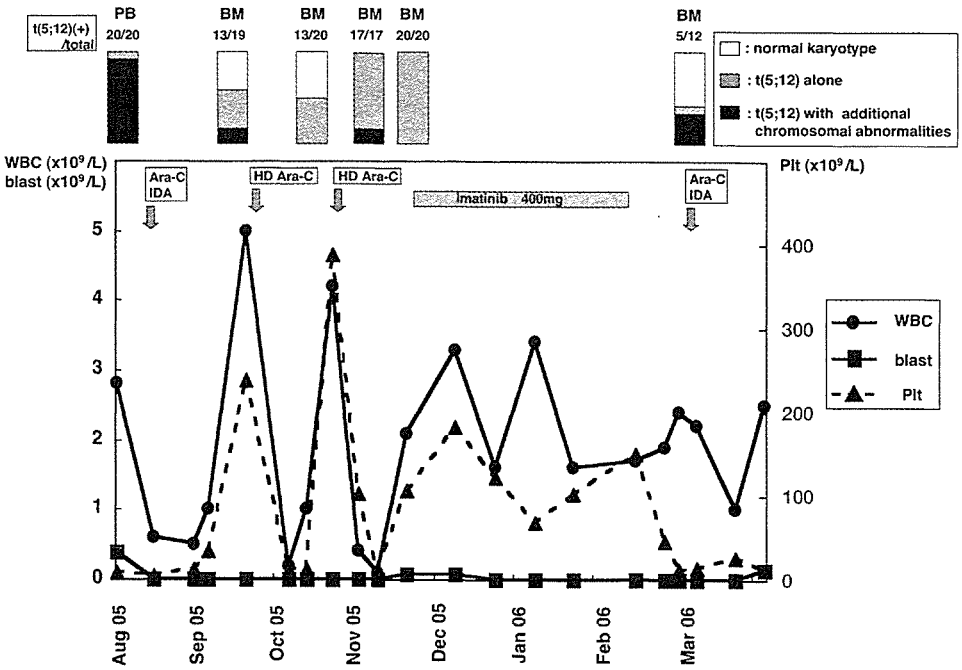

Figure 1 Clinical course. Imatinib was commenced in November 2005 following 3 courses of chemotherapy. At this point, although complete remission of acute leukaemia was achieved morphologically, all dividing cells analysed cytogenetically had t(5;12). After 3 months of imatinib therapy, partial cytogenetic response was obtained with the appearance of cells having normal karyotype. Boxes in the top represent the results of cytogenetic analysis and numbers indicate the ratio of the t(5;12)-positive cells in the total. Abbreviations: PB, peripheral blood; BM, bone marrow; HD, high dose; Ara-C, cytarabine; IDA, idarubicin; WBC, white blood cell; Plt, platelet.

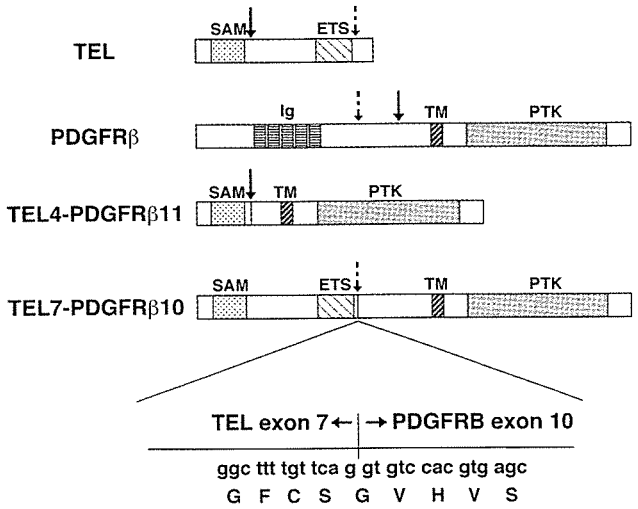

Figure 2 Structure of novel TEL-PDGFRβ fusion protein. Schematic diagrams of TEL, PDGFRβ and previously reported (TEL4-PDGFRβ11) and novel (TEL7-PDGFRβ10) TEL-PDGFRβ chimaeric proteins are depicted. The nucleotides and amino-acid sequences at the TEL7-PDGFRβ10 fusion junction are shown below. The dotted and solid arrows indicate the breakpoints in TEL7-PDGFRβ10 and TEL4-PDGFRβ11, respectively. Abbreviations: SAM, sterile-alpha-motif domain; ETS, ETS domain; Ig, immunoglobulin-like domain; TM, transmembrane region; PTK, protein-tyrosine kinase domain.

homo-oligomerization and thus make oncogenic conversion of the catalytic domain provided from PDGFRβ.[4] In the fusion protein encoded by *TEL7-PDGFRB10*, the internal and ETS domains of TEL are also included in the chimaeric protein, which differs from previously reported TEL4-PDGFRβ11. Co-repressor N-CoR and histone deacetylase-3 are known to bind to TEL via this internal domain and have important roles in transcription-repressive activity of TEL and its chimaeric proteins. On the other hand, it is interesting to note that no other TEL-related chimaeric molecules fused to tyrosine kinase possess the ETS DNA-binding domain. These additional internal and ETS domains in TEL7-PDGFRβ10 may modify the original function of TEL4-PDGFRβ11.

There is an example presenting that the internal domain of TEL in TEL-tyrosine kinase chimaera may have an impact on the disease phenotype. TEL-TRKC fusion gene is formed as a result of t(12;15)(p13;q25) and encodes activated kinase. It is unique in that it is found in both solid tumours and leukaemia,[5] with a single exon difference, and codes for the internal domain of TEL between the two sets of cancer phenotypes. Mouse models have suggested that the presence of the internal domain of TEL may alter the phenotype of the tumours.[6] Given the fact that this novel TEL7-PDGFRβ10 fusion was observed in a CIMF case, unknown function of the internal and ETS domains of TEL in the context of chimaera may be responsible for the disease phenotype.

It is important to stress that the t(5;12)(q33;p13)-carrying clone without additional chromosomal abnormalities remained throughout the clinical course, even when morphological complete remission of AML was obtained. This indicates that t(5;12)(q33;p13) may have been the first hit in stem cell to cause CIMF, which later transformed into AML with additional genetic hit(s).

Imatinib is a biochemical inhibitor for PDGFR[7] as well as ABL and c-kit. It has recently been reported that imatinib is clinically effective for MPD with aberrant activation of PDGFR kinase such as HES with *FIP1L1-PDGFRA*[3] and CMMoL or other MPDs with *TEL-PDGFRB*.[8] Although the treatment period may not have been enough in this case, some cytogenetic response after three-month administration of imatinib was observed. This case suggests that tyrosine kinase activation other than JAK2^{V617F} mutation may also be responsible for the pathogenesis of CIMF and such cases may respond to imatinib therapy.

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References

1 Cross NC, Reiter A. Tyrosine kinase fusion genes in chronic myeloproliferative diseases. *Leukemia* 2002; **16**: 1207–1212.
2 Golub TR, Barker GF, Lovett M, Gilliland DG. Fusion of PDGF receptor β to a novel ets-like gene, tel, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. *Cell* 1994; **77**: 307–316.
3 Cools J, DeAngelo DJ, Gotlib J, Stover EH, Legare RD, Cortes J *et al.* A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. *N Engl J Med* 2003; **348**: 1201–1214.
4 Carroll M, Tomasson MH, Barker GF, Golub TR, Gilliland DG. The TEL/platelet-derived growth factor β receptor (PDGFβR) fusion in chronic myelomonocytic leukemia is a transforming protein that self-associates and activates PDGFβR kinase-dependent signaling pathways. *Proc Natl Acad Sci USA* 1996; **93**: 14845–14850.
5 Eguchi M, Eguchi-Ishimae M, Tojo A, Morishita K, Suzuki K, Sato Y *et al.* Fusion of ETV6 to neurotrophin-3 receptor TRKC in acute myeloid leukemia with t(12;15)(p13;q25). *Blood* 1999; **93**: 1355–1363.
6 Eguchi M, Eguchi-Ishimae M, Green A, Enver T, Greaves M. Directing oncogenic fusion genes into stem cells via an SCL enhancer. *Proc Natl Acad Sci USA* 2005; **102**: 1133–1138.
7 Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ *et al.* Inhibition of the Abl protein-tyrosine kinase *in vitro* and *in vivo* by a 2-phenylaminopyrimidine derivative. *Cancer Res* 1996; **56**: 100–104.
8 Apperley JF, Gardembas M, Melo JV, Russell-Jones R, Bain BJ, Baxter EJ *et al.* Response to imatinib mesylate in patients with chronic myeloproliferative diseases with rearrangements of the platelet-derived growth factor receptor beta. *N Engl J Med* 2002; **347**: 481–487.

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IgE-activated mast cells in combination with pro-inflammatory factors induce T_h2-promoting dendritic cells

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Abstract

Dendritic cells (DCs) and mast cells (MCs) co-localize in peripheral tissues of antigen entry, i.e. skin and mucosa. Due to the proximity of these two cell types, activation of MCs may affect DC functions. Here, we co-cultured human monocyte-derived DCs with cord blood-derived MCs activated by cross-linking of FcεRI to elucidate the net effect of the whole MC products on DCs. Activated MCs induced maturation of DCs, and potently suppressed IL-12p70 production by the DCs. Whereas co-culture of DCs with activated MCs alone did not significantly influence the type of $CD4^+$ T cell responses induced by the DCs, DCs co-cultured with activated MCs in the presence of pro-inflammatory or T_h1-inducing factors caused T_h2 polarization. Although histamine was involved in the induction of DC maturation and T_h2 polarization by activated MCs, a combinatorial effect of various MC-derived factors, including those acting in a cell contact-dependent manner, was required for the optimal induction of T_h2-promoting DCs. Furthermore, we demonstrated that clusters of DCs are located closely with MCs in lesions of atopic dermatitis. Collectively, this study suggests that the interaction between DCs and IgE-activated MCs in a pro-inflammatory or even T_h1-prone environment is instrumental in maintaining and augmenting T_h2 responses in allergy, and that disruption of the DC–MC interaction may constitute an effective strategy to treat ongoing allergic diseases.

Introduction

Immature dendritic cells (DCs) are located mainly in peripheral tissues through which antigens invade, particularly in skin and mucosa (1). At the initial stage of an immune response, immature DCs are activated directly by pathogens and indirectly by various inflammation-associated factors produced by tissue resident cells in the microenvironment (2). Activation of DCs induces their maturation and migration to secondary lymphoid organs, where the mature DCs prime antigen-specific naive T cells. During the process of maturation, DCs integrate signals from both pathogens and tissue-derived factors and acquire the capacity of inducing different types of $CD4^+$ T cell responses, prototypes of which are T_h1 and T_h2 types. Thereby, DCs induce appropriate types of adaptive immune responses for efficient elimination of the given pathogens.

Another abundant cell type present in skin and mucosa is mast cells (MCs) (3–6). MCs are well recognized as key effector cells in IgE-associated, T_h2-type immune responses. Upon activation by cross-linking of a high-affinity IgER, FcεRI, MCs immediately undergo degranulation and secrete a vast array of humoral mediators (reviewed in 5, 6). These include preformed granule-associated molecules [e.g. neutral proteases, tumor necrosis factor (TNF)-α and histamine], *de novo* synthesized lipid mediators [e.g. prostaglandin D_2 (PGD_2), leukotriene C_4 and B_4], cytokines and chemokines. In addition to FcεRI, MCs express a diverse spectrum of receptors for 'danger' signals, such as pathogens and endogenous inflammatory mediators (5, 6). By virtue of their ability to directly sense 'dangers' and to immediately secrete a vast array of humoral mediators, MCs play an important role in the first line of defense against microbial invasions as well as in immediate allergic reactions.

Recent studies have been revealing critical roles of MCs in inducing optimal adaptive T cell responses (6). First, MCs, by immediately secreting preformed TNF-α upon bacterial invasion, induce hypertrophy of draining lymph nodes and recruitment of circulating T cells (7). Second, MCs appear to be critical for the full manifestation of experimental autoimmune

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encephalomyelitis (8, 9). Third, several studies suggest that MCs migrate to secondary lymphoid organs and influence T cell responses (9–11). Thus, other than an established role as immediate effector cells in allergic responses, MCs are likely to be critically involved in determining the strength and quality of adaptive immune responses.

MCs and immature DCs co-localize at antigen entry sites, i.e. skin and mucosa. Both human and mouse MCs activated by cross-linking of FcεRI have been shown to express markedly high levels of chemokines that attract immature DCs: CCL2, CCL3 and CCL4 (12, 13). Reciprocally, DCs have been shown to produce CCL5 and CCL8 (14) that can interact with CCR3 on MCs (15). Due to such apparent interaction between the two cell types, a vast array of humoral and possibly membrane-associated molecules derived from MCs may influence DC functions in peripheral inflamed tissues, which leads to modulation of adaptive T cell responses in draining lymphoid organs (6). Indeed, several molecules secreted by MCs have been shown to affect DC functions. First, histamine, which is stored in MC granules and is immediately released upon activation, induce human monocyte-derived dendritic cells (MoDCs) to transiently express CD86 expression (16), to produce more IL-10 and less IL-12 and to differentiate into T_h2-promoting DCs (17–20). Second, PGD_2, a major eicosanoid from MCs, reduces IL-12 production by MoDCs and favors T_h2 development (21, 22). Third, thymic stromal lymphopoietin (TSLP), whose mRNA is expressed in MCs, promote maturation of $CD11c^+$ blood DCs and their differentiation into T_h2-promoting DCs (23–25). Lastly, MC-derived exosomes have been shown to induce DC maturation (26). These studies suggest that MCs influence DC functions via different mechanisms. However, MCs express many other secretory and membrane-associated molecules that potentially affect DCs, and the net effect of the whole MC-derived factors on DC function, which will occur in a physiological situation, remains to be determined.

Here, to investigate the effects of the whole MC products on DC functions, we co-cultured human MoDCs with cord blood-derived MCs activated by cross-linking of FcεRI, and examined DC maturation, cytokine production and naive $CD4^+$ T cell differentiation primed by the DCs. Significantly, whereas co-culture of DCs with activated MCs alone did not have any effect on polarization of T cell differentiation, DCs co-cultured with activated MCs in the presence of other DC maturation-inducing factors polarized T cell responses toward a T_h2 type. Although histamine was involved in inducing T_h2-promoting DCs, combinatorial effects of other MC-derived factors, including those acting in a cell contact-dependent manner, were required for the optimal induction of T_h2-promoting DCs.

Methods

Media and reagents

RPMI 1640 (Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated FCS (ThermoTrace, Victoria, Australia), 2 mM L-glutamine, penicillin G, streptomycin (GIBCO BRL, Carlsbad, CA, USA) and 10 mM HEPES (Nacalai Tesque, Japan) was used (referred to as complete medium). Recombinant human cytokines, IL-3, IL-4, IL-6, IFN-γ, TNF-α and IL-1β were purchased from PeproTech (London, UK), and stem cell factor (SCF) was obtained from Amgen (Thousand Oaks, CA, USA). Granulocyte–macrophage colony-stimulating factor (GM-CSF) was obtained from Schering–Plough.

Generation of human umbilical cord blood-derived MCs

Umbilical cord blood was obtained from healthy volunteers in local obstetrics hospitals. Written informed consent was obtained from mothers from whom the cord blood was got, and the procedures were approved by the Human Studies Internal Review Board of Kyoto University. Cord blood-derived MCs were obtained as previously described (27). Briefly, mononuclear cells were isolated from cord blood by centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden), and the cells were cultured in AIM-V medium containing 5% FCS in the presence of 100 ng ml^{-1} SCF and 50 ng ml^{-1} IL-6 for >10 weeks. Half of the medium was exchanged weekly for fresh medium supplemented with the cytokines. Then, the cells were further incubated with 1 μg ml^{-1} IgE (Biosource International, Camarillo, CA, USA), 5 ng ml^{-1} IL-3, 10 ng ml^{-1} IL-4 for 5 days in the presence of SCF and IL-6. These factors have been shown to act synergistically on cord blood-derived MCs, and prolong survival, induce maturation, enhance FcεRI expression and optimize secretion of histamine, PGD_2 and leukotriene C_4 when MCs are activated by cross-linking of FcεRI (15, 28–32). For the last 3 h of incubation, IgE was added again to assure that IgE binds to FcεRI, and then IgE-sensitized mature MCs were harvested. MCs obtained by this method were positively stained with toluidine blue and expressed FcεRI (stained with anti-FcεRI mAb: clone CRA-1). The purity of MCs was >98% as assessed by the expressions of CD117 (eBioscience, San Diego, CA, USA) and CD203c (Beckman Coulter Immunotech, Marseille, France) by flow cytometry.

Generation of human MoDCs

Buffy coats were obtained from healthy donors in the local blood bank (Red Cross Blood Center, Kyoto, Japan). PBMCs were isolated by centrifugation on Ficoll–Paque. Monocytes were purified from PBMCs by positive selection using anti-CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were cultured for 6–7 days in complete medium in the presence of 40 ng ml^{-1} IL-4, and 50 ng ml^{-1} GM-CSF to induce immature MoDCs. Every 3 days, half of the medium was exchanged for fresh medium supplemented with the cytokines.

Co-culture of DCs and MCs

Immature MoDCs and IgE-sensitized mature MCs were co-cultured in complete medium at a density of 5×10^5 DCs ml^{-1} per well in a 24-well microplate in the presence of 50 ng ml^{-1} GM-CSF, 40 ng ml^{-1} IL-4 and 100 ng ml^{-1} SCF at a DC:MC ratio of 2:1, unless otherwise indicated. For MC activation by cross-linking of FcεRI, goat anti-human IgE antibody (Biosource International) was added at a concentration of 3 μg ml^{-1}, and the co-culture was performed for 24 h. Where indicated, 10 μg ml^{-1} mouse anti-human TNF-α mAb (clone: MAb1, BD PharMingen, San Diego, CA, USA), a mixture of histamine receptor antagonists or 10 μM indomethacin

(Sigma–Aldrich) (33), was added. The following histamine receptor antagonists were combined: 10^{-7} M pyrilamine (Sigma–Aldrich), 10^{-4} M cimetidine (a gift from Sumitomo Pharmaceuticals, Osaka, Japan) and 10^{-6} M thioperamide (Sigma–Aldrich) for H1, H2 and H3 plus H4 receptor blocking, respectively (18, 34). Separation of DCs and MCs by a porous membrane in the co-culture was performed by using transwell culture plates with polycarbonate membrane insert with 0.4-μm pore size (Costar, Corning, NY, USA). In some experiments, DCs were stimulated with 100 ng ml^{-1} LPS (from *Escherichia coli* O111:B4, Sigma–Aldrich), 1000 IU ml^{-1} IFN-γ, 10 ng ml^{-1} TNF-α, 10 ng ml^{-1} IL-1β and/or 10^{-5} M histamine (Sigma–Aldrich), with or without MCs. Concentrations of IL-12p70 in 24-h supernatants were measured by the ELISA kits (BD PharMingen). Stimulation of DCs with CD40 ligand (CD40L) was done as described (35), using irradiated (55 Gy) CD40L-transduced L cells.

Measurement of concentrations of MC-derived humoral mediators

IgE-sensitized mature MCs were plated alone at the same density and under the same cytokine condition as the DC–MC co-culture, and were activated by cross-linking of FcεRI. Concentrations of TNF-α in 24-h supernatants were measured by an ELISA kit (Biosource International), and those of histamine and PGD_2 by enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA).

Phenotypic analysis of DCs and MCs

The expression of surface markers was analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA, USA) using the following mAbs: FITC-labeled anti-CD80, CD83, CD40, CD54, HLA-ABC and HLA-DR (Beckman Coulter Immunotech); FITC-labeled anti-CD86 (BD PharMingen) and FITC-labeled anti-CCR7 mAbs (R&D Systems, Minneapolis, MN, USA). For cells in DC–MC co-culture, the cells were stained with PE-labeled anti-CD11c mAb (Becton Dickinson), and $CD11c^{bright}$ cells and $CD11c^{dim}$ cells were gated as DCs and MCs, respectively. For OX40 ligand (OX40L), cells were stained with ik-5 mAb (mouse IgG2a) (36) and FITC-labeled $F(ab')_2$ goat anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA, USA).

Analysis of cytokine production by primed T cells

Naive $CD4^+$ T cells were isolated from human cord blood mononuclear cells with the CD4 isolation kit II or from adult PBMCs with CD4 Multisort kit and CD45RA microbeads (Miltenyi Biotec). This method yielded highly purified (>92%) $CD4^+$ $CD45RA^+$ naive T cells as assessed by flow cytometry (data not shown). Naive T cells (5×10^4 cells) were co-cultured with allogeneic DCs (5×10^3 cells) in complete medium in 96-well round-bottom microplates. DCs co-cultured with MCs were purified by FACSAria cell sorter (Becton Dickinson) by gating $CD11c^{bright}$ population as DCs before they were used to stimulate T cells. On day 3, 10 ng ml^{-1} IL-2 (teceleukin, Takeda Pharmaceuticals, Japan) was added. T cells were further expanded and subjected to analysis of cytokine production on days 12–14. For intracellular cytokine staining, primed T cells were re-stimulated with 50 ng ml^{-1} phorbol myristate acetate (PMA) (Sigma–Aldrich) and 500 ng ml^{-1} A23187 (Calbiochem) for 6 h. Brefeldin A (10 μg ml^{-1}) (Sigma–Aldrich) was added during the last 3 h. The cells were fixed, permeabilized and stained with FITC-labeled anti-IFN-γ mAb (BD PharMingen) plus PE-labeled anti-IL-4 mAb (BD PharMingen). For ELISA, T cells were re-stimulated with PMA/A23187 at 1×10^6 cells ml^{-1} for 24 h, and the supernatants were harvested. For IFN-γ, a matched antibody pair (clone 2G1 and B133.5; Pierce Biotechnology, Rockford, IL, USA) was used. For IL-4, IL-5, IL-10 and IL-13, commercially available ELISA kits (Biosource International) were used.

Real-time reverse transcription–PCR analysis of Notch ligands

Total RNA was isolated from FACS-sorted DCs after DC–MC co-culture using RNeasy Mini kit (Qiagen, Hilden, Germany). Total RNA (0.5 μg) was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol. Real-time PCR was performed using qPCR Mastermix Plus (Eurogentec, Belgium) and TaqMan Gene Expression Assays for JAG1, JAG2 and DLL4 (Applied Biosystems) on the ABI PRISM 7700 Sequence Detection System. Relative quantitations of mRNA expressions were performed by the relative standard curve method and mRNA expression levels of each gene were normalized to those of β-glucronidase.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded sections were prepared from biopsied specimens of lesional skins from patients with atopic dermatitis. After deparaffinization, endogeneous peroxidase activity was blocked by 0.3% H_2O_2 in methyl alcohol. The slides were pre-incubated with 1% normal horse serum and incubated with anti-human MC tryptase mAb (clone: G3, Chemicon International, Temecula, CA, USA). Subsequently, they were incubated with biotinylated horse anti-mouse serum, and the development of staining was performed using avidin–biotin–peroxidase complex (ABC-Elite, Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine. After incubation with 0.1 M glycine–HCl (pH 2.2) and antigen retrieval by autoclave methods (37), fascin was stained with mouse anti-human fascin mAb (clone: 55K-2, DakoCytomation, Carpinteria, CA, USA) by the same procedure as the above, using avidin–biotin–alkaline phosphatase and New fuchsin in the development step. Using isotype-matched control mAbs instead of the anti-tryptase or anti-fascin mAb did not show non-specifically stained cells.

Results

Activated MC-derived factors induce DC maturation

First, we examined whether the *in vitro*-generated MCs secrete humoral factors upon activation that have been reported to affect DC functions. IgE-sensitized mature MCs were plated at the same cell density and under the same cytokine condition as the DC–MC co-culture, and activated by cross-linking of FcεRI. They secreted considerable amounts of TNF-α (256 ± 0.6 pg ml^{-1}), histamine (6.7 ± 0.6 μM) and PGD_2

(21.3 ± 0.86 ng ml^{-1}) for 24 h. As expected, addition of a cyclooxygenase inhibitor, indomethacin, inhibited the production of PGD_2 by activated MCs, whereas a mixture of histamine receptor antagonists did not affect the secretion of the three factors (data not shown). Thus, the *in vitro*-generated MCs are physiologically relevant in that they secrete major humoral factors produced by MCs, including the ones which can affect DC functions.

To examine the net effect of the whole activated MC-derived factors on DC functions, we co-cultured IgE-sensitized mature MCs with immature MoDCs, and then activated MCs by cross-linking of FcεRI by adding goat anti-human IgE antibody. GM-CSF, IL-4 and SCF were added to the co-culture to maintain the viability of DCs and MCs and to optimize mediator release from MCs (32, 38, 39). SCF alone did not induce DC maturation or affect DC maturation induced by LPS (data not shown). First, we analyzed phenotypes of DCs after 24 h of co-culture with activated MCs by flow cytometry, gating CD11c^{bright} population as DCs (Fig. 1). Without activation, MCs had no effect on the phenotypes of the DCs. In contrast, MCs activated by cross-linking of FcεRI induced up-regulation of CD80, CD86, CD83, CCR7, HLA-ABC and HLA-DR on the co-cultured DCs. Activated MCs did not affect maturation of DCs induced by LPS (Fig. 1) or LPS/IFN-γ (data not shown) added at the same time as the MC activation. These data indicate that activated MC-derived factors induce DC maturation, and that they do not have antagonistic effects on Toll-like receptor 4-mediated maturation of DCs.

To investigate relative contributions of each MC-derived factor to DC maturation, we added histamine receptor antagonists, neutralizing anti-TNF-α mAb or indomethacin (33) to the DC–MC co-culture to block the actions of histamine or TNF-α, or to inhibit the synthesis of PGD_2 in activated MCs, respectively. Because immature MoDCs express histamine H1, H2, H3 and H4 receptors (18, 20, 40), a mixture of antagonists against all the receptors (pyrilamine, cimetidine and thioperamide) was used. In addition, to evaluate the effect of cell contact on DC maturation, we separated DCs from MCs by a porous membrane using transwell plates. Although MCs can also produce IL-4 (4–6), which affects DC function, the addition of exogenous IL-4 to the co-culture precludes us from evaluating the influence of MC-derived IL-4 on DCs. As shown in Fig. 2, up-regulation of co-stimulatory molecules, CD80 and CD86, on DCs was largely inhibited by histamine receptor antagonists, whereas anti-TNF-α mAb or indomethacin did not show a considerable effect. Separation of DCs and MCs diminished the levels of the up-regulation. These data indicate that histamine is responsible for the up-regulation of co-stimulatory molecules on DCs, whereas TNF-α and PGD_2 are not involved. Cell contact has an augmenting effect on the up-regulation. It remains to be determined whether this augmentation is mediated by membrane-associated molecules on MCs that act in combination with histamine or by possible increases in local concentrations of MC-derived soluble factors around DCs.

IL-12p70 production by DCs is potently suppressed by activated MCs

IL-12p70 is a key DC-derived cytokine that plays a crucial role in induction of T_h1 responses (41). Thus, we next examined the effects of activated MC-derived factors on IL-12p70 production by DCs. We co-cultured DCs and MCs, and stimulated DCs with either LPS or CD40L in the presence or absence of IFN-γ to induce IL-12p70 production by DCs (42, 43). At the same time as the DC stimulation, MCs were activated by cross-linking of FcεRI. After 24 h of culture, concentrations of IL-12p70 in the supernatants were measured by ELISA (Table 1). Stimulation with either LPS or CD40L in the presence or absence of IFN-γ induced variable amounts of IL-12p70

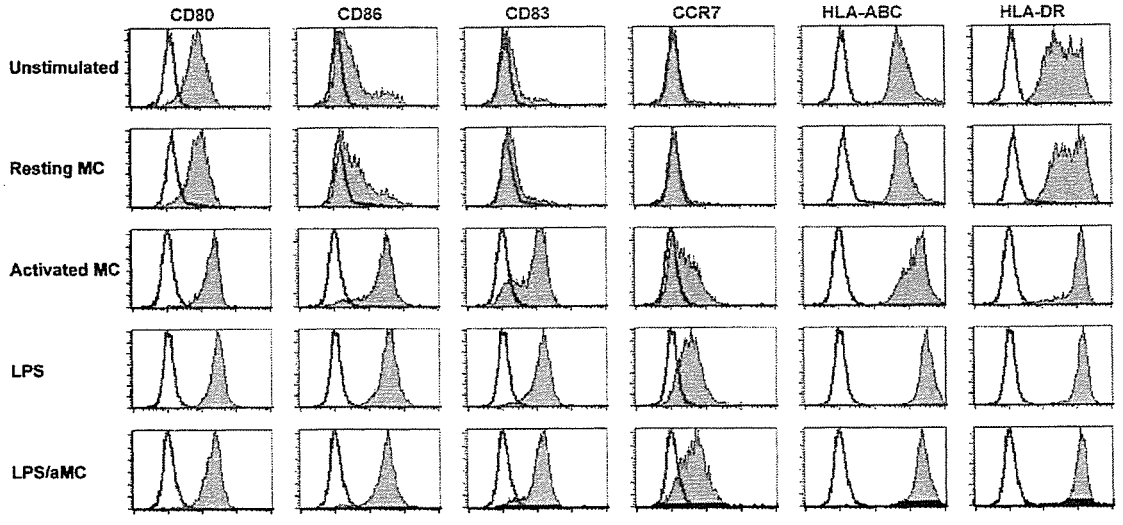

Fig. 1. Activated MC-derived factors induce DC maturation. Immature MoDCs were either cultured alone or co-cultured with IgE-sensitized mature MCs at a DC:MC ratio of 2:1. To activate IgE-sensitized MCs, FcεRI was cross-linked with goat anti-human IgE antibody. Where indicated, 100 ng ml^{-1} LPS was added to immature DCs either alone or with activated MCs (at the same time as FcεRI cross-linking). After 24 h of co-culture, surface phenotypes of DCs were analyzed by flow cytometry. CD11c^{bright} cells were gated and analyzed as DCs. Open histograms indicate background staining with an isotype-matched control mAb. These results are representative of three independent experiments.

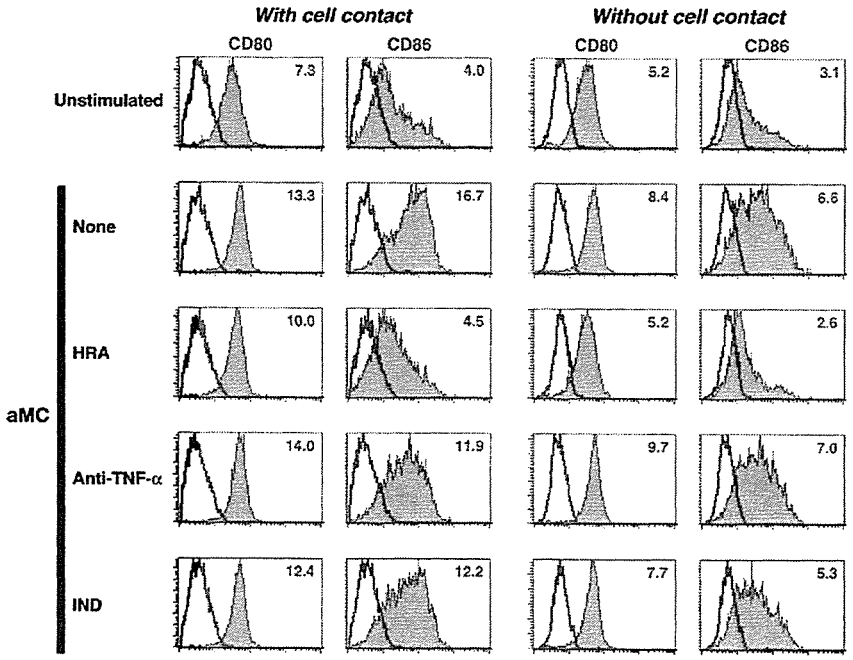

Fig. 2. Contribution of each activated MC-derived factor to the induction of DC maturation. Immature MoDCs and activated MCs were co-cultured, in the same chamber (with cell contact) or separated by a porous membrane (without cell contact) in transwell culture plates. A mixture of histamine receptor antagonists (10^{-7} M pyrilamine, 10^{-4} M cimetidine and 10^{-6} M thioperamide), 10 μg ml^{-1} neutralizing anti-TNF-α mAb or 10 μM indomethacin was added to the co-culture as indicated. After 24 h of co-culture, cells were analyzed by flow cytometry as above. Open histograms indicate background staining with an isotype-matched control mAb. Numbers indicate the relative fluorescent intensity of each marker, obtained by dividing the mean fluorescent intensity of each marker by that of the isotype control. aMC, activated mast cells; HRA, histamine receptor antagonists; IND: indomethacin. These results are representative of three independent experiments.

production by DCs, depending on donors. Remarkably, IL-12p70 production by DCs was potently suppressed by activated MCs irrespective of the type of DC stimulation, indicating that activated MCs suppress IL-12p70 production whether DCs are activated by a pathogen-derived signal in the peripheral tissues or by a T cell-derived signal during interaction with T cells. Separation of DCs and MCs only slightly diminished the suppression, indicating that soluble factors play a major role. Histamine receptor antagonists only partially reversed the suppression, and addition of histamine at 10^{-5} M, the concentration comparable to that produced by activated MCs in this system, was not sufficient to reproduce the suppressive effect of activated MCs, indicating that although histamine plays an important role, combinatorial effects of other MC-derived soluble factors are also present. Although PGD_2 has been reported to suppress IL-12p70 production by MoDCs (21, 22), indomethacin did not have a considerable effect on the suppression, even when combined with histamine receptor antagonists, indicating that PGD_2 is not a critical factor for the suppression in this culture system. Thus, activated MCs potently suppress IL-12p70 production by DCs mainly through histamine and other synergistic soluble factors except PGD_2. Cell contact appears to play only a minor role in this suppression.

DCs co-cultured with activated MCs in combination with other maturation-inducing factors induce T_h2-promoting DCs

The cytokine profile of $CD4^+$ T cells primed by mature DCs is profoundly affected by signals given to DCs during maturation (2). Thus, we primed allogeneic naive $CD4^+$ T cells with DCs that had been co-cultured with activated MCs in the presence or absence of other maturation-inducing factors: LPS or TNF-α/IL-1β. Some of MC-derived factors such as IL-4, histamine and OX40L have been shown to have direct effects on T cells (11, 44). To eliminate direct effects of activated MCs on T cell priming, we purified DCs from the DC–MC co-culture by a cell sorter before they were used to prime T cells. The purity of DCs was always >98% as assessed by the CD11c expression by flow cytometry. After 12–14 days of expansion, the T cells were re-stimulated with PMA/A23187 and their cytokine profiles were analyzed by intracellular cytokine staining for IFN-γ and IL-4 (Fig. 3A).

Immature DCs or DCs stimulated with LPS or TNF-α/IL-1β mainly induced IFN-γ single-producing T_h1 cells with a minor population of T cells exhibiting an IFN-γ/IL-4 double-positive pattern of uncommitted 'T_h0-like' T cells. Co-culturing DCs with activated MCs in the absence of other maturation-inducing factors did not affect the cytokine profile of T cells. Remarkably, however, when DCs were co-cultured with activated MCs in the presence of LPS or TNF-α/IL-1β, the frequency of IL-4 single-producing T_h2 cells considerably increased, while the frequency of both T_h1 and T_h0-like cells decreased. The degree of the T_h2-polarizing effect was correlated with the DC:MC ratio, while this effect was still observed at the DC:MC ratio of 32:1 (Fig. 3B). Moreover, the T_h2-polarizing effect was observed even when DCs were co-cultured with activated MCs in the presence of LPS/IFN-γ, a combination that strongly

Table 1. Effects of MC-derived factors on IL-12p70 production by DCs[a]

		Experiment 1	Experiment 2	Experiment 3
Unstimulated		<7.8	<15.6	<15.6
LPS/IFN-γ	None	12067 ± 34	393 ± 5	3403 ± 27
	Histamine	2810 ± 66	138 ± 5	1103 ± 16
	aMC (contact)	816 ± 14	<15.6	187 ± 3
	aMC (transwell)	1320 ± 53	<15.6	542 ± 15
	aMC (contact) + HRA	4320 ± 29	301 ± 2	375 ± 1
	aMC (contact) + IND	1210 ± 41	<15.6	131 ± 3
	aMC (contact) + HRA/IND	4490 ± 226	348 ± 28	ND
LPS	None	313 ± 1	ND	ND
	Histamine	53 ± 1	ND	ND
	aMC (contact)	<7.8	ND	ND
	aMC (transwell)	36 ± 1	ND	ND
	aMC (contact) + HRA	96 ± 1	ND	ND
	aMC (contact) + IND	<7.8	ND	ND
	aMC (contact) + HRA/IND	84 ± 1	ND	ND
		Experiment 4	Experiment 5	Experiment 6
Unstimulated		<7.8	<7.8	<7.8
CD40L/IFN-γ	None	55914 ± 3150	61335 ± 1438	63327 ± 2879
	aMC (contact)	18793 ± 833	24169 ± 250	15420 ± 411
CD40L	None	21537 ± 627	10047 ± 120	16863 ± 725
	aMC (contact)	6415 ± 260	5172 ± 116	5157 ± 42

[a]Immature DCs (5×10^5 cells ml^{-1}) were cultured without stimulation, or stimulated with LPS or CD40L in the presence or absence of IFN-γ for 24 h. Histamine, activated MCs (aMCs), histamine receptor antagonists (HRA) and/or indomethacin (IND) were also added as indicated. The concentrations of IL-12p70 (pg ml^{-1}) in culture supernatants were measured by ELISA. Results shown are means ± SD of duplicate ELISA values. ND: not done.

induces T_h1-promoting DCs (42; Fig. 3C). Thus, IgE-activated MCs, when combined with additional DC maturation factors, induce DCs that diminish T_h1 and promote T_h2 differentiation.

Activated MCs induce T_h2-promoting DCs by combinatorial effects of different factors

We then investigated relative contributions of each MC-derived factor to the induction of T_h2-promoting DCs (Fig. 3C). DCs stimulated with LPS/IFN-γ induced IFN-γ single-producing T_h1 cells with a minor population of IFN-γ/IL-4 double-producing T cells, as observed with DCs stimulated with LPS or TNF-α/IL-1β. DCs co-cultured with activated MCs in the presence of LPS/IFN-γ increased IL-4 single-producing T_h2 cells and decreased T_h1 and T_h0-like cells. Both addition of histamine receptor antagonists and separation of DCs and MCs considerably reduced the T_h2 polarization of DCs, indicating that both histamine and cell contact contribute to the induction of T_h2-promoting DCs. Importantly, addition of histamine at 10^{-5} M only slightly antagonized the T_h1 induction by DCs stimulated with LPS/IFN-γ. Indomethacin did not have any considerable effect on T cell polarization in accordance with the absence of its effect on activated MC-induced suppression of IL-12p70 production by DCs (data not shown).

We also examined cytokine production by T cells by ELISA (Fig. 3D). $CD4^+$ T cells primed by LPS/IFN-γ-stimulated DCs produced a high level of IFN-γ and low or undetectable levels of T_h2 cytokines (IL-4, IL-5 and IL-13) as well as IL-10. Addition of histamine alone slightly suppressed the T_h1 induction, as shown by a decrease in IFN-γ production and slight increases in IL-5, IL-10 and IL-13 production. Activated MCs decreased IFN-γ production more potently than histamine, and remarkably increased the production of T_h2 cytokines and IL-10 far more than histamine did. Histamine receptor antagonists or separation of DCs and MCs considerably suppressed the production of T_h2 cytokines and IL-10, whereas did not significantly increase the IFN-γ production.

The intracellular staining data (Fig. 3A and C) indicate that a considerable number of naive T cells differentiated toward IFN-γ/IL-4 double-producing T_h0-like cells in the absence of MCs. Consequently, the overall frequency of IL-4-producing T cells (i.e. T_h2 cells plus T_h0-like cells) does not change much irrespective of the presence or absence of MCs. However, the ELISA data (Fig. 3D) suggest that IL-4 single-producing T_h2 cells induced by MC-stimulated DCs are qualitatively distinguished from IFN-γ/IL-4 double-producing T_h0-like cells induced without MCs, because the former T cells appear to produce large amounts of other T_h2 cytokines (IL-5 and IL-13) and IL-10, whereas the latter T cells produce little amounts of these cytokines (Fig. 3D). These data indicate that the $CD4^+$ T cells induced by MC-stimulated DCs appear to be truly T_h2-polarized cells, and thus such DCs have T_h2-promoting activity as well as T_h1-suppressing activity.

Collectively, activated MCs, even in the presence of T_h1-promoting stimuli (LPS/IFN-γ), induce DCs that suppress T_h1 and promote T_h2 differentiation by combinatorial effects of different factors, including histamine, other soluble factors except PGD_2 and cell contact-dependent factors.

Close anatomical associations between DCs and MCs in atopic dermatitis

Finally, to obtain insights into the DC–MC interactions *in vivo*, we examined the anatomical relationship between DCs and MCs in inflammatory skin lesions. We visualized DCs and MCs in lesional skins of chronic atopic dermatitis by

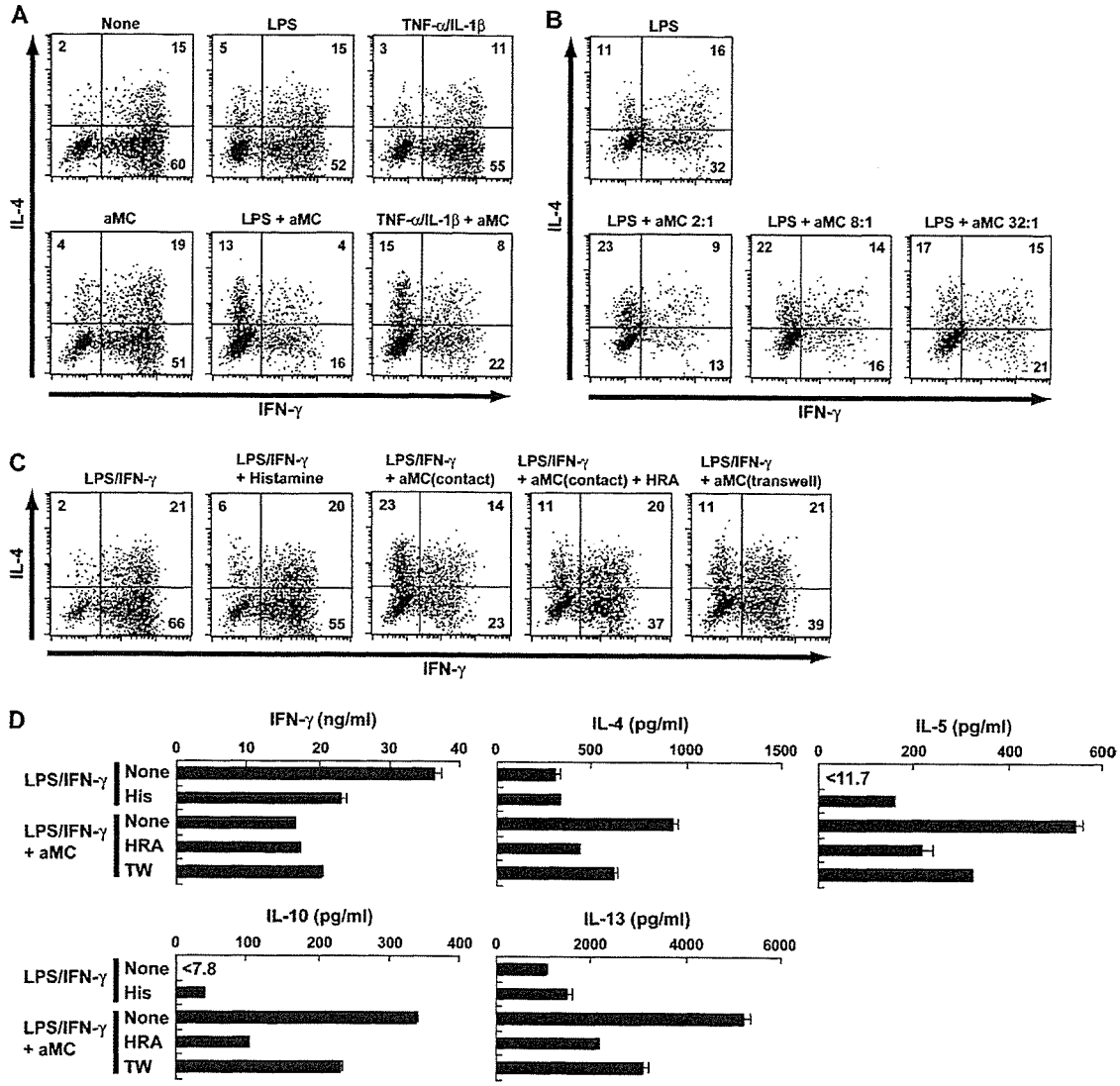

Fig. 3. DCs were polarized toward T_h2 by co-culture with activated MCs. (A) Immature DCs were cultured with or without activated MCs in the presence or absence of LPS or TNF-α/IL-1β. After 24 h, $CD11c^{birght}$ cells were sorted as DCs by a cell sorter, and were used to prime cord blood allogeneic naive $CD4^+$ T cells. After 12- to 14-day expansion, T cells were re-stimulated with PMA/A23187, and were analyzed by intracellular cytokine staining for IFN-γ and IL-4. Percentages of cytokine-producing cells are indicated in each quadrant. (B) Immature DCs were cultured with activated MCs at different DC:MC ratios in the presence of LPS for 24 h. The cytokine profile of adult allogeneic naive $CD4^+$ T cells primed by the DCs was analyzed as in (A). (C) Immature DCs were stimulated with LPS/IFN-γ in the presence or absence of either histamine or activated MCs for 24 h. In some co-culture, a mixture of histamine receptor antagonists was added, or DCs and MCs were separated by a porous membrane in transwell plates. The cytokine profiles of cord blood allogeneic naive $CD4^+$ T cells primed by the DCs were analyzed as in (A). (D) Cord blood allogeneic naive $CD4^+$ T cells were primed with the DCs as in (C), and were re-stimulated with PMA/A23187 at 1×10^6 cells ml^{-1} for 24 h. The concentrations of cytokines in supernatants were measured by ELISA. Error bars indicate standard deviation of duplicate measurements. aMC, activated MCs; His, Histamine; HRA, histamine receptor antagonists; TW, transwell. The results in (A–D) are representatives of three independent experiments.

immunohistochemical staining using anti-fascin mAb for DCs and anti-tryptase mAb for MCs (Fig. 4). Anti-fascin mAb has been reported to react with DCs, endothelial cells and some neuronal cells in central nervous system (45–47). In all of four patients examined, both fascin-positive cells with DC morphology and tryptase-positive cells were detected. Fascin-positive cells were present in the superficial layer of dermis, forming aggregates (Fig. 4A, arrows). Tryptase-positive cells were scattered throughout the dermis, and some of them surrounded the aggregates of fascin-positive cells with a few intermingled with fascin-positive cells (Fig. 4B and C). The anatomical proximity of the two cell types was observed in all patients, suggesting functional associations between DCs and MCs in inflammatory skin lesions.

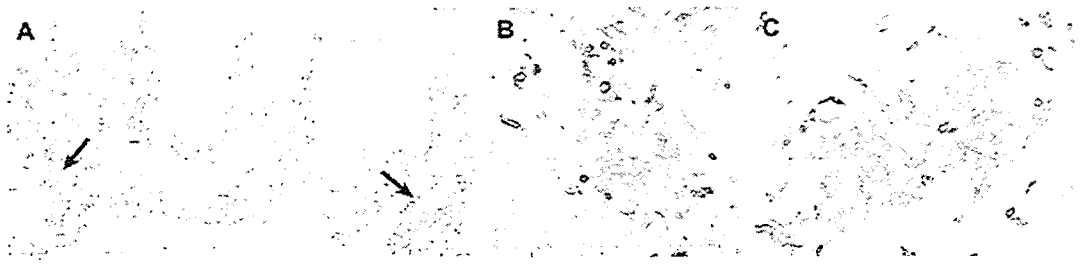

Fig. 4. Close anatomical associations between DCs and MCs in atopic dermatitis. A biopsied specimen of lesional skin of atopic dermatitis was immunohistochemically stained by anti-fascin mAb for DCs (red) and anti-tryptase mAb for MCs (brown). (A) Arrows indicate aggregates of fascin-positive cells in the superficial layer of the dermis. Original magnification: ×100. (B and C) Magnified views of the aggregates of fascin-positive cells shown in (A). Note that tryptase-positive cells surround the aggregates, and some of them are intermingled with fascin-positive cells. Original magnification: ×400. These results are representative of four different patients.

Discussion

Co-localization of DCs and MCs in sub-epithelial areas as sentinels for invading antigens, and immediate production of a variety of inflammatory mediators by activated MCs, suggest that MCs may influence the type of adaptive T cell immune responses through modulating the function of maturing DCs in inflamed tissues. However, no studies have directly addressed this question by co-culturing the two cell types *in vitro*. In this study, we utilized *in vitro* cultured human MCs, and examined the net effect of activated MCs as a whole on DCs. We found that (i) IgE-activated MCs induce DC maturation, as shown by the up-regulation of several surface molecules (Fig. 1), and potently suppress IL-12p70 production by DCs (Table 1), (ii) activated MCs alone do not have the ability to polarize DCs, but when combined with other DC maturation-inducing factors, such as LPS, TNF-α/IL-1β (pro-inflammatory cytokines) or even LPS/IFN-γ (potent IL-12-inducing factors), activated MCs induce DCs that suppress T_h1 differentiation and promote T_h2 differentiation (Fig. 3A–D) and (iii) histamine is an important mediator of these effects as reported (16–20), but a combinatorial effect of different MC-derived factors, including other soluble and cell contact-dependent factors, is required for the optimal induction of T_h2-polarizing DCs (Fig. 3C and D).

There have been several MC-derived molecules reported to affect DC functions. First, histamine has been shown to up-regulate CD86 on DCs, suppress IL-12p70 production by DCs and polarize DCs toward a T_h2-inducing type (16–20). In line with these reports, up-regulation of CD86 appeared to be almost totally dependent on histamine (Fig. 2). However, the addition of histamine together with LPS/IFN-γ or LPS only partially suppressed IL-12p70 production, compared with the stronger suppressive effect of activated MCs (Table 1). In addition, histamine receptor antagonists only partially reversed the suppression of IL-12p70 production by activated MCs. Accordingly, the T_h2-promoting effect of histamine and the T_h2-attenuating effect of histamine receptor antagonists in the DC–MC co-culture were also partial (Fig. 3C and D). These data indicate that histamine alone is not sufficient to reproduce the IL-12-suppressing and T_h2-promoting effects of activated MCs.

PGD_2 is another mediator synthesized by activated MCs (4–6). Stimulation of MoDCs with PGD_2 has been shown to diminish IL-12p70 production and favors a T_h2 response (21, 22). In our study, however, inhibition of PGD_2 synthesis by indomethacin (a cyclo-oxygenase inhibitor) did not influence the MC-induced suppression of IL-12p70 production (Table 1) and T_h2 polarization of DCs (data not shown), even when it was combined with histamine receptor antagonists. Thus, PGD_2 is not likely to be involved in T_h2 induction by MCs in our system.

As other potential MC-derived soluble factors inducing T_h2-promoting DCs, we examined two recently reported cytokines, IL-25 and TSLP. It has been reported that IL-25 is produced by a murine *in vitro* cultured MCs (48), and is implicated in induction of T_h2 responses (49, 50). However, we could not detect IL-25 mRNA expressions in the DC–MC co-culture at several time points within 24 h (data not shown). Human *in vitro* cultured MCs have been shown to express TSLP mRNA, and TSLP induces maturation and T_h2 polarization of human myeloid $CD11c^+$ DCs (23, 25). However, stimulation of MoDCs with TSLP did not affect their phenotypes, indicating that they do not express a receptor for TSLP, and moreover, addition of anti-TSLP-blocking antibody to the DC–MC co-culture did not diminish the induction of T_h2 responses by the DCs (data not shown). Thus, it is unlikely that IL-25 or TSLP is involved in the induction of T_h2-promoting DCs in our co-culture system.

Due to localization of DCs and MCs in anatomical proximity and high-level expressions of DC-attracting chemokines by IgE-activated MCs (12, 13), MCs may well have chances to directly contact with DCs in inflamed tissues. When DCs and MCs were co-cultured in direct contact, suppression of IL-12p70 production and T_h2 polarization of DCs were maximal, whereas separation of DCs and MCs by a porous membrane diminished these effects (Table 1 and Fig. 3C and D). These data suggest the presence of membrane-associated molecules on the surface of activated MCs that exert these effects in cooperation with MC-derived soluble factors.

Interestingly, DCs exhibited T_h1-suppressing as well as T_h2-promoting capacities only when DCs were co-cultured with activated MCs in the presence of other exogenous pro-inflammatory factors (Fig. 3A), including strong T_h1-inducing factors (LPS/IFN-γ) (Fig. 3C). It has been shown that helminth antigens, which induce T_h2 responses, more profoundly altered gene expressions in DCs when mixed with LPS than used alone (51). This and our observations suggest that cooperation of T_h2-inducing factors with pro-inflammatory, or even

T_h1-inducing, factors results in a full-blown T_h2 response. This is consistent with the observations in mice where LPS can promote T_h2 responses (52, 53), and may explain exacerbation of allergic symptoms by superimposed bacterial infections (54). These findings may also give a warning to an anti-allergy vaccine strategy that attempts to treat allergies by deviating the immune response toward T_h1 (55); simple application of T_h1-inducing factors may not alleviate a T_h2 response, but may rather exacerbate it.

Whereas IL-12 plays a dominant role in T_h1 development, the absence of IL-12 does not appear to be sufficient for T_h2 development (25, 56). Using different experimental systems, several molecules inducing naive $CD4^+$ T cells to differentiate into a T_h2 type have been reported to be expressed by DCs (57). OX40L (25, 58–62) is one of the candidates of such T_h2-inducing molecules. Moreover, Amsen *et al.* (63) demonstrated in a murine system that different Notch ligands on antigen-presenting cells instruct naive T cells to differentiate into different effector T cells; Delta promotes T_h1 responses, while Jagged promotes T_h2 responses, suggesting that DCs polarized by activated MCs may express high levels of Jagged. However, OX40L (Supplementary Fig. 1, available at *International Immunology* Online) was not detected on the T_h2-inducing, LPS/IFN-γ-stimulated DCs co-cultured with activated MCs by flow cytometry. In addition, there was no correlation between expression levels of Notch ligands (Jagged-1 and Jagged-2) measured by real-time reverse transcription–PCR and T_h2-inducing capacity of DCs stimulated with different stimuli (Supplementary Fig. 2, available at *International Immunology* Online). These data indicate that neither OX40L nor Notch ligands are responsible for the T_h2 polarization in our system.

de Jong *et al.* (64) demonstrated that T_h1- or T_h2-promoting DCs express diverse T_h-polarizing signals according to types of microbial stimuli, and some T_h2-promoting DCs exert its function via an OX40L-dependent mechanism, while others via an OX40L-independent, unknown mechanisms. Thus, T_h2-inducing molecules on DCs may be diverse, depending on types of stimuli and DCs. It is possible that an unspecified T_h2-inducing molecule, other than OX40L and Notch ligands, is expressed on T_h2-promoting DCs co-cultured with activated MCs.

Finally, we examined anatomical relationships between DCs and MCs in inflammatory skin lesions. We chose biopsied specimens from patients with chronic atopic dermatitis, because we thought that DC–MC interactions would be most prominently visualized in T_h2-type inflammatory lesions. In all specimens, both DCs and MCs were located in proximity in the dermis (Fig. 4). Intriguingly, MCs surrounded and entered the aggregates of DCs, suggesting production of MC-attracting chemokine by the DC aggregates, such as CCL5 and CCL8 (14) that can interact with CCR3 on MCs (15). These histological findings, together with the *in vitro* data that a relatively few MCs can influence T cell polarization induced by DCs (Fig. 3B), suggest that interactions of MCs with DCs are physiologically relevant *in vivo*, and that MCs may affect DC function through soluble and also possibly membrane-associated factors in the dermis of atopic lesions.

An IgE antibody is an end product of a T_h2 response, and thus, IgE-mediated activation of MCs occurs as a consequence of a previous T_h2 response. Our observation that IgE-activated MCs polarizes DCs toward T_h2 implies that a DC–MC interaction may constitute a positive feedback loop to maintain or augment T_h2 responses. Recently, Kalinski and Moser (65) proposed a 'success-driven consensual immunity' model, wherein outcomes of a 'successful' adaptive immune response induce polarization of DCs toward the same type of responses, constituting a positive feedback loop that stabilizes the type of adaptive immune responses. Our study is consistent with this model, demonstrating a successful T_h2 response where IgE-activated MCs deliver signals to DCs results in the stabilization of the T_h2 response. This mechanism may favor a defense against extracellular parasites by augmenting a T_h2 response, but may also lead to persistence of unwanted T_h2 responses, such as allergies. Thus, disruption of the DC–MC interaction may constitute an effective strategy to treat ongoing allergic reactions.

Supplementary data

Supplementary figures are available at *International Immunology* Online.

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Abbreviations

CD40L	CD40 ligand
COE	Center of Excellence
DC	dendritic cell
GM-CSF	granulocyte–macrophage colony-stimulating factor
MC	mast cell
MoDC	monocyte-derived dendritic cell
OX40L	OX40 ligand
PGD2	prostaglandin D_2
PMA	phorbol myristate acetate
SCF	stem cell factor
TNF	tumor necrosis factor
TSLP	thymic stromal lymphopoietin

References

1 Banchereau, J., Briere, F., Caux, C. *et al.* 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18:767.
2 Kapsenberg, M. L. 2003. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat. Rev. Immunol.* 3:984.
3 Kitamura, Y. 1989. Heterogeneity of mast cells and phenotypic change between subpopulations. *Annu. Rev. Immunol.* 7:59.
4 Metcalfe, D. D., Baram, D. and Mekori, Y. A. 1997. Mast cells. *Physiol. Rev.* 77:1033.
5 Marshall, J. S. 2004. Mast-cell responses to pathogens. *Nat. Rev. Immunol.* 4:787.
6 Galli, S. J., Nakae, S. and Tsai, M. 2005. Mast cells in the development of adaptive immune responses. *Nat. Immunol.* 6:135.
7 McLachlan, J. B., Hart, J. P., Pizzo, S. V. *et al.* 2003. Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection. *Nat. Immunol.* 4:1199.
8 Secor, V. H., Secor, W. E., Gutekunst, C. A. and Brown, M. A. 2000. Mast cells are essential for early onset and severe disease

in a murine model of multiple sclerosis. *J. Exp. Med.* 191:813.
9 Tanzola, M. B., Robbie-Ryan, M., Gutekunst, C. A. and Brown, M. A. 2003. Mast cells exert effects outside the central nervous system to influence experimental allergic encephalomyelitis disease course. *J. Immunol.* 171:4385.
10 Wang, H. W., Tedla, N., Lloyd, A. R., Wakefield, D. and McNeil, P. H. 1998. Mast cell activation and migration to lymph nodes during induction of an immune response in mice. *J. Clin. Invest.* 102:1617.
11 Kashiwakura, J., Yokoi, H., Saito, H. and Okayama, Y. 2004. T cell proliferation by direct cross-talk between OX40 ligand on human mast cells and OX40 on human T cells: comparison of gene expression profiles between human tonsillar and lung-cultured mast cells. *J. Immunol.* 173:5247.
12 Sallusto, F., Schaerli, P., Loetscher, P. *et al.* 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol.* 28:2760.
13 Nakajima, T., Inagaki, N., Tanaka, H. *et al.* 2002. Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcepsilon receptor I cross-linking: an interspecies comparison. *Blood* 100:3861.
14 Sallusto, F., Palermo, B., Lenig, D. *et al.* 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol.* 29:1617.
15 Ochi, H., De Jesus, N. H., Hsieh, F. H., Austen, K. F. and Boyce, J. A. 2000. IL-4 and -5 prime human mast cells for different profiles of IgE-dependent cytokine production. *Proc. Natl Acad. Sci. USA* 97:10509.
16 Caron, G., Delneste, Y., Roelandts, E. *et al.* 2001. Histamine induces CD86 expression and chemokine production by human immature dendritic cells. *J. Immunol.* 166:6000.
17 Caron, G., Delneste, Y., Roelandts, E. *et al.* 2001. Histamine polarizes human dendritic cells into Th2 cell-promoting effector dendritic cells. *J. Immunol.* 167:3682.
18 Mazzoni, A., Young, H. A., Spitzer, J. H., Visintin, A. and Segal, D. M. 2001. Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. *J. Clin. Invest.* 108:1865.
19 Gutzmer, R., Langer, K., Lisewski, M. *et al.* 2002. Expression and function of histamine receptors 1 and 2 on human monocyte-derived dendritic cells. *J. Allergy Clin. Immunol.* 109:524.
20 Idzko, M., la Sala, A., Ferrari, D. *et al.* 2002. Expression and function of histamine receptors in human monocyte-derived dendritic cells. *J. Allergy Clin. Immunol.* 109:839.
21 Gosset, P., Bureau, F., Angeli, V. *et al.* 2003. Prostaglandin D2 affects the maturation of human monocyte-derived dendritic cells: consequence on the polarization of naive Th cells. *J. Immunol.* 170:4943.
22 Gosset, P., Pichavant, M., Faveeuw, C. *et al.* 2005. Prostaglandin D2 affects the differentiation and functions of human dendritic cells: impact on the T cell response. *Eur. J. Immunol.* 35:1491.
23 Soumelis, V., Reche, P. A., Kanzler, H. *et al.* 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol.* 3:673.
24 Gilliet, M., Soumelis, V., Watanabe, N. *et al.* 2003. Human dendritic cells activated by TSLP and CD40L induce proallergic cytotoxic T cells. *J. Exp. Med.* 197:1059.
25 Ito, T., Wang, Y. H., Duramad, O. *et al.* 2005. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J. Exp. Med.* 202:1213.
26 Skokos, D., Botros, H. G., Demeure, C. *et al.* 2003. Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses *in vivo*. *J. Immunol.* 170:3037.
27 Nakahata, T., Tsuji, K., Tanaka, R. *et al.* 1995. Synergy of stem cell factor and other cytokines in mast cell development. In Kitamura, Y., Yamamoto, S. and Galli, S. J., eds., *Biological and Molecular Aspect of Mast Cell and Basophil Differentiation and Function*, p. 13. Raven Press, New York.
28 Yanagida, M., Fukamachi, H., Ohgami, K. *et al.* 1995. Effects of T-helper 2-type cytokines, interleukin-3 (IL-3), IL-4, IL-5, and IL-6 on the survival of cultured human mast cells. *Blood* 86:3705.
29 Toru, H., Ra, C., Nonoyama, S., Suzuki, K., Yata, J. and Nakahata, T. 1996. Induction of the high-affinity IgE receptor (Fc epsilon RI) on human mast cells by IL-4. *Int. Immunol.* 8:1367.
30 Toru, H., Eguchi, M., Matsumoto, R., Yanagida, M., Yata, J. and Nakahata, T. 1998. Interleukin-4 promotes the development of tryptase and chymase double-positive human mast cells accompanied by cell maturation. *Blood* 91:187.
31 Yamaguchi, M., Sayama, K., Yano, K. *et al.* 1999. IgE enhances Fc epsilon receptor I expression and IgE-dependent release of histamine and lipid mediators from human umbilical cord blood-derived mast cells: synergistic effect of IL-4 and IgE on human mast cell Fc epsilon receptor I expression and mediator release. *J. Immunol.* 162:5455.
32 Hsieh, F. H., Lam, B. K., Penrose, J. F., Austen, K. F. and Boyce, J. A. 2001. T helper cell type 2 cytokines coordinately regulate immunoglobulin E-dependent cysteinyl leukotriene production by human cord blood-derived mast cells: profound induction of leukotriene C(4) synthase expression by interleukin 4. *J. Exp. Med.* 193:123.
33 Obata, T., Nagakura, T., Kanbe, M., Masaki, T., Maekawa, K. and Yamashita, K. 1996. IgE-anti-IgE-induced prostaglandin D2 release from cultured human mast cells. *Biochem. Biophys. Res. Commun.* 225:1015.
34 Hough, L. B. 2001. Genomics meets histamine receptors: new subtypes, new receptors. *Mol. Pharmacol.* 59:415.
35 Rissoan, M. C., Soumelis, V., Kadowaki, N. *et al.* 1999. Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 283:1183.
36 Imura, A., Hori, T., Imada, K. *et al.* 1996. The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. *J. Exp. Med.* 183:2185.
37 Toda, Y., Kono, K., Abiru, H. *et al.* 1999. Application of tyramide signal amplification system to immunohistochemistry: a potent method to localize antigens that are not detectable by ordinary method. *Pathol. Int.* 49:479.
38 Bischoff, S. C. and Dahinden, C. A. 1992. c-kit ligand: a unique potentiator of mediator release by human lung mast cells. *J. Exp. Med.* 175:237.
39 Bischoff, S. C., Sellge, G., Lorentz, A., Sebald, W., Raab, R. and Manns, M. P. 1999. IL-4 enhances proliferation and mediator release in mature human mast cells. *Proc. Natl Acad. Sci. USA* 96:8080.
40 Gutzmer, R., Diestel, C., Mommert, S. *et al.* 2005. Histamine H4 receptor stimulation suppresses IL-12p70 production and mediates chemotaxis in human monocyte-derived dendritic cells. *J. Immunol.* 174:5224.
41 Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3:133.
42 Hilkens, C. M., Kalinski, P., de Boer, M. and Kapsenberg, M. L. 1997. Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. *Blood* 90:1920.
43 Snijders, A., Kalinski, P., Hilkens, C. M. and Kapsenberg, M. L. 1998. High-level IL-12 production by human dendritic cells requires two signals. *Int. Immunol.* 10:1593.
44 Jutel, M., Watanabe, T., Klunker, S. *et al.* 2001. Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. *Nature* 413:420.
45 Mosialos, G., Yamashiro, S., Baughman, R. W. *et al.* 1994. Epstein-Barr virus infection induces expression in B lymphocytes of a novel gene encoding an evolutionarily conserved 55-kilodalton actin-bundling protein. *J. Virol.* 68:7320.
46 Mosialos, G., Birkenbach, M., Ayehunie, S. *et al.* 1996. Circulating human dendritic cells differentially express high levels of a 55-kd actin-bundling protein. *Am. J. Pathol.* 148:593.
47 Pinkus, G. S., Pinkus, J. L., Langhoff, E. *et al.* 1997. Fascin, a sensitive new marker for Reed-Sternberg cells of Hodgkin's disease. Evidence for a dendritic or B cell derivation? *Am. J. Pathol.* 150:543.
48 Ikeda, K., Nakajima, H., Suzuki, K. *et al.* 2003. Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. *Blood* 101:3594.

49 Fort, M. M., Cheung, J., Yen, D. *et al.* 2001. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity* 15:985.

50 Kim, M. R., Manoukian, R., Yeh, R. *et al.* 2002. Transgenic overexpression of human IL-17E results in eosinophilia, B-lymphocyte hyperplasia, and altered antibody production. *Blood* 100:2330.

51 Kane, C. M., Cervi, L., Sun, J. *et al.* 2004. Helminth antigens modulate TLR-initiated dendritic cell activation. *J. Immunol.* 173:7454.

52 Kaisho, T., Hoshino, K., Iwabe, T., Takeuchi, O., Yasui, T. and Akira, S. 2002. Endotoxin can induce MyD88-deficient dendritic cells to support T(h)2 cell differentiation. *Int. Immunol.* 14:695.

53 Piggott, D. A., Eisenbarth, S. C., Xu, L. *et al.* 2005. MyD88-dependent induction of allergic Th2 responses to intranasal antigen. *J. Clin. Invest.* 115:459.

54 Kamradt, T., Goggel, R. and Erb, K. J. 2005. Induction, exacerbation and inhibition of allergic and autoimmune diseases by infection. *Trends Immunol.* 26:260.

55 Wohlleben, G. and Erb, K. J. 2001. Atopic disorders: a vaccine around the corner? *Trends Immunol.* 22:618.

56 Jankovic, D., Kullberg, M. C., Hieny, S., Caspar, P., Collazo, C. M. and Sher, A. 2002. In the absence of IL-12, CD4(+) T cell responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an IL-10(−/−) setting. *Immunity* 16:429.

57 Eisenbarth, S. C., Piggott, D. A. and Bottomly, K. 2003. The master regulators of allergic inflammation: dendritic cells in Th2 sensitization. *Curr. Opin. Immunol.* 15:620.

58 Ohshima, Y., Yang, L. P., Uchiyama, T. *et al.* 1998. OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. *Blood* 92:3338.

59 Chen, A. I., McAdam, A. J., Buhlmann, J. E. *et al.* 1999. Ox40-ligand has a critical costimulatory role in dendritic cell: T cell interactions. *Immunity* 11:689.

60 Murata, K., Ishii, N., Takano, H. *et al.* 2000. Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand. *J. Exp. Med.* 191:365.

61 Tanaka, H., Demeure, C. E., Rubio, M., Delespesse, G. and Sarfati, M. 2000. Human monocyte-derived dendritic cells induce naive T cell differentiation into T helper cell type 2 (Th2) or Th1/Th2 effectors. Role of stimulator/responder ratio. *J. Exp. Med.* 192:405.

62 Ito, T., Amakawa, R., Inaba, M. *et al.* 2004. Plasmacytoid dendritic cells regulate Th cell responses through OX40 ligand and type I IFNs. *J. Immunol.* 172:4253.

63 Amsen, D., Blander, J. M., Lee, G. R., Tanigaki, K., Honjo, T. and Flavell, R. A. 2004. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 117:515.

64 de Jong, E. C., Vieira, P. L., Kalinski, P. *et al.* 2002. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells *in vitro* with diverse Th cell-polarizing signals. *J. Immunol.* 168:1704.

65 Kalinski, P. and Moser, M. 2005. Consensual immunity: success-driven development of T-helper-1 and T-helper-2 responses. *Nat. Rev. Immunol.* 5:251.

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Type I Interferons Attenuate T Cell Activating Functions of Human Mast Cells by Decreasing TNF- Production and OX40 Ligand Expression While Increasing IL-10 Production

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Recent studies have demonstrated that mast cells not only mediate inflammatory reactions in type I allergy but also play an important role in adaptive immunity. In the present study, we investigated the effects of interferon- , which shares the same receptor as IFN- , on human cord blood-derived mast cells. Mast cells produced TNF- , and IL-10, and expressed OX40 ligand upon activation by crosslinking of Fc RI. When treated with interferon- , TNF- production was decreased while IL-10 and TGF- productions were increased. Furthermore, flow cytometric analysis revealed that interferon- downregulated expression OX40 ligand on mast cells which is crucial for mast cell-T cell interaction. We confirmed that the viability of mast cells was not affected by interferon- treatment. Accordingly, interferon- -treated mast cells induced lower levels of $CD4^+$ T cell proliferation compared with those without interferon- treatment. These results suggest that type I interferons suppress T cell immune responses through their regulatory effects on mast cells.

KEY WORDS: Mast cells; $CD4^+$ T cells; Type I interferons; OX40 ligand; .

INTRODUCTION

Mast cells (MCs) are well known for their ability to mediate inflammatory reactions in pathological processes of allergic diseases (1). Upon recognition of specific antigens by IgE bound to Fc RI, MCs secrete various kinds of cytokines, chemokines, and chemical mediators, which trigger allergic reactions (2). This IgE-dependent activation of MCs has been emphasized as a major causative event of harmful type I hypersensitivity. However, MCs are originally engaged in the first line defense against bacterial as well as parasite infections. Recent evidence has indicated that in mice, MCs recruit and activate T cells in the draining lymph nodes during bacterial infection and thus positively regulate adaptive immunity (3). TNF- has been shown to play a pivotal role in this enforcement of T cell responses (3, 4). In addition to TNF- secretion, MCs can directly interact with T cells and even present antigens to T cells in the context of MHC class I and class II (5–7), resulting in enhanced T cell proliferation. It has been reported that human MCs and murine BMMCs express OX40 ligand (OX40L) upon activation and the OX40/OX40L system plays a crucial role in MCs-mediated T cell proliferation (8, 9).

In clinical settings, MCs are implicated in the pathogenesis of most allergic diseases in which Th2-type immune responses appear to be predominant such as bronchial asthma and atopic dermatitis (10). On the other hand, recent reports have revealed that MCs also play important roles in some Th1 dominant diseases such as inflammatory bowel diseases (11), multiple sclerosis (MS) (12), and experimental autoimmune encephalomyelitis (EAE), an animal model of MS. MS is a demyelinating disorder of the central nervous system that preferentially affects young adults. Although myelin-specific $CD4^+$ T cells are principal effector cells, other cell types are also likely to be involved in the pathogenesis of the disease. It is noted that MCs are required for the development and aggravation of EAE because MC-deficient W/W^v mice are less sensitive to EAE than wild-type mice and reconstitution of MCs by injection of bone marrow-derived MCs from wild-type mice restore the severity of EAE(13). In humans, MCs are found in the CNS plaques of MS patients (14) and

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the concentrations of histamine and tryptase were higher in MS patients compared with normal healthy individuals (15, 16), suggesting that MCs are also involved in MS.

Treatment of MS remains to be a challenging problem. Apart from several immunosuppressive drugs, type I IFNs have been shown to be effective in a proportion of MS patients (17), although the precise mechanism of their effects has not been fully elucidated. In a study using specimens from MS patients, IFN- has been reported to correct Th1/Th2 imbalance by increasing IL-4 production and lowering IFN- production (18). Since type I IFNs do not have such a direct effect on $CD4^+$ T cells, these reports suggest the presence of other target cells of type I IFNs that mediate alleviation of pathological T cell responses. Based on the roles of MCs in EAE and MS as discussed above, it is to be determined whether type I IFNs could modify MC-mediated T cell activation processes. As far as we know, effects of IFNs on MCs have not been studied in detail. In the present study, we addressed this question by using human cord blood-derived MCs and IFN- , a type I IFN. Herewith we report that IFN- did not affect the viability of MCs but decreased TNF- production and OX40 ligand expression while increased TGF- and IL-10 production. Thus, type I IFNs indirectly attenuate T cell responses through the regulatory effects on MCs. The possible involvement of MCs in the effectiveness of IFN- therapy for MS and other Th1 diseases is discussed.

MATERIALS AND METHODS

Antibodies and Cytokines

SCF and IL-6 were purchased from Wako (Tokyo, Japan) and IL-3 and IL-4 were from Peprotech (Rock Hill, NJ). Human myeloma IgE was from Chemicon International (Temecula, CA) and goat anti-human IgE from Biosource (Camarillo, CA). Phycoerythrin (PE)-conjugated anti-human OX40 ligand monoclonal antibody (mAb) (clone ik-1) and PE-conjugated mouse IgG1 was obtained from Becton-Dickinson (Franklin Lakes, NJ). Anti-OX40L blocking mAb (mouse IgG2a, clone ik-5) was generated in our laboratory (19). Control IgG2a (clone UPC10) was purchased from Sigma (St. Louis, MO).

Preparation of MCs

Human cord blood-derived MCs (CBMCs) were generated as described (20). In brief, mononuclear cells were isolated from cord blood of healthy neonates with the informed consent of the parents and subsequently cultured in AIM-V (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies, Rockville, MD), 100 U/ml penicillin, 100 g/ml streptomycin, 292 g/ml L-glutamine (penicillin–streptomycin–glutamine; Life Technologies), 100 ng/ml SCF and 50 ng/ml IL-6. Half medium change was performed once a week. Cells cultured for more than 8 weeks were used which consisted of 95% pure MCs measured as c-kit-positive cells by flow cytometry.

Activation of MCs

Naïve MCs were cultured with 5 ng/ml IL-3 and 10 ng/ml IL-4 for 5 days. Human IgE was added at 1 g/ml for the last 24 h to be presensitized. For the aggregation of Fc RI, presensitized MCs were collected, washed three times, and cultured with 2 g/ml goat anti-human IgE Ab in SCF-containing medium (without IL-6) in the presence or absence of IFN- for the indicated periods of time.

RT-PCR

Presensitized MCs were cultured with anti-IgE Ab and with or without IFN- (Biosource, Camarillo, CA) for 6 h. Cells were then collected and washed three times. Total RNA was isolated with RNeasy mini kit (QIAGEN, Hilden, Germany). 1 g of RNA was used in reverse transcription using ImProm-II ™ reverse transcription system (Promega, Madison, WI). The same amount of complementary DNA was used in the subsequent PCR. The following primers were used: -actin (sense 5 -TCAGCCTCTTCTCCTTCCTGATCG-3 ; antisense 5 -TGAAGAGGACCTGGGAGTAGATGA-3) (21), TNF- (5 -GTTCCTCAGCCTCTTCTCCT-3 ; antisense 5 -ATCTATCTGGGAGGGGTCT T-3) (22), IL-10 (sense 5 -ATGCCCCAAGCTGAGAAC CAAGACC CA-3 ; antisense 5 -AAGGGGCTGGGTCAGCTATCCCA-3) (23), and TGF- (sense 5 -CTACTAGGCCAAGGA GGTCAC-3 ; antisense 5 -TTGCTGAGGTATGGCCAG GAA-3) (24). PCR products were separated through 1.5% agarose gel, stained with ethidium bromide, and visualized with an UV transilluminator.

Measurement of Cytokine Production

Cytokine production was measured by ELISA according to the manufacture's protocol. Presensitzed MCs were treated with goat anti-human IgE Ab and IFN- for 6 h at 1.0 10^6/ml. Culture supernatants were then collected and stored at 80 C until they were subjected to analysis. Concentrations of TNF- , IL-10, and TGF- in the supernatants were measured by using the respective ELISA kits (Biosource).