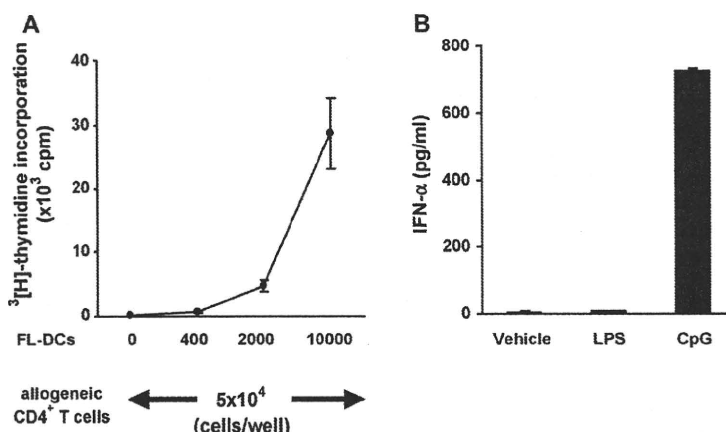

Fig. 2. FL-DCs from LSKs are functional. (A) Allogeneic CD4$^+$ T cells (5×10^4 cells) from BALB/c mice were co-cultured with graded numbers of irradiated (30Gy) FACS-sorted FL-DCs (CD11c$^+$ cells) from C57BL/6 mice for 4 days. Proliferation was measured by [^{3}H]-thymidine incorporation. Mean ± SD is shown. Experiments were repeated at least three times with similar results. (B) After 9 days in culture, FL-DCs were stimulated with CpG (1 μM), LPS (1 μg/ml), or left unstimulated in a total volume of 100 μl. After 24 h, the supernatants were harvested and assayed using ELISA.

differentiation is crucially regulated by STAT3/5 and in part associated with myeloid differentiation [3,44]. From these observations, we hypothesised that myeloid neoplasm-related gene abnormalities themselves might cause the quantitative imbalance found in *in vivo* leukemic DC subsets. Therefore, we determined whether myeloid neoplasm-related gene abnormalities affected FL-DC differentiation from LSKs. We selected FLT3-ITD, FLT3-TKD, CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα as representatives of class I mutations, and AML1/ETO, PML/RARα, CBFβ/MYH11, and AML1dC as representatives of class II mutations. We investigated how myeloid neoplasm-related gene abnormalities affected the absolute cell numbers of EGFP$^+$ cells (Fig. 3A). Compared with the mock population, all class I mutations except for FIP1L1/PDGFRα increased the number of EGFP$^+$ cells to various extents ($p < 0.01$). AML1/ETO and CBFβ/MYH11 yielded less EGFP$^+$ cells than mock population ($p < 0.05$). PML/RARα and AML1dC yielded comparable EGFP$^+$ cells to the mock population. We then focused on the proportion of whole FL-DCs (EGFP$^+$CD11c$^+$ cells in EGFP$^+$ cells). Compared to the mock population, all myeloid neoplasm-related gene abnormalities showed a significant decrease in the proportion of whole FL-DCs from LSKs ($p < 0.00001$) (Fig. 3B and C). Class II mutations uniformly displayed a mild decrease in the proportion of whole FL-DCs from LSKs (the proportion of EGFP$^+$CD11c$^+$ cells in EGFP$^+$ cells: 35.6–55.8%). By contrast, class I mutations exhibited variability (from mild to severe) in the decrease of the proportion of whole FL-DCs from LSKs (the proportion of EGFP$^+$CD11c$^+$ cells in EGFP$^+$ cells: 9.4–55.2%). From these data, we calculated the fold increase in whole FL-DC yields by multiplying the fold increase in EGFP$^+$ cells by the proportion of FL-DCs (the mean fold increase in EGFP$^+$ cells × the mean proportion of whole FL-DCs). FLT3-ITD and CA-N-Ras showed increased FL-DC yields than the mock population (Fig. 3D). The remaining class I mutations and all class II mutations exhibited decreased FL-DC yields. Lastly, we focused on the DC subsets, pDCs and cDCs. Compared with mock, CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα mutations displayed a severe decrease in the proportion of pDCs, with almost all EGFP$^+$CD11c$^+$ cells being cDCs, indicating a severe decrease in the pDC/cDC ratio (Fig. 3B and E). By contrast, FLT3-WT, FLT3-ITD, FLT3-TKD, and all class II mutations displayed comparable pDC/cDC ratios as the mock population. Taken together, class II mutations consistently yielded fewer FL-DCs from LSKs, and exhibited comparable pDC/cDC ratios with the control population. In contrast, class I mutations exhibited a variety of patterns regarding the whole FL-DC yields and their pDC/cDC ratios. FLT3-ITD and FLT3-TKD exhibited a comparable pDC/cDC ratio with the control, regardless of the difference in whole FL-DC yields. CA-N-Ras displayed a marked increase in the FL-DC yield and a severe decrease in the pDC/cDC ratio. c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα exhibited less whole FL-DC yields and a severe decrease in the pDC/cDC ratio.

3.4. CA-N-Ras-, c-Kit-TKD-, TEL/PDGFRβ-, and FIP1L1/PDGFRα-expressing FL-DCs from LSKs showed distinct differentiation patterns from FL-DCs derived from LSKs

Two distinct types of DCs exist, steady-state and inflammatory DCs, the equivalents of which can be induced *in vitro* by FL or GM-CSF/IL-4 and are termed FL-DCs and GM/IL-4-DCs, respectively [3,19]. GM/IL-4-DCs are considered to be monocyte-derived DCs and composed of only cDCs. By contrast, FL-DCs include both pDCs and cDCs. Moreover, precursors of steady state DCs and FL-DCs are distinct from monocytes [19,45], suggesting that FL-DC and GM/IL-4-DC differentiation are distinct pathways. We focused on the differentiation pattern of FL-DCs and GM/IL-4-DCs from LSKs (Fig. 4A). In the presence of FL, almost all cultured cells began to express CD11c and Mac-1$^{-/dim}$ during 4–7 days in culture, and then they began to differentiate into CD11c$^+$Mac-1$^-$ and CD11c$^+$Mac-1$^+$ cells. In the presence of GM-CSF and IL-4, almost all cultured cells initially expressed Mac-1 and subsequently expressed CD11c after 7 days in culture. Consistent with previous reports, GM/IL4-DCs lacked pDCs (B220$^+$CD11c$^+$ cells) throughout the culture period (Fig. 4A). We next investigated the effects of CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα on the differentiation pattern of FL-DCs from LSKs (Fig. 4B). FL-DC differentiation from LSKs expressing CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, or FIP1L1/PDGFRα was characterised by the initial expression of Mac-1 during 4–7 days in culture, followed by the delayed expression of CD11c on Mac-1$^+$ cells. Among these four mutations, CA-N-Ras induced a significant proportion of CD11c$^+$Mac-1$^+$ cells at 7 days in culture. FL-DCs from LSKs expressing CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, or FIP1L1/PDGFRα generated few pDCs throughout the culture period.

3.5. Active forms of STAT5 and MEK1 severely impaired pDC differentiation from LSKs

We next examined why class I, but not class II, mutations caused variable patterns of FL-DC differentiation from LSKs. Class I, but not class II, mutations constitutively activated various sig-

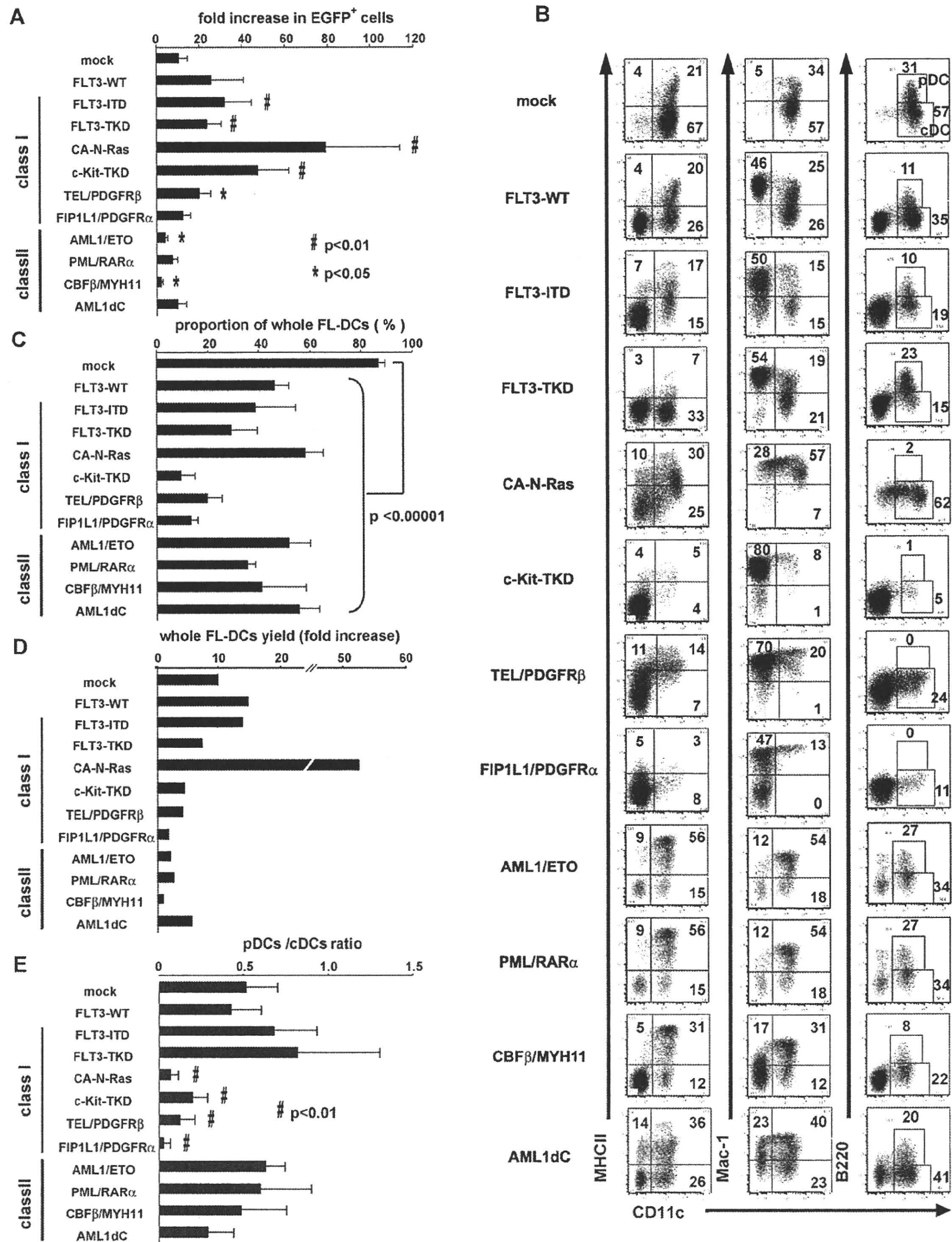

Fig. 3. FL-DC differentiation from LSKs is deregulated in a myeloid neoplasm-related gene abnormality-specific manner. During the pre-culture phase, cells were transduced with the indicated myeloid neoplasm-related gene abnormality. After 9 days in culture, cells were collected, and the absolute cell numbers and expressions of the indicated surface markers were analysed by gating on the EGFP+ cells by flow cytometry (A–C, and E). Numbers in the dot plots represent percentage of cells. The bars represent fold increase in EGFP+ cells (fold increase in absolute cell numbers of EGFP+ cells from 3 to 9 days in culture) (A), the proportion of FL-DCs (EGFP+CD11c+ cells in EGFP+ cells) (C), FL-DC yields (the mean fold increase in EGFP+ cells × the mean proportion of whole FL-DCs) (D), and pDCs (EGFP+CD11c+B220+ cells)/cDCs (EGFP+CD11c+B220− cells) ratio (E), respectively. Data are representative of at least three experiments with similar results. Mean ± SD is shown. * and # indicate $p < 0.05$ and $p < 0.01$ respectively.

Fig. 4. CA-N-Ras-, c-Kit-TKD-, TEL/PDGFRβ-, and FIP1L1/PDGFRα-expressing FL-DCs from LSKs showed distinct differentiation patterns. (A) After the indicated days in culture in the presence of FL or GM-CSF with IL-4, cells were collected and the expression of the indicated surface markers was analysed by flow cytometry. (B) During the pre-culture phase, cells were transduced with the indicated myeloid neoplasm-related gene abnormality. After the indicated days of culture in the presence of FL, cells were collected and the expression of the indicated surface markers was analysed on the gated EGFP+ cell population by flow cytometry. Numbers in the dot plots represent percentage of cells.

nal transduction pathways [43,46]. Therefore, we hypothesised that constitutively activated signals might generate the heterogeneous patterns of FL-DC differentiation from LSKs. We evaluated the influence of activated forms of signaling molecules on FL-DC differentiation from LSKs. We selected STAT3c, membrane-targeted p110, 1*6-STAT5A, and CA-MEK1 as a constitutively active form of STAT3, PI 3-kinase, STAT5, and MAP-kinase pathways, respectively. Compared with control cells, CA-STAT3 and CA-PI 3-kinase displayed a slight decrease in the proportion of whole FL-DCs from LSKs and was associated with a modest increase in the pDC/cDC ratio (Fig. 5). By contrast, CA-STAT5 and CA-MEK1 displayed a severe and mild decrease in the proportion of whole FL-DCs from LSKs, respectively. Both mutations resulted in a, severe decrease in the pDC/cDC ratio (Fig. 5).

3.6. Expression patterns of MHC II and costimulatory molecules on FL-DCs from LSKs are heterogeneous among class I mutations

We determined whether this heterogeneity in DC differentiation seen in class I mutations influenced the DC maturation state. DC maturation is associated with up-regulation of MHC II and costimulatory molecules [47]. We therefore screened the surface expression of MHC II and costimulatory molecules such as CD40, CD80, and CD86 on FL-DCs from LSKs bearing myeloid neoplasm-related gene abnormalities. Overall, expressions of MHC II, CD80, and CD86 on whole FL-DCs induced by class I mutations were higher than those in control cells with several exceptions such as MHC II expression on DCs induced by FLT3-TKD and FIP1L1/PDGFRα (Fig. 6A and B). None of the class I mutations induced expression

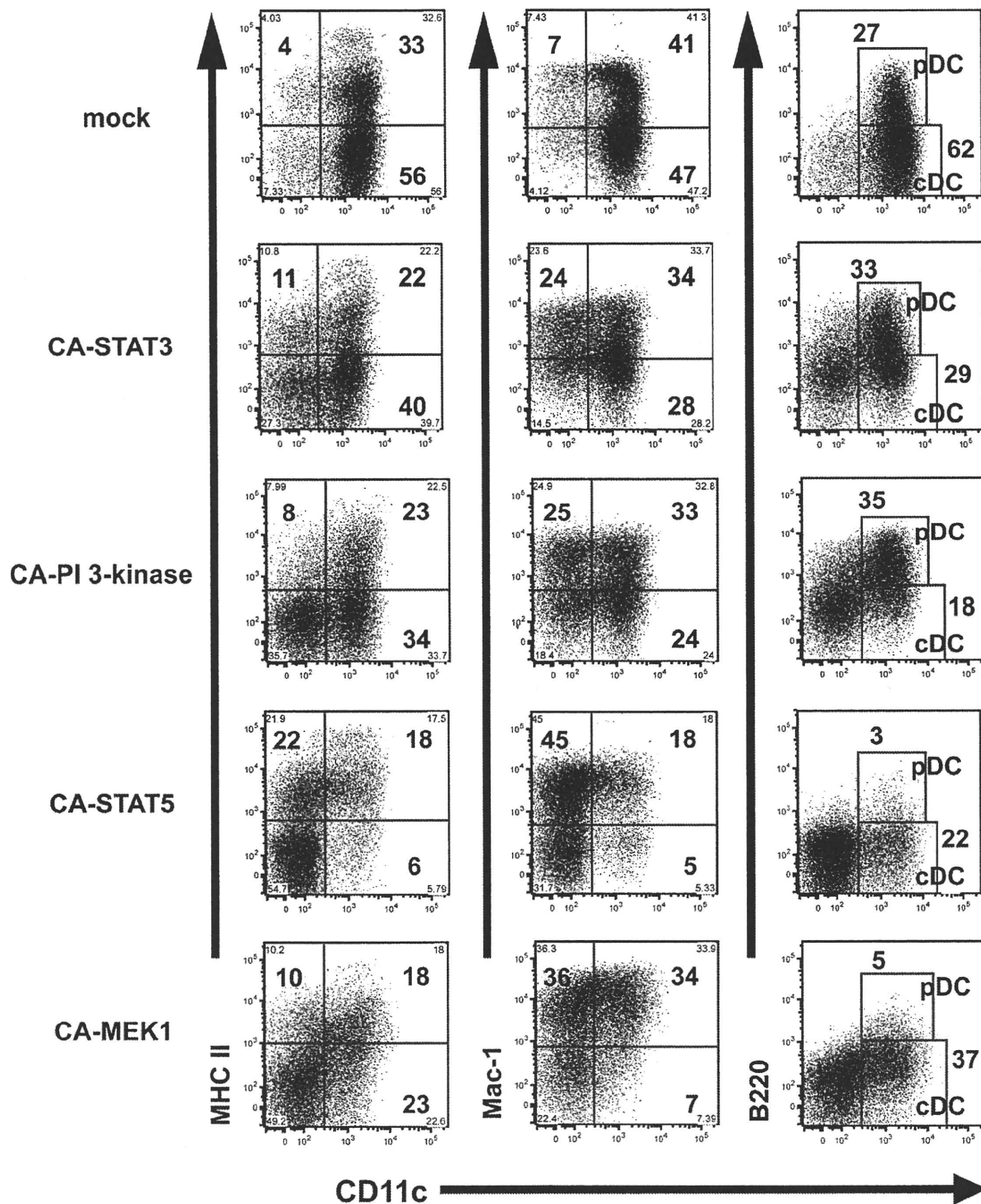

Fig. 5. Both active forms of STAT5 and MEK1 severely impaired pDC differentiation from LSKs. During the pre-culture phase, cells were transduced with the indicated activated form of signaling molecule. After 9 days in culture, cells were collected and the expression of the indicated surface markers was analysed by flow cytometry on the gated EGFP+ cell population. Data are representative of at least two experiments with similar results. Numbers in the dot plots represent percentage of cells.

of CD40 on whole FL-DCs from LSKs (data not shown). In addition, we determined whether these differences in expression of MHC II and costimulatory molecules on FL-DCs from LSKs between class I mutations affected the ability to stimulate allogeneic $CD4^+$ T cells. Among class I mutations, we selected FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ as representatives for an allogeneic MLR assay, because they showed contrasting patterns of whole FL-DC yields (Fig. 3D), pDC/cDC ratios (Fig. 3E), differentiation patterns (Fig. 4), and expression patterns of MHC II and costimulatory molecules (Fig. 6A and B). Both CA-N-Ras- and TEL/PDGFRβ-expressing FL-DCs from LSKs efficiently stimulated allogeneic $CD4^+$ T cells, possibly resulting from high expressions of MHC II and costimulatory molecules (Fig. 6C). By contrast, despite FLT3-ITD-expressing FL-DCs from LSKs exhibiting a relatively higher expression of MHC II and costimulatory molecules than those of mock cells, FLT3-ITD-expressing FL-DCs stimulated allogeneic $CD4^+$ T cells at comparable

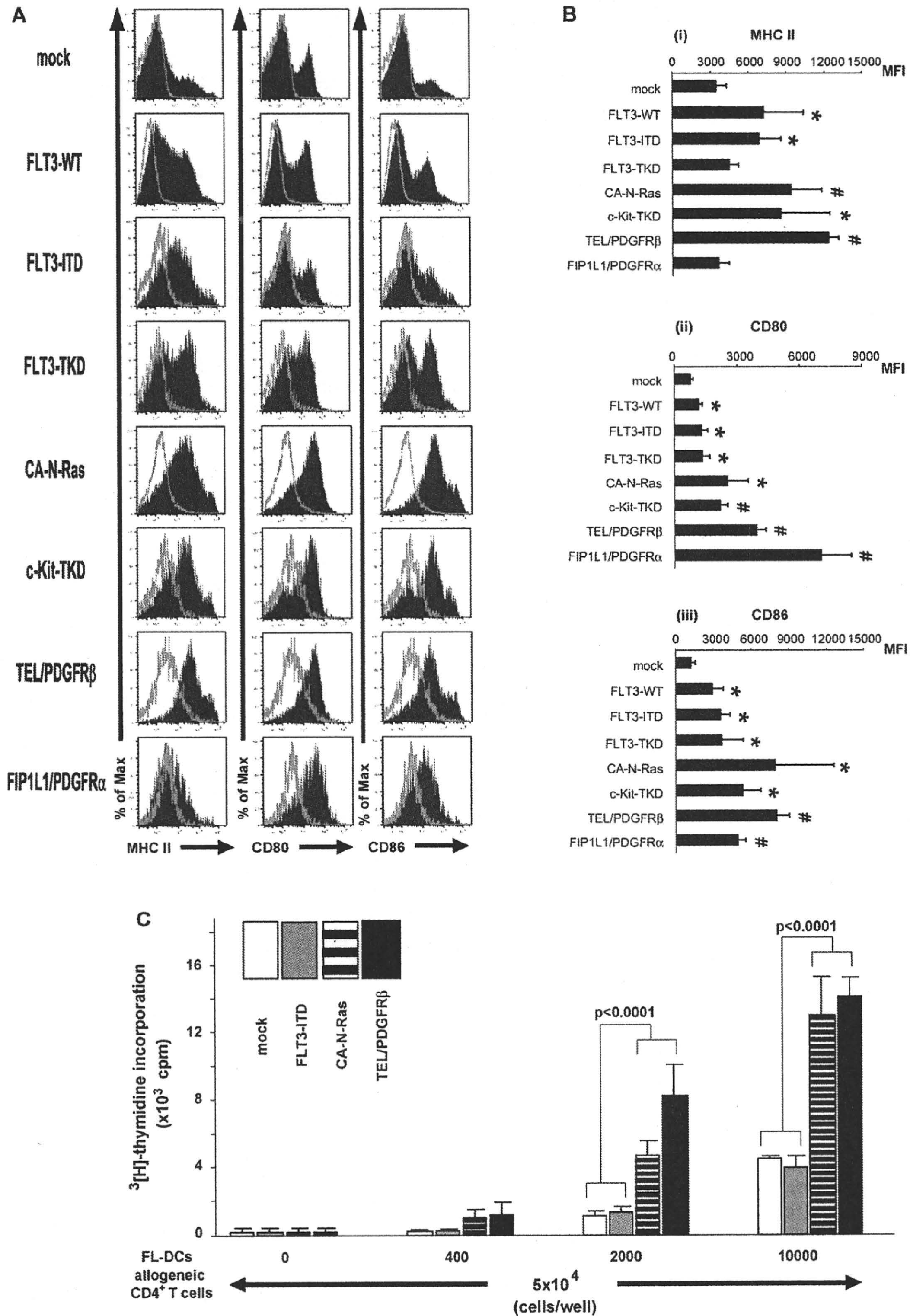

Fig. 6. Expression patterns of MHC II and costimulatory molecules on FL-DCs from LSKs are heterogeneous among class I mutations. During the pre-culture phase, cells were transduced with the indicated class I mutation. After 9 days in culture, cells were collected and the expression of MHC II and costimulatory molecules was analysed by flow cytometry on the gated EGFP$^+$CD11c$^+$ cell population. (A) Filled and open histograms show specific staining and back ground staining respectively. (B) The bars represent mean fluorescence intensity (MFI) of surface expression of MHC II (i), CD80 (ii), and CD86 (iii) respectively. (C) Allogeneic CD4$^+$ T cells from BALB/c mice were co-cultured with graded numbers of irradiated (30 Gy) FACS-sorted EGFP$^+$ FL-DCs from LSKs from C57BL/6 mice for 4 days. Proliferation was measured by [^{3}H]-thymidine incorporation. Data are representative of at least three experiments with similar results. Mean ± SD is shown. * and # indicate $p < 0.05$ and $p < 0.01$ respectively.

level as control cells (Fig. 6C). Taken together, the class I mutations tested displayed heterogeneous expression patterns of MHC II and costimulatory molecules on FL-DCs from LSKs.

3.7. FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ aberrantly induced PD-L1-expressing DCs

The interaction between programmed death-1 (PD-1) and PD-L1 results in diminished anti-tumor T-cell responses in both solid tumors [10,48–51] and hematological malignancies [52–54]. Therefore, we determined whether class I mutations induced expression of PD-L1 on FL-DCs from LSKs. We selected FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ as representatives of class I mutations. FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ induced PD-L1-expressing DCs, whereas the mock population did not induce PD-L1-expressing DCs (Fig. 7). In FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ, the proportion of FL-DCs expressing PD-L1 was increased in an EGFP-intensity dependent manner, suggesting that the induction of PD-L1-expressing DCs correlated with the expression of each myeloid neoplasm-related gene abnormality.

4. Discussion

Little is known about *in vivo* DCs in patients with myeloid leukemia [13–17,55–57] compared with *ex vivo* leukemic DCs [11,18]. This may be a result from several obstacles in the study of *in vivo* DCs [58]. DCs are a relatively rare population *in vivo* compared with other hematopoietic cells [59]. In addition, the expression of aberrant markers such as CD7, CD19, or CD56 is often detected [60–62], and may be retained on *in vivo* leukemic DCs in AML cases. Therefore, it is difficult to phenotypically identify *in vivo* leukemic DCs, which are identified by the negative selection using surface markers such as CD3, CD14, CD16, CD19, and CD56 [13,16]. Furthermore, in hematological malignancies, two distinct types of DCs *in vivo* exist that differ in their origin, "*in vivo* leukemic DCs" and "*in vivo* normal-origin DCs". To date it is not possible to isolate viable *in vivo* leukemic DCs from *in vivo* normal-origin DCs until genetic analysis for leukemia-specific markers such as fluorescence *in situ* hybridisation, sequence, or polymerase chain reaction is performed. Thus, study into *in vivo* DCs in leukemia cases is challenging and may explain the relatively small number of studies that have investigated the significance of *in vivo* leukemic DCs.

In order to address this problem, we established a reproducible FL-mediated *in vitro* DC differentiation system from LSKs, which imitates the differentiation process of *in vivo* leukemic DCs. Our system is characterised by two points distinct from previous studies. First, the system recapitulates steady-state DC differentiation. In tumor immunology, once tolerance to tumor-associated antigens (TAAs) has been established, immunisation with TAA, even with the use of mature DCs merely enhances TAA-specific immunosuppression [63–66], suggesting that early TAA-specific immune responses critically affect subsequent tumor control. We believed it was important to analyse the properties of *in vivo* leukemic DCs during the early phase of the disease or at the phase of minimal residual disease after therapy, which is regarded as the steady-state condition in DC development. Therefore, in our DC differentiation system, we selected FL, a crucial cytokine in steady-state DC differentiation [3]. Second, we developed a DC differentiation system from murine LSKs, but not from whole bone marrow cells. Leukemic cells develop from hematopoietic stem cells or progenitor cells that have acquired various genetic abnormalities. These myeloid neoplasm-related gene abnormalities are characterised primarily as growth/survival promoting abnormalities or differentiation blocking abnormalities, and are described as class I or class II mutations, respectively [43]. These mutations classes have also been identified as prognostic factors, as demonstrated for FLT3-ITD [67,68]. Obviously, *in vivo* leukemic DCs should have the same origin and genetic abnormalities as leukemic blasts. Therefore, we transduced abnormal myeloid neoplasm-related abnormal genes into hematopoietic stem cells or progenitor cells (which are target cells for leukemic transformation) and subsequently induced these cells into DCs in the presence of FL. For this purpose, we used murine LSKs as the initial cell population for DC differentiation. At present, there is a well-established culture method to generate FL-DCs from whole bone marrow cells [19,20,39], which are equivalent with steady-state splenic DCs [3,19,20]. Because the cell density is important for this culture method [39], we selected a 96-well round-bottom rather than flat-bottom culture plate during the DC-induction phase to maintain a constant cell density. Consequently, we were able to establish a simple, efficient, and reproducible FL-DC differentiation system from LSKs without additional cytokines or feeder cells (Fig. 1A–F), thereby enabling a comparative study between various genetic manipulations.

In this study, we have found that class I mutations differentially affected FL-DC differentiation from LSKs regarding the fold increase in whole FL-DCs, and the pDC/cDC ratio (Fig. 3A–E). The effects of these mutations on FL-DC differentiation differed from those on myeloid differentiation, a process that is inhibited primarily by class II mutations [43]. A time course study of FL-DC differentiation showed that CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα induced a transition from Mac-1^{+}CD11c^{-} to Mac-1^{+}CD11c^{+} to varying degrees, which differed from the control FL-mediated DC differentiation pathway (Fig. 4A and B). These findings suggest that class I mutations deregulate FL-mediated DC differentiation or may convert it into GM-CSF/IL-4-mediated DC differentiation to various degrees despite the cells being cultured in the presence of FL. Previous studies showed that DC differentiation is regulated by FL and GM-CSF primarily through the activation of STAT3 via FLT3 and STAT5 via the GM-CSF receptor, respectively [3,69]. Thus, steady-state DC differentiation is composed of both pDCs and cDCs and is maintained by FL, of which activation of STAT3 is indispensable. By contrast, DC differentiation in the inflammatory state which produces only cDC, is mediated primarily by GM-CSF, in which the activation of STAT5 by GM-CSF plays an important role in promoting GM-CSF-mediated DC differentiation and inhibiting FL-mediated DC differentiation. In DC differentiation in the inflammatory state, activation of STAT3 is dispensable. In addition, it is postulated that FL- or GM-CSF-mediated DC differentiation is determined by the balance between the activations of STAT3 and STAT5 [69,70], which leads to the difference in pDC/cDC ratio. Consistent with these observations, CA-STAT5, but not CA-STAT3, impaired FL-DC, particularly pDC, differentiation in our system (Fig. 4). In addition, CA-MEK1 inhibited FL-DC, particularly pDC, differentiation. This inhibition is similar to that seen by CA-N-Ras, suggesting that the Ras/MAP kinase pathway plays a role in the impairment of pDC differentiation (Fig. 4). Because class I mutations constitutively and simultaneously activate multiple signal pathways [43], we speculate that these mutations differentially regulate FL-DC differentiation, possibly via their specific targets and extent of activation of each signal transduction pathway. Further studies will be required to investigate how each class I mutation and its deregulated signal transduction pathway is involved in FL-DC differentiation.

Mohty et al. [13] reported that patients with AML showed various patterns of quantitative imbalances in the proportions of circulating myeloid DCs (mDCs) (the human counterpart for murine cDCs) and pDCs. They classified these various patterns into three groups. Group I showed similar proportions with healthy volunteers. Group II included three subgroups: mDC expansion, pDC expansion, and mDC and pDC expansion. Group III showed no detectable DC subsets. The heterogeneous DC proportions in

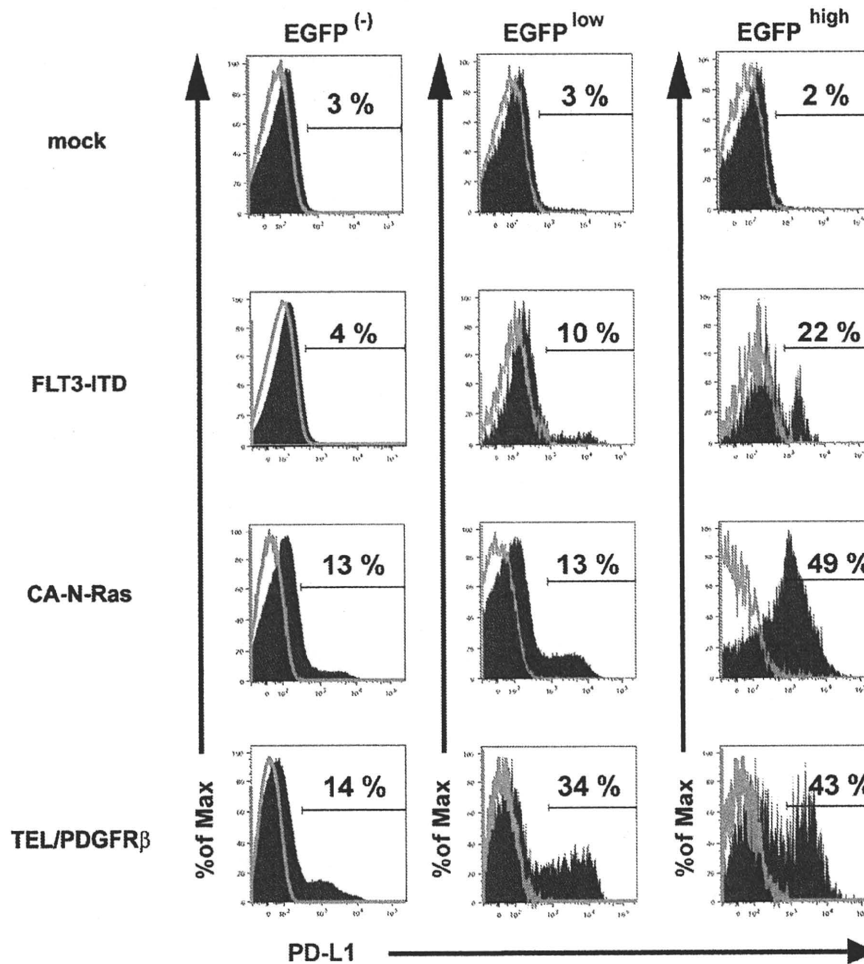

Fig. 7. FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ aberrantly induce PD-L1-expressing DCs. During the pre-culture phase, cells were transduced with the indicated gene. After 9 days in culture, cells were collected and the expression of PD-L1 was analysed by flow cytometry on the gated $EGFP^{-}CD11c^{+}$, $EGFP^{low}CD11c^{+}$ and $EGFP^{high}CD11c^{+}$ cell population. Filled and open histograms show specific staining and back ground staining respectively. $EGFP^{low}$ and $EGFP^{high}$ cells were defined as those showing lower and higher fluorescence intensity than the MFI of all $EGFP^{+}$ cells, respectively. Data are representative of at least three experiments with similar results.

AML patients may partly reflect the various effects that myeloid neoplasm-related gene abnormalities have on DC differentiation as we have shown. However, particularly during the manifestation of the disease, *in vivo* DC differentiation may be affected by various mechanisms/factors such as abnormal secreted cytokine levels [7,56,71,72] and DC distributions [73]. Therefore, we should reassess the changes in *in vivo* DCs in relation to myeloid neoplasm-related gene abnormalities in patients with myeloid neoplasms.

DCs play a pivotal role in determining the balance between T cell immunity and tolerance to tumor cells [5,6,74]. Generally, mature and immature DCs contribute to T cell immunity and tolerance, respectively. pDCs usually display an immature phenotype and often facilitate tumor progression through various mechanisms [6,7,9] such as induction of T cell anergy or deletion, induction of T cells with regulatory property, IDO-mediated tryptophan catabolism, or induction of PD-L on IDO-negative DCs by IDO-positive pDCs. In this study, we have shown that FLT3-ITD specifically retained pDC differentiation from LSKs and showed relatively immature phenotype among class I mutations tested. In addition, FLT3-ITD aberrantly induced PD-L1-expressing DCs. Therefore, FLT3-ITD may work as an inducer of *in vivo* leukemic DCs with tolerogenic function among class I mutations, which may be one reason for it being a poor prognostic factor [67,68]. Therefore, whether leukemic DCs bearing FLT3-ITD have tolerogenic property needs to be further investigated. In contrast, both CA-N-Ras- and TEL/PDGFRβ-expressing FL-DCs exhibited a mature phenotype among the class I mutations tested and efficiently stimulated allogeneic T cells. Therefore, these mutations may have immunogenic properties. However, in certain immunological contexts, mature DCs can also contribute to tolerance by inducing T cells with regulatory properties [47,64]. In addition, both CA-N-Ras and TEL/PDGFRβ aberrantly induced PD-L1-expressing DCs. Therefore, how *in vivo* leukemic DCs bearing CA-N-Ras or TEL/PDGFRβ affect host immune responses needs to be further elucidated. In summary, the differentiation, maturation, and function of FL-DCs from LSKs are deregulated by myeloid neoplasm-related gene abnormalities, particularly class I mutations. Therefore, in patients with myeloid neoplasms, how myeloid neoplasm-related gene abnormalities (particularly class I mutations and associated deregulated signal transduction pathways), affect host immune system, tolerance and immunity, through *in vivo* leukemic DCs needs to be further examined. If *in vivo* leukemic DCs have tolerogenic prop-

erties, they may be candidate targets for therapy. Conversely, if *in vivo* leukemic DCs have immunogenic property, their inhibition may lead to disease progression.

In conclusion, here we have found the possible novel function inherent in myeloid neoplasm-related gene abnormalities, that is, the differentiation, maturation, and function of *in vivo* leukemic DCs may be differentially affected by myeloid neoplasm-related gene abnormalities themselves.

Acknowledgements

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture and Technology of Japan. The authors thank Noriko Kikunaga and Yoko Habuchi for their professional assistance; Jun Ishiko, Isao Takahashi, Tetsuo Maeda, and Takafumi Yokota for their helpful advice and discussion.

References

[1] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245–52.
[2] Steinman RM, Hawiger D, Liu K, Bonifaz L, Bonnyay D, Mahnke K, et al. Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. Ann N Y Acad Sci 2003;987:15–25.
[3] Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 2007;7:19–30.
[4] Steinman RM, Banchereau J. Taking dendritic cells into medicine. Nature 2007;449:419–26.
[5] Dhodapkar MV, Dhodapkar KM, Palucka AK. Interactions of tumor cells with dendritic cells: balancing immunity and tolerance. Cell Death Differ 2008;15:39–50.
[6] Melief CJ. Cancer immunotherapy by dendritic cells. Immunity 2008;29:372–83.
[7] Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol 2004;4:941–52.
[8] Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med 2001;194:769–79.
[9] Sharma MD, Baban B, Chandler P, Hou DY, Singh N, Yagita H, et al. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. J Clin Invest 2007;117:2570–82.
[10] Curiel TJ, Wei S, Dong H, Alvarez X, Cheng P, Mottram P, et al. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. Nat Med 2003;9:562–7.
[11] Houtenbos I, Westers TM, Ossenkoppele GJ, van de Loosdrecht AA. Feasibility of clinical dendritic cell vaccination in acute myeloid leukemia. Immunobiology 2006;211:677–85.
[12] Li L, Reinhardt P, Schmitt A, Barth TF, Greiner J, Ringhoffer M, et al. Dendritic cells generated from acute myeloid leukemia (AML) blasts maintain the expression of immunogenic leukemia associated antigens. Cancer Immunol Immunother 2005;54:685–93.
[13] Mohty M, Jarrossay D, Lafage-Pochitaloff M, Zandotti C, Briere F, de Lamballeri XN, et al. Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairment. Blood 2001;98:3750–6.
[14] Fujii S, Shimizu K, Koji F, Kawano F. Malignant counterpart of myeloid dendritic cell (DC) belonging to acute myelogenous leukemia (AML) exhibits a dichotomous immunoregulatory potential. J Leukoc Biol 2003;73:82–90.
[15] Orsini E, Calabrese E, Maggio R, Pasquale A, Nanni M, Trasarti S, et al. Circulating myeloid dendritic cell directly isolated from patients with chronic myelogenous leukemia are functional and carry the bcr-abl translocation. Leuk Res 2006;30:785–94.
[16] Ma L, Delforge M, van Duppen V, Verhoef G, Emanuel B, Boogaerts M, et al. Circulating myeloid and lymphoid precursor dendritic cells are clonally involved in myelodysplastic syndromes. Leukemia 2004;18:1451–6.
[17] Micheva I, Thanopoulou E, Michalopoulou S, Kakagianni T, Kouraklis-Symeonidis A, Symeonidis A, et al. Impaired generation of bone marrow CD34-derived dendritic cells with low peripheral blood subsets in patients with myelodysplastic syndrome. Br J Haematol 2004;126:806–14.
[18] Schmitt M, Casalegno-Garduno R, Xu X, Schmitt A. Peptide vaccines for patients with acute myeloid leukemia. Expert Rev Vaccines 2009;8:1415–25.
[19] Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. J Immunol 2007;179:7577–84.
[20] Naik SH, Proietto AI, Wilson NS, Dakic A, Schnorrer P, Fuchsberger M, et al. Cutting edge: generation of splenic CD8+ and CD8− dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. J Immunol 2005;174:6592–7.
[21] Spiekermann K, Bagrintseva K, Schwab R, Schmieja K, Hiddemann W. Overexpression and constitutive activation of FLT3 induces STAT5 activation in primary acute myeloid leukemia blast cells. Clin Cancer Res 2003;9:2140–50.
[22] Fenski R, Flesch K, Serve S, Mizuki M, Oelmann E, Kratz-Albers K, et al. Constitutive activation of FLT3 in acute myeloid leukaemia and its consequences for growth of 32D cells. Br J Haematol 2000;108:322–30.
[23] Delgado MD, Vaque JP, Arozarena I, Lopez-Ilasaca MA, Martinez C, Crespo P, et al. H-K- and N-Ras inhibit myeloid leukemia cell proliferation by a p21WAF1-dependent mechanism. Oncogene 2000;19:783–90.
[24] Hashimoto K, Matsumura I, Tsujimura T, Kim DK, Ogihara H, Ikeda H, et al. Necessity of tyrosine 719 and phosphatidylinositol 3′-kinase-mediated signal pathway in constitutive activation and oncogenic potential of c-kit receptor tyrosine kinase with the Asp814Val mutation. Blood 2003;101:1094–102.
[25] Stover EH, Chen J, Lee BH, Cools J, McDowell E, Adelsperger J, et al. The small molecule tyrosine kinase inhibitor AMN107 inhibits TEL-PDGFRbeta and FIP1L1-PDGFRalpha in vitro and in vivo. Blood 2005;106:3206–13.
[26] Shimizu K, Kitabayashi I, Kamada N, Abe T, Maseki N, Suzukawa K, et al. AML1-MTG8 leukemic protein induces the expression of granulocyte colony-stimulating factor (G-CSF) receptor through the up-regulation of CCAAT/enhancer binding protein epsilon. Blood 2000;96:288–96.
[27] Alcalay M, Tomassoni L, Colombo E, Stoldt S, Grignani F, Fagioli M, et al. The promyelocytic leukemia gene product (PML) forms stable complexes with the retinoblastoma protein. Mol Cell Biol 1998;18:1084–93.
[28] Zhao L, Cannons JL, Anderson S, Kirby M, Xu L, Castilla LH, et al. CBFB-MYH11 hinders early T-cell development and induces massive cell death in the thymus. Blood 2007;109:3432–40.
[29] Satoh Y, Matsumura I, Tanaka H, Ezoe S, Fukushima K, Tokunaga M, et al. AML1/RUNX1 works as a negative regulator of c-Mpl in hematopoietic stem cells. J Biol Chem 2008;283:30045–56.
[30] Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, et al. Stat3 as an oncogene. Cell 1999;98:295–303.
[31] Matsumura I, Kitamura T, Wakao H, Tanaka H, Hashimoto K, Albanese C, et al. Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. EMBO J 1999;18:1367–77.
[32] Doornbos RP, Theelen M, van der Hoeven PC, van Blitterswijk WJ, Verkleij AJ, van Bergen en Henegouwen PM. Protein kinase Czeta is a negative regulator of protein kinase B activity. J Biol Chem 1999;274:8589–96.
[33] Satoh Y, Matsumura I, Tanaka H, Ezoe S, Sugahara H, Mizuki M, et al. Roles for c-Myc in self-renewal of hematopoietic stem cells. J Biol Chem 2004;279:24986–93.
[34] Nakano H, Yanagita M, Gunn MD. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. J Exp Med 2001;194:1171–8.
[35] O'Keeffe M, Hochrein H, Vremec D, Caminschi I, Miller JL, Anders EM, et al. Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8(+) dendritic cells only after microbial stimulus. J Exp Med 2002;196:1307–19.
[36] Nikolic T, Dingjan GM, Leenen PJ, Hendriks RW. A subfraction of B220(+) cells in murine bone marrow and spleen does not belong to the B cell lineage but has dendritic cell characteristics. Eur J Immunol 2002;32:686–92.
[37] Pelayo R, Hirose J, Huang J, Garrett KP, Delogu A, Busslinger M, et al. Derivation of 2 categories of plasmacytoid dendritic cells in murine bone marrow. Blood 2005;105:4407–15.
[38] Blasius AL, Barchet W, Cella M, Colonna M. Development and function of murine B220+CD11c+NK1.1+ cells identify them as a subset of NK cells. J Exp Med 2007;204:2561–8.
[39] Brasel K, De Smedt T, Smith JL, Maliszewski CR. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. Blood 2000;96:3029–39.
[40] Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K, et al. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. J Exp Med 1996;184:1953–62.
[41] Naik SH, Sathe P, Park HY, Metcalf D, Proietto AI, Dakic A, et al. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. Nat Immunol 2007;8:1217–26.
[42] Naik SH. Generation of large numbers of pro-DCs and pre-DCs in vitro. Methods Mol Biol 2010;595:177–86.
[43] Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood 2002;100:1532–42.
[44] Waskow C, Liu K, Darrasse-Jeze G, Guermonprez P, Ginhoux F, Merad M, et al. The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. Nat Immunol 2008;9:676–83.
[45] Naik SH, Metcalf D, van Nieuwenhuijze A, Wicks I, Wu L, O'Keeffe M, et al. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. Nat Immunol 2006;7:663–71.
[46] Scholl C, Gilliland DG, Frohling S. Deregulation of signaling pathways in acute myeloid leukemia. Semin Oncol 2008;35:336–45.
[47] Rutella S, Danese S, Leone G. Tolerogenic dendritic cells: cytokine modulation comes of age. Blood 2006;108:1435–40.
[48] Blank C, Brown I, Peterson AC, Spiotto M, Iwai Y, Honjo T, et al. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. Cancer Res 2004;64:1140–5.

[49] Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. Cancer Res 2005;65:1089–96.
[50] Strome SE, Dong H, Tamura H, Voss SG, Flies DB, Tamada K, et al. B7-H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. Cancer Res 2003;63:6501–5.
[51] Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci U S A 2002;99:12293–7.
[52] Mumprecht S, Schurch C, Schwaller J, Solenthaler M, Ochsenbein AF. Programmed death 1 signaling on chronic myeloid leukemia-specific T cells results in T-cell exhaustion and disease progression. Blood 2009;114:1528–36.
[53] Zhang L, Gajewski TF, Kline J. PD-1/PD-L1 interactions inhibit antitumor immune responses in a murine acute myeloid leukemia model. Blood 2009;114:1545–52.
[54] Liu J, Hamrouni A, Wolowiec D, Coiteux V, Kuliczkowski K, Hetuin D, et al. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. Blood 2007;110: 296–304.
[55] Mohty M, Isnardon D, Vey N, Briere F, Blaise D, Olive D, et al. Low blood dendritic cells in chronic myeloid leukaemia patients correlates with loss of CD34+/CD38– primitive haematopoietic progenitors. Br J Haematol 2002;119:115–8.
[56] Boissel N, Rousselot P, Raffoux E, Cayuela JM, Maarek O, Charron D, et al. Defective blood dendritic cells in chronic myeloid leukemia correlate with high plasmatic VEGF and are not normalized by imatinib mesylate. Leukemia 2004;18:1656–61.
[57] Floisand Y, Normann AP, Heim S, Lund-Johansen F, Tjonnfjord GE. High expression of CD7 on CD34+ cells is not linked to deletion of derivative chromosome 9 or lack of dendritic cells in chronic myeloid leukaemia. Scand J Clin Lab Invest 2008;68:93–8.
[58] Panoskaltsis N. Dendritic cells in MDS and AML—cause, effect or solution to the immune pathogenesis of disease? Leukemia 2005;19:354–7.
[59] Robinson SP, Patterson S, English N, Davies D, Knight SC, Reid CD. Human peripheral blood contains two distinct lineages of dendritic cells. Eur J Immunol 1999;29:2769–78.
[60] Reading CL, Estey EH, Huh YO, Claxton DF, Sanchez G, Terstappen LW, et al. Expression of unusual immunophenotype combinations in acute myelogenous leukemia. Blood 1993;81:3083–90.
[61] Bahia DM, Yamamoto M, Chauffaille Mde L, Kimura EY, Bordin JO, Filgueiras MA, et al. Aberrant phenotypes in acute myeloid leukemia: a high frequency and its clinical significance. Haematologica 2001;86:801–6.
[62] Bhushan B, Chauhan PS, Saluja S, Verma S, Mishra AK, Siddiqui S, et al. Aberrant phenotypes in childhood and adult acute leukemia and its association with adverse prognostic factors and clinical outcome. Clin Exp Med 2010;10:33–40.
[63] Zhou G, Drake CG, Levitsky HI. Amplification of tumor-specific regulatory T cells following therapeutic cancer vaccines. Blood 2006;107:628–36.
[64] Maksimow M, Miiluniemi M, Marttila-Ichihara F, Jalkanen S, Hanninen A. Antigen targeting to endosomal pathway in dendritic cell vaccination activates regulatory T cells and attenuates tumor immunity. Blood 2006;108:1298–305.
[65] Wei S, Kryczek I, Zou L, Daniel B, Cheng P, Mottram P, et al. Plasmacytoid dendritic cells induce CD8+ regulatory T cells in human ovarian carcinoma. Cancer Res 2005;65:5020–6.
[66] Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. J Clin Invest 2007;117:1147–54.
[67] Schlenk RF, Dohner K, Krauter J, Frohling S, Corbacioglu A, Bullinger L, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med 2008;358:1909–18.
[68] Yanada M, Matsuo K, Suzuki T, Kiyoi H, Naoe T. Prognostic significance of FLT3 internal tandem duplication and tyrosine kinase domain mutations for acute myeloid leukemia: a meta-analysis. Leukemia 2005;19:1345–9.
[69] Onai N, Manz MG. The STATs on dendritic cell development. Immunity 2008;28:490–2.
[70] Cohen PA, Koski GK, Czerniecki BJ, Bunting KD, Fu XY, Wang Z, et al. STAT3- and STAT5-dependent pathways competitively regulate the pan-differentiation of CD34pos cells into tumor-competent dendritic cells. Blood 2008;112:1832–43.
[71] Tao M, Li B, Nayini J, Andrews CB, Huang RW, Devemy E, et al. SCF, IL-1beta, IL-1ra and GM-CSF in the bone marrow and serum of normal individuals and of AML and CML patients. Cytokine 2000;12:699–707.
[72] Panoskaltsis N, Reid CD, Knight SC. Quantification and cytokine production of circulating lymphoid and myeloid cells in acute myelogenous leukaemia. Leukemia 2003;17:716–30.
[73] Mumprecht S, Claus C, Schurch C, Pavelic V, Matter MS, Ochsenbein AF. Defective homing and impaired induction of cytotoxic T cells by BCR/ABL-expressing dendritic cells. Blood 2009;113:4681–9.
[74] Sotomayor EM, Borrello I, Rattis FM, Cuenca AG, Abrams J, Staveley-O'Carroll K, et al. Cross-presentation of tumor antigens by bone marrow-derived antigen-presenting cells is the dominant mechanism in the induction of T-cell tolerance during B-cell lymphoma progression. Blood 2001;98:1070–7.

Int J Hematol (2011) 93:36–46
DOI 10.1007/s12185-010-0748-9

ORIGINAL ARTICLE

Safety and efficacy of the terminal complement inhibitor eculizumab in Japanese patients with paroxysmal nocturnal hemoglobinuria: the AEGIS Clinical Trial

Yuzuru Kanakura · Kazuma Ohyashiki · Tsutomu Shichishima · Shinichiro Okamoto · Kiyoshi Ando · Haruhiko Ninomiya · Tatsuya Kawaguchi · Shinji Nakao · Hideki Nakakuma · Jun-ichi Nishimura · Taroh Kinoshita · Camille L. Bedrosian · Marye Ellen Valentine · Gus Khursigara · Keiya Ozawa · Mitsuhiro Omine

Received: 26 October 2010 / Revised: 9 December 2010 / Accepted: 12 December 2010 / Published online: 12 January 2011
© The Japanese Society of Hematology 2011

Abstract Paroxysmal nocturnal hemoglobinuria (PNH) is a progressive and life-threatening disease characterized by complement-mediated chronic hemolysis, resulting in serious life-threatening complications and early mortality. Eculizumab, a humanized anti-C5 monoclonal antibody that inhibits terminal complement activation, has been shown to reduce hemolysis in PNH patients. The pivotal open-label, 12-week phase II registration study (AEGIS) was designed to evaluate the efficacy and safety of eculizumab in Japanese patients with PNH. This trial achieved its primary endpoint of reducing intravascular hemolysis with high statistical significance. Twenty-seven of the 29 patients responded to eculizumab treatment, resulting in an 87% reduction in hemolysis ($P < 0.0001$) and subsequent improvement in anemia ($P = 0.0003$) despite reduction in transfusion requirements ($P = 0.006$). Fatigue and dyspnea significantly improved within 1–2 weeks of eculizumab treatment and the improvement was independent of changes in hemoglobin. Chronic kidney disease (CKD) was common (66%) and eculizumab treatment improved CKD in 41% of patients at 12 weeks ($P < 0.001$). Elevated thrombotic risk was evident in Japanese PNH patients and eculizumab treatment normalized D-dimer levels in 45% of patients with elevated D-dimers at baseline ($P < 0.001$). The AEGIS results demonstrate that eculizumab is effective, safe and well tolerated in Japanese patients with PNH.

Y. Kanakura (✉) · J. Nishimura
Department of Hematology and Oncology,
Osaka University Hospital, Suita, Japan
e-mail: kanakura@bldon.med.osaka-u.ac.jp

K. Ohyashiki
Tokyo Medical University Hospital, Tokyo, Japan

T. Shichishima
Fukushima Medical University, Fukushima, Japan

S. Okamoto
Division of Hematology,
Keio University School of Medicine, Tokyo, Japan

K. Ando
Department of Hematology and Oncology,
Tokai University, Isehara, Japan

H. Ninomiya
University of Tsukuba, Tsukuba, Japan

T. Kawaguchi
Kumamoto University, Kumamoto, Japan

S. Nakao
Cellular Transplantation Biology,
Kanazawa University, Kanazawa, Japan

H. Nakakuma
Department of Hematology/Oncology,
Wakayama Medical University, Wakayama, Japan

T. Kinoshita
Research Institute for Microbial Diseases,
Osaka University Hospital, Suita, Japan

C. L. Bedrosian · M. E. Valentine · G. Khursigara
Alexion Pharmaceuticals, Cheshire, CT, USA

G. Khursigara
e-mail: khursigarag@alxn.com

K. Ozawa
Jichi Medical University Hospital, Tochigi, Japan

M. Omine
Showa University Fujigaoka Hospital, Yokohama, Japan

Keywords Paroxysmal nocturnal hemoglobinuria · Complement-inactivating agents · Hemolysis · Eculizumab · Hematopoietic stem cell

1 Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a progressive and life-threatening disease characterized by chronic hemolysis [20, 28, 36]. The 5-year mortality in patients presenting with hemolysis ranges from 15 to 35% and median survival ranges from 10 to 22 years after diagnosis [10, 20, 27, 36]. The disease can present at any age with the median age ranging from early 30s to mid-40s [27]. PNH arises from an acquired genetic mutation in the X-linked phosphatidylinositol glycan-complementation class A (*PIGA*) of hematopoietic progenitor cells leading to clonal deficiency of glycosylphosphatidylinositol (GPI)-linked proteins on the surface membrane of blood cells [38, 41]. This GPI deficiency results in the loss of the complement inhibitor proteins CD55 and CD59 from the surface of hematopoietic cells in PNH patients leading to complement-mediated red cell lysis [3, 28], platelet activation [2], and hemostatic activation with inflammation [40].

Historically, physicians viewed and treated PNH as a disease of anemia. However, the demonstration of the underlying phenotype—deficiency of GPI-linked complement inhibitors CD55 and CD59—indicates that the primary clinical manifestation is the terminal complement activation causing not only lysis of PNH red blood cells but also in parallel platelet, monocyte, and leukocyte activation with consequent inflammation and hemostatic activation [13, 40]. Thus, anemia is only a single consequence of the underlying chronic intravascular hemolysis. Patients suffer severe morbidities and early mortality as a direct result of terminal complement activation with chronic hemolysis including kidney disease, thrombosis, pulmonary hypertension, hemoglobinuria, debilitating fatigue, severe dyspnea, disabling pain, and a poor quality of life (QoL) [15, 18, 27–29, 31, 32]. Thromboembolism (TE) accounts for 40–67% of PNH-related deaths and renal failure accounts for 8–18% of PNH-related deaths [15, 18, 27–29, 31, 32].

Eculizumab is a humanized monoclonal antibody that specifically targets the terminal complement protein C5, thereby inhibiting terminal complement-mediated hemolysis [33]. The efficacy and safety of eculizumab have been evaluated in two multinational phase III studies and a multinational extension trial performed predominantly in the North America, Europe and Australia [4, 21, 22]. These studies demonstrate that eculizumab significantly reduces hemolysis, thrombotic events, renal impairment, pulmonary hypertension, and improves fatigue, QoL, and anemia, while reducing transfusion requirements.

While the clinical course of PNH in both the Western and Asian populations is associated with similar mortality rates and both populations suffer significant hemolysis-mediated symptoms, the clinical manifestation of the disease is perceived to differ between the two populations [27]. The current study was an open-label, single-arm, phase II registration study (AEGIS) designed to evaluate the safety, efficacy, pharmacokinetics and pharmacodynamics of eculizumab in Japanese patients with PNH and to examine the consistency of these results with the previously reported multinational phase III and extension studies of eculizumab.

2 Methods

AEGIS was an open-label, single-arm, multi-center study in Japanese patients who were 12 years of age or older with a diagnosis of PNH for at least 6 months and was conducted at 9 medical centers in Japan. Additional inclusion criteria included: the presence of a population of GPI-deficient red blood cells (PNH Type III RBCs) by flow cytometry $\geq$ 10% at screening; and lactate dehydrogenase (LDH) $\geq$ 1.5 times the upper limit of normal (ULN) within 12 weeks of screening or during the screening period, and platelet count $\geq 30 \times 10^9$/L. Enrolled patients were judged by the physician to require at least one red blood cell transfusion within the past 2 years, although enrollment of patients who had received no red blood cell transfusions was permitted provided that the physician had judged the patient to have required transfusion. All patients were required to give written informed consent. Females of child-bearing potential were required to have a negative pregnancy test (serum HCG) at screening. Sexually active females had to agree to use a reliable and medically approved method of contraception. Exclusion criteria included: platelet count $< 30 \times 10^9$/L at screening; absolute neutrophil count $\leq$ 500/μL at screening; known or suspected hereditary complement deficiency; history of hematopoietic stem cell transplantation; and participation in any other investigational drug trial or exposure to other investigational agent, device, or procedure within 30 days prior to screening. Patients who were pregnant or breast-feeding, or who could become pregnant or intended to conceive during the course of the study (including the post-treatment period and the follow-up visits for early termination) were excluded. Additional exclusion criteria included a history of meningococcal disease and, in the opinion of the investigator, the presence or suspicion of active bacterial infection 2 weeks prior to first dose of eculizumab, or recurrent bacterial infections.

Patients received 600 mg of eculizumab intravenously every 7 $\pm$ 2 days for 4 weeks, followed by 900 mg 1 week

later followed by 900 mg every 14 ± 2 days for a total of 12 weeks. All patients were vaccinated with a meningococcal vaccine at least 2 weeks before receiving the first dose of eculizumab.

The primary efficacy endpoint in the AEGIS study was the change in intravascular hemolysis (as measured by change in LDH) at study Week 12 from baseline. Secondary endpoints included change from baseline in the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) [5] scale at study Week 12, change in PNH Type III RBC count at study Week 12, change in transfusion requirements (number of units of packed RBCs transfused), change in plasma-free hemoglobin (free-Hgb) at study Week 12, area under the curve (AUC) for change of LDH, and change in the European Organization for Research and Treatment of Cancer Quality of Life Core 30 (EORTC QLQ-C30) [1] questionnaire score at study Week 12. D-Dimer levels were measured at the central laboratory at baseline, Week 4 and Week 12.

The effect of eculizumab on renal function as measured by an improvement or worsening in chronic kidney disease (CKD) stage during treatment was also evaluated. CKD stages were determined for each patient according to Kidney Disease Outcomes Quality Initiative (KDOQI) CKD published guidelines classification (Stage 5: glomerular filtration rate, GFR < 15 mL/min/1.73 m^2; Stage 4: GFR 15–30 mL/min/1.73 m^2; Stage 3: GFR 30–60 mL/min/1.73 m^2; Stage 2: GFR 60–90 mL/min/1.73 m^2 and evidence of proteinuria; Stage 1: GFR ≥ 90 mL/min/1.73 m^2 and evidence of proteinuria; no CKD: GFR > 60 mL/min/1.73 m^2 and no evidence of proteinuria) [26]. An improvement in renal function was defined as a categorical reduction in CKD stage level or fulfilling the criteria of no CKD. Worsening in renal function was defined as a categorical increase in CKD stage level.

Evaluation of the safety of eculizumab included assessment of adverse events (AEs), assessment of thrombotic events, laboratory measurements, vital signs, ECG, and chest X-ray.

The pharmacokinetics of eculizumab was determined with a validated enzyme-linked immunosorbent assay that detects both free and C5-bound eculizumab [39]. The analytical range of the assay was 10–640 μg/mL. The pharmacodynamics of eculizumab was determined by measuring the capacity of the patient's serum to lyse chicken erythrocytes in a validated standard total human serum-complement hemolytic assay [30].

2.1 Statistical analysis

Changes in LDH, hemoglobin (Hgb), RBC type III, free-Hgb and PRBC transfusion units from baseline to Week 12 were analyzed by Wilcoxon's signed rank test. Transfusion avoidance was evaluated with a McNemar test.

Changes in scores on the FACIT-Fatigue instrument and the EORTC QLQ-C30 instrument from baseline through Week 12 were analyzed with the use of a mixed-effects model, with baseline scores as the covariate, time as the fixed effect, and the patient identifier as the random effect. The proportion of patients returning to normal range of D-dimer was analyzed by exact binomial test.

Changes in the proportion of patients in each CKD stage from baseline were compared using Chi-square analyses and the hypothesis tested the probability of worsening CKD stage was equal to the probability of improving CKD stage.

For each subgroup of patients with a history of bone marrow dysfunction (BMD) that includes aplastic anemia (AA) or myelodysplastic syndrome (MDS), or no history of BMD, the change in LDH, Hgb, FACIT-Fatigue, EORTC from baseline was analyzed by a t test in mixed-effects model and between the two subgroups was analyzed by F test in mixed-effects model with baseline as covariate, subgroup and time as fixed effect, and patient as random effect.

3 Results

3.1 AEGIS patient characteristics

Eculizumab was administered to 29 Japanese patients (14 men and 15 women; median patient age 47 years; range 26–70 years) at 9 institutions (see Table 1). Forty-five percent (45%; 13/29) of patients had a history of AA or MDS. Forty-eight percent (48%; 14/29) of patients were receiving concomitant corticosteroids. The cohort also included 2 patients who were never transfused. These two patients demonstrated clinical signs and symptoms of PNH comparable to patients who had received transfusions. Twenty-seven of 29 patients completed the study.

3.2 Hemolysis

The primary endpoint—reduction of intravascular hemolysis—was achieved with a high level of statistical significance with eculizumab treatment. Eculizumab treatment reduced LDH 87% from a median of 1814 U/L at baseline to 244 U/L at 12 weeks of treatment ($P < 0.0001$; normal range 103–223 U/L). Mean LDH levels decreased from 1845 ± 115 U/L at baseline to 399 ± 99 U/L at 12 weeks (Table 1). A significant reduction in LDH was observed within 1 week of treatment ($P < 0.0001$) and this reduction was sustained throughout the 12-week study (Fig. 1). In two patients, eculizumab serum concentrations were maintained above the effective level of 35 μg/ml but

Table 1 Baseline characteristics, effects of eculizumab on hematologic parameters

Parameter	Baseline		
Median age (years) (range)	47 (26–70)		
Gender, female	52%		
History of AA or MDS	45%		
History of thrombosis	17%		
Elevated D-dimer	38%		
Elevated D-dimer or thrombosis	52%		
Chronic kidney disease (CKD)	66%		
Concomitant antithrombotic (%)	31%		
Concomitant steroids (%)	48%		
Parameter	**Mean ± SE (median)**		**P value**
	Baseline	**12 weeks**	
LDH (U/L)	1845 ± 115 (1814)	399 ± 100 (244)	<0.0001
RBC type III (%)	43.9 ± 4.5 (39.2)	57.3 ± 4.9 (56.7)	<0.0001
PNH RBC mass ($\times 10^{12}/\mu L$)	1.2 ± 0.1 (1.2)	1.8 ± 0.2 (1.7)	<0.0001
Transfusion (units/12 weeks)	5.2 ± 1.0 (2.0)	1.5 ± 0.7 (0.0)	0.006
Hemoglobin (g/dL)	7.9 ± 0.3 (7.6)	8.9 ± 0.4 (9.0)	0.0003
Free hemoglobin (mg/dL)	22.6 ± 2.6 (20.0)	2.8 ± 1.0 (1.0)	<0.0001

P value was calculated using signed rank test

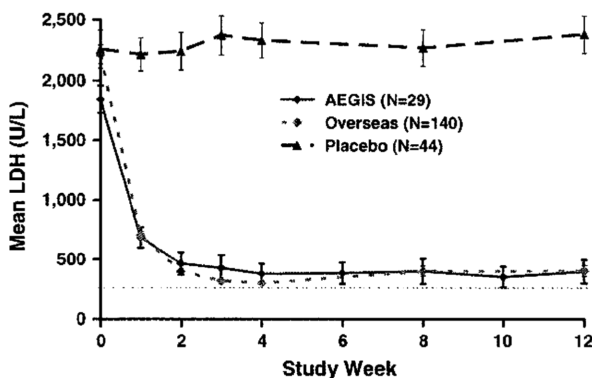

Fig. 1 Comparison of the effects of eculizumab on LDH in AEGIS versus multinational studies (units, U/L). Treatment with eculizumab reduced mean LDH levels from baseline (Week 0) within 1 week of treatment ($P < 0.001$ from baseline) and was sustained for 12 weeks ($P < 0.001$ from baseline) in Japanese-treated patients (AEGIS, *red diamonds*). The 87% reduction in the Japanese patients was similar to the treated patients in multinational overseas study ($P < 0.001$; overseas, *orange diamonds*). Patients not treated with eculizumab did not demonstrate a reduction in LDH (placebo, *blue triangles*). The overseas data consist of the placebo-controlled TRIUMPH and open-label SHEPHERD registration studies [4, 21, 22]. The placebo group is from the TRIUMPH study

without reduction in LDH, although a partial drug response was observed with a reduction in complement activation as measured by in vitro CH50 assays.

3.3 Red blood cell mass and anemia

Eculizumab treatment significantly increased the proportion of PNH type III RBCs from a median of 39.2% at baseline to 56.7% at Week 12 ($P < 0.001$; Table 1). Similarly, the proportion of type II RBCs was also significantly increased from a median of 4.2% at baseline to a median of 5.6% at Week 12 ($P < 0.0001$). Eculizumab treatment significantly increased PNH RBC mass from a mean at baseline $1.2 \times 10^{12}/\mu L$ to $1.8 \times 10^{12}/\mu L$ at 12 weeks ($P < 0.0001$; Table 1).

Eculizumab treatment significantly reduced the number of PRBC transfusion units from a median 2.0 units (mean of 5.2 ± 1.0 units) per patient in the 12 weeks prior to eculizumab treatment to a median 0.0 units (mean 1.5 ± 0.7) per patient during the 12-week treatment phase ($P = 0.006$; Table 1). Of the 21 patients that had received at least one or more transfusion in the 12-week period prior to treatment, 66% (14/21) did not require transfusion during the 12 weeks of treatment ($P = 0.001$). Despite the decrease in transfusion requirements, Hgb levels increased from a median of 7.6 g/dL at baseline to 9.0 g/dL at Week 12 ($P = 0.0003$; Table 1). However, the statistical improvement in Hgb was first observed 8 weeks after treatment initiation (median 8.7 g/dL at Week 8 vs. 7.6 g/dL at baseline, $P = 0.007$).

Table 2 Effects of eculizumab on fatigue, dyspnea, hemoglobin and free hemoglobin during first 4 weeks of treatment

	Baseline	Change from baseline [mean ± SE, median (*P* value)]			
		Week 1	Week 2	Week 4	Week 12
Fatigue (FACIT Score)	38.5 ± 1.9, 41.0	2.1 ± 1.1, 2.0 (0.04)	4.2 ± 1.0, 4.0 (<0.001)	4.9 ± 1.4, 4.0 (<0.001)	4.1 ± 2.3, 5.0 (<0.001)
Dyspnea (EORTC Score)[a]	37.9 ± 5.2, 33.3	−11.5 ± 4.1, 0.0 (0.006)	−13.8 ± 3.9, 0.0 (<0.001)	−13.8 ± 4.5, 0.0 (<0.001)	−13.8 ± 4.5, 0.0 (<0.001)
Hemoglobin (g/dL)	7.9 ± 0.3, 7.6	N/A	0.2 ± 0.2, 0.1 (NS)	0.4 ± 0.2, 0.25 (NS)	1.0 ± 0.25, 1.0 (<0.001)
Free hemoglobin (mg/dL)	22.6 ± 2.6, 22	−18.3 ± 2.7, −12.0 (<0.001)	−18.6 ± 3.0, −17.0 (<0.001)	−11.9 ± 9.1, −16.0 (0.006)	−19.8 ± 2.7, −17.0 (<0.001)

NS not significant: *P* value based on *t* test

[a] A negative change in EORTC score of fatigue and dyspnea indicates improvement

There was an immediate reduction in median free-Hgb levels at 1 week of eculizumab treatment (from 20.0 mg/dL at baseline to 4.2 mg/dL; $P < 0.001$) compared to baseline levels and this reduction was sustained throughout the study to 1.0 mg/dL at Week 12 ($P < 0.0001$).

3.4 Improvement in FACIT-Fatigue and QoL

Fatigue levels, as measured by the FACIT-Fatigue instrument and confirmed by the EORTC-Fatigue instrument, significantly improved within 2 weeks of eculizumab treatment. Thirty-eight percent of treated patients experienced a clinically meaningful improvement in fatigue (at least a 3-point increase on the FACIT-Fatigue scale) [6, 7] at Week 1 of treatment, 62% at Week 2 and 66% at Week 12. Eculizumab treatment improved fatigue, a mean of 2.1 and 4.2 points on the FACIT-Fatigue scale after 1 and 2 weeks of treatment ($P = 0.04$ and $P < 0.001$ compared to baseline, respectively; Table 2). The improvement in fatigue was sustained, a mean increase of 4.1 points at 12 weeks ($P < 0.001$). The rapid improvement in fatigue measured by FACIT-Fatigue was confirmed by a large mean improvement in EORTC QLQ-C30 Fatigue of 7.1 and 11.1 points by Weeks 1 and 2 ($P < 0.001$ compared to baseline each week).

Treatment with eculizumab demonstrated improvements in QoL ($P = 0.02$) as measured by EORTC QLQ-C30, with statistically significant improvements in global health status ($P = 0.02$), role ($P < 0.001$), physical ($P = 0.02$) and emotional functioning ($P = 0.002$), fatigue ($P < 0.0001$), dyspnea ($P < 0.0001$), and appetite loss symptoms ($P < 0.0001$). Fifty percent of Japanese patients treated with eculizumab improved by at least 10% in global health status at Week 12, a degree of improvement considered clinically meaningful. Eculizumab treatment was associated with a large and rapid improvement in patient-reported dyspnea symptoms with an 11.5 points improvement at Week 1 ($P = 0.02$). Furthermore, 41% of patients reported a major improvement (10% or greater) in dyspnea with eculizumab treatment that was sustained through Week 12.

3.5 Improvement in CKD

Renal dysfunction was common in the study population, with 66% (19 of 29 patients; Table 1; Fig. 3a) of patients demonstrating CKD at baseline. Patients treated with eculizumab for 12 weeks were more likely to improve (41%; 12/29) rather than worsen (3%; 1/29) CKD stage ($P = 0.0002$ improved compared to worsen) and 55% (16/29) of patients had no change in their CKD stage (Fig. 3b). Of the 16 patients with CKD Stage 1–2 at baseline, 11 patients (69%) improved with eculizumab; of the 3 patients with CKD Stage 3–5 at baseline, 1 patient (33%) improved with eculizumab treatment (Fig. 3).

3.6 Thrombotic events and D-dimer levels

There were five patients with a history of TEs (one patient had a cerebrovascular accident and 4 patients had a deep vein thrombosis) prior to study enrollment and there were no reported TEs during eculizumab treatment. At baseline, 11/29 (38%) of patients had D-dimer levels above the ULN. Seven of the 11 patients with elevated D-dimer levels had evidence of CKD. Eculizumab treatment was associated with the normalization of D-dimer levels in 5 of the 11 (45%) of patients with elevated D-dimer ($P < 0.001$).

3.7 AA or MDS subpopulation analysis

Forty-five percent (13/29) of the PNH patients enrolled in the study were also diagnosed with a history of BMD (AA or MDS). Patients with or without BMD showed similar levels of hemolysis, as measured by LDH ($P = 0.74$ between both subpopulations), Hgb ($P = 0.60$), transfusions ($P = 0.82$), FACIT-Fatigue score ($P = 0.66$),

EORTC-Fatigue ($P = 0.62$), and EORTC-Dyspnea ($P = 0.32$) at study entry. Both PNH patient subgroups showed an immediate and sustained reduction in LDH from baseline within the first week of treatment ($P < 0.001$ in each group). Eculizumab treatment improved QoL measures in both patient groups as indicated by significant improvements in FACIT-Fatigue ($P < 0.001$ and $P = 0.008$, respectively), EORTC-Fatigue ($P = 0.001$ and $P = 0.008$, respectively) and EORTC-Dyspnea ($P < 0.001$ and $P = 0.003$, respectively). Hgb was improved with eculizumab treatment at Week 12 from baseline in both groups ($P < 0.05$ and $P = 0.006$, respectively). There was no difference in improvement between the two groups during eculizumab treatment: LDH reduction ($P = 0.51$), increase Hgb ($P = 0.26$), improvement in FACIT-Fatigue ($P = 0.95$), EORTC-Global health ($P = 0.90$), EORTC-Fatigue ($P = 0.94$), and EORTC-Dyspnea ($P = 0.49$).

PNH patients with PNH and BMD experienced significant improvement in renal function. At baseline, 12/13 (92%) of patients with a history of BMD demonstrated CKD. Eculizumab treatment led to 7/13 patients improving CKD, 6/13 patients with no change, and no patients with worsening of CKD ($P = 0.0001$). At baseline, 7/16 (44%) of patients without a history of BMD demonstrated CKD. Eculizumab treatment was associated with a strong trend for improvement ($P = 0.07$) with 5/16 patients improving to a level of no CKD, 10/16 patients with no change, and one patient with worsening of CKD from Stage 0 to Stage 1.

3.8 Never transfused patients

Despite 2 patients never being transfused at baseline, these patients were hemolytic (LDH approximately 7- and 11-fold above normal, respectively), demonstrated significant organ damage with evidence of renal disease (CKD stage 2 and 1, respectively) and thrombosis (1 patient with DVT), and suffered disabling QoL as measured by FACIT-Fatigue and EORTC QLQ-C30 Dyspnea. In both patients, eculizumab treatment resulted in substantial 78–88% reductions in LDH, significant improvements in fatigue (improvements of 5 and 23 points, respectively), improvement in dyspnea in one patient (improvement of 33 points from baseline), and elimination of CKD with no subsequent TE in both patients.

3.9 Pharmacokinetics and pharmacodynamics of eculizumab

Blood samples for PK/PD assessments were collected at all dosing visits in the AEGIS study. Pharmacokinetic analysis showed that eculizumab trough levels reached a median of 85.8 μg/mL (range 20.4–172.5 μg/mL) and peak levels reached a median of 189.9 μg/mL (range 90.6–297.9 μg/mL) at study Week 2. Over the course of the study, both peak and trough levels were maintained above the levels reached at Week 2. No patients at Week 4, and 1 patient each at Weeks 6, 8, and 12 showed serum eculizumab levels below 35 μg/mL, a minimal level required to completely inhibit complement-related hemolysis in serum samples [17]. After the induction period (study Week 8), 93.1% (27/29) of patients showed strong inhibition of hemolysis.

3.10 Safety

Eculizumab was safe and well tolerated in all patients. The majority of AEs (98.3%) were reported as mild or moderate. There were no patient deaths during the study, and no patients withdrew participation due to an AE. There was a single infection-related serious AE (pyrexia) which was not reported as probably or definitely related to drug.

The most frequent treatment-emergent AEs were headache (52%), nasopharyngitis (41%), and nausea (21%). Notably, of 15 patients who reported headache, 14 did so within 1 day of study drug infusion. All headaches were reported as mild or moderate in severity and were effectively treated with over-the-counter medications. The frequency of headaches reduced from 45% during the first 4 weeks to only 14% during the following 8 weeks. Other AEs that were reported with ≥10% incidence were diarrhea (14%), eczema (10%), pyrexia (10%), and vomiting (10%) (Table 3). Most (88%) infection-related AEs were mild and no infection-related events were reported as probably or definitely related to drug. No meningococcal infections were reported during the treatment period. There were no deaths and no pregnancies, major adverse vascular events, or serious hemolysis events during the study.

Table 3 Frequently (≥10%) reported treatment-emergent adverse events

Preferred term	Patients, *n* (%)	
	Eculizumab ($N = 29$)	Placebo[a] ($N = 44$)
Total patient reporting	28 (96.6%)	37 (84.1%)
Serious AE	1 (3.5%)	5 (11.4%)
Withdrawal due to AE	0 (0.0%)	0 (0.0%)
AEs that were mild or moderate	(98.3%)	(97.0%)
Headache	15 (51.7%)	9 (20.5%)
Nasopharyngitis	12 (41.4%)	5 (11.4%)
Nausea	6 (20.7%)	3 (6.8%)
Diarrhea	4 (13.8%)	3 (6.8%)
Eczema	3 (10.3%)	0 (0%)
Pyrexia	3 (10.3%)	2 (4.5%)
Vomiting	3 (10.3%)	3 (6.8%)

[a] First 12 weeks of placebo from the multinational study

4 Discussion

The AEGIS trial results demonstrate that eculizumab is safe, effective and well tolerated in Japanese patients with PNH. The primary endpoint of the study, reduction of hemolysis, was achieved with a high level of statistical significance, demonstrating that treatment with eculizumab significantly suppresses chronic intravascular hemolysis 87% in Japanese patients with PNH. The response to eculizumab was immediate (within 1 week of treatment) and sustained for at least the current 12-week observation period (Fig. 1). These results are consistent with those of the multinational phase III trials of eculizumab which showed a similar 87% reduction in LDH from a median of 2042 U/L at baseline to 265 U/L at 12 weeks ($N = 140$; $P < 0.001$). The reduction in hemolysis in the multinational trials was sustained for at least the 54-month observation period in these studies (2165 U/L at baseline to 274 U/L at 18 months, $P < 0.001$, $n = 171$ and 277 U/L at 54 months, $P = 0.002$, $n = 10$) [37]. Given the globally consistent response to therapy in other multinational studies, it is expected that long-term eculizumab treatment will continue to result in sustained inhibition of hemolysis and reduction in the hemolysis-driven morbidities in Japanese patients.

At baseline, and despite ongoing immunosuppressive therapy in 4/13 patients, levels of hemolysis, fatigue, dyspnea, global health, CKD, anemia, and transfusion requirements were similar in patients with or without a history of AA or MDS. These results demonstrate that intravascular hemolysis significantly contributes to disabling symptoms in patients with PNH, irrespective of whether the patient has or does not have a history of AA or MDS. Further, our data demonstrate that chronic treatment with eculizumab effectively suppressed intravascular hemolysis and significantly improved signs and symptoms of PNH similarly in patients with or without a history of AA or MDS.

The immediate and sustained improvements in fatigue in Japanese PNH patients treated with eculizumab are similar to the improvements observed in the multinational trials. We observed that the significant improvements in fatigue are independent of any improvement in anemia, since the fatigue improvement at Week 1 preceded any observable change in Hgb levels (which did not change until Week 8, Fig. 2) [22]. The burden of fatigue in PNH is frequently underappreciated, and has historically been ascribed to concomitant anemia. The results of the current study as well as the parallel results of the multinational studies demonstrate, however, that in both Japanese and non-Japanese patients with PNH, terminal complement activation leading to intravascular hemolysis causes fatigue, and that fatigue in patients with PNH is independent of the level of

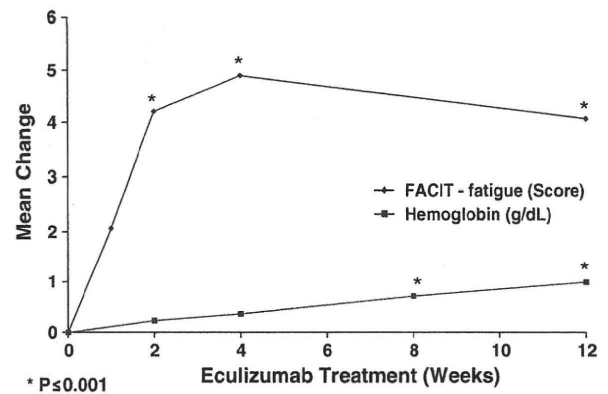

Fig. 2 Effects of eculizumab on FACIT-Fatigue scores. Treatment with eculizumab improved fatigue (as measured by FACIT-Fatigue) within 2 weeks of treatment ($P < 0.001$; *blue diamonds*). A change of 3 points is considered clinically significant. Significant improvement in hemoglobin was not seen in until Week 8 of treatment (*red squares*)

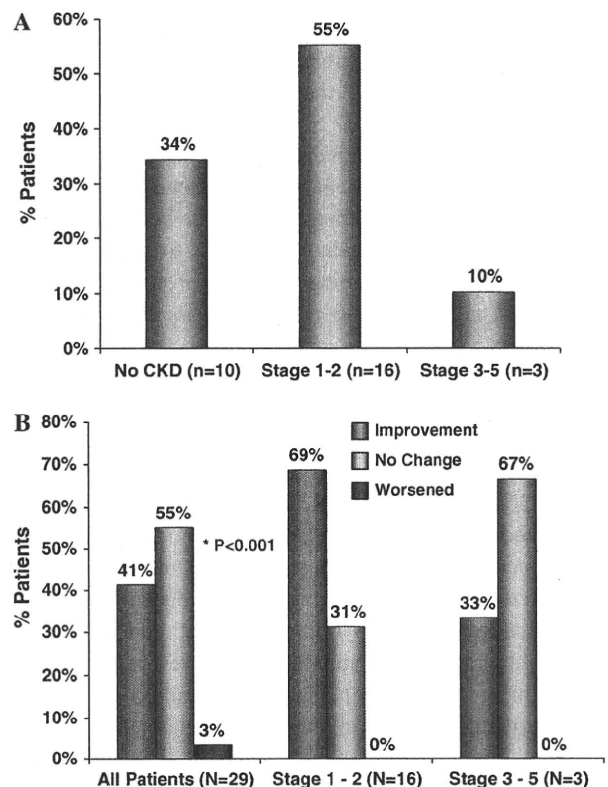

Fig. 3 Baseline and change in chronic kidney disease after first 12 weeks of eculizumab treatment. **a** CKD at baseline. **b** Treatment with eculizumab improved CKD in 41% of all patients ($P < 0.001$). 69% of patients with CKD Stage 1–2 at baseline improved and 33% of patients with CKD Stage 3–5 at baseline improved with eculizumab treatment

anemia. Hence, in patients with PNH, Hgb level alone will not accurately reflect the full burden of the disease.

Similar to fatigue, eculizumab treatment was associated with rapid improvements in dyspnea (within 1 week of eculizumab treatment, $P = 0.006$ from baseline) independent of anemia, as dyspnea improved well before any observable changes in Hgb in Japanese PNH patients (Table 2). Dyspnea is considered a manifestation of pulmonary hypertension and cardiac overload. N-terminal pro-brain natriuretic peptide (NT-proBNP), a measure of pulmonary vascular resistance and right ventricular dysfunction, is elevated in PNH patients, possibly due to elevated hemolysis and subsequent NO depletion [14, 15]. Treatment with eculizumab has been shown to reduce NT-proBNP and the reduction correlated with an increase of NO availability and improvement in dyspnea, both independent of anemia [14, 15]. Taken together, these data further demonstrate that symptoms historically associated with anemia such as fatigue and dyspnea are in fact independent of Hgb levels in patients with PNH. The reduction of terminal complement activation and chronic intravascular hemolysis appears to be directly responsible, independent of any possible improvement in anemia, for significantly reducing the severe morbidities in patients with PNH.

Transfusion requirement does not accurately reflect the disease burden or clinical risks associated with PNH. In our cohort, 2 Japanese patients had never been transfused yet demonstrated hemolysis comparable to patients that had been transfused including evidence of hemolysis, TE and CKD. The response to eculizumab in these never transfused patients was comparable to the benefits obtained with eculizumab treatment of patients who had been previously transfused in our study. Consistent with our data, a separate multinational study demonstrated that never-transfused PNH patients experience elevated hemolysis (median LDH of 1360 U/L) and that 87% had impaired QoL as documented by the patient or physician and 28% of the multinational group had clinical evidence of thrombosis [24]. Eculizumab treatment significantly reduced hemolysis and was associated with improved QoL, and reductions in thrombotic events, comparable to the results observed with treatment of the never-transfused Japanese patients in the current study. Taken together, it is apparent that hemolysis drives the signs and symptoms of PNH in both Japanese and multinational PNH patients independent of transfusions and transfusions do not appear to be a useful measure of the risks or clinical burden suffered by PNH patients.

Renal failure is a consequence of hemolysis and has a significant impact on survival in Japanese patients with PNH, accounting for 18% of deaths [27]. The incidence of renal failure is reported at 10.5% in the Japanese PNH population, similar to that reported in the US PNH population 9.6% [27]. Repetitive exposure to elevated cell-free Hgb causes renal hemosiderin accumulation, tubulointerstitial inflammation, and kidney damage [25]. In addition, NO depletion due to excess free-Hgb leads to alterations in renal blood flow and can have a direct effect on the GFR and renal plasma flow [11, 12, 34, 35]. There is also evidence of microscopic infarction playing a role in chronic renal failure [9, 19]. We found that 37% (7/19) of patients with CKD had elevated D-dimer levels, suggestive that microthrombotic infarctions may also contribute to CKD, but only in some PNH patients. Elevated D-dimer levels are not specific to CKD patients as elevated D-dimer levels were evident in 40% (4/10) of patients with no CDK. We determined that renal dysfunction or damage, as defined by stages of CKD, is common (66%) in Japanese PNH patients enrolled in the study (Fig. 3), similar to the 64% of PNH patients with CKD in the PNH multinational studies and to the 68% of PNH patients with reduced creatinine clearance studied separately [9, 19]. Treatment with eculizumab led to improvement of CKD in 41% of Japanese patients at 12 weeks. While there is no placebo control arm in the current study, the placebo group in multinational PNH did not demonstrate any likelihood of CKD improvement compared to baseline ($P = 0.78$) at 26 weeks [19, 23]. Treatment of Japanese patients with milder CKD Stage 1–2 at baseline was associated with a higher likelihood of improvement in renal function. This result is similar to the PNH multinational trials in which 64% with Stage 1–2 at baseline improved with eculizumab treatment. There were very few patients in the current trial with CKD Stage 3–5 to determine the beneficial impact, although 1 of the 3 patients improved with eculizumab treatment, and no patient worsened. In the PNH multinational clinical trials, 20% of PNH patients with CKD Stage 3–5 showed improvement and 75% remained stable over 18 months. Taken together, these data suggest that eculizumab had a pronounced and beneficial effect on pre-existing CKD within 12 weeks of chronic treatment initiation and initiation of eculizumab treatment earlier in the disease course was more likely to be associated with significant improvement in renal function.

The standard dosing regimen is designed to maintain eculizumab serum levels > 35 μg/mL, which is sufficient to completely and consistently block complement-mediated hemolysis in patients with PNH. In the multinational studies ($N = 195$), 100% of patients showed a strong response to the standard eculizumab dosing regimen as measured by a significant reduction in LDH [4, 21, 22]. In the current trial, two patients treated with eculizumab were not observed to show a rapid and strong reduction in LDH despite eculizumab serum concentration above 35 μg/mL. In these two patients, a partial drug response was observed with a reduction in complement activation as measured by

in vitro CH50 assays. This is an extremely rare event, perhaps unique to Japanese patients. Efforts are underway to examine the molecular linkage between CH50 and LDH in these two patients. Twenty-seven patients who each showed a strong response to treatment were enrolled in an extension trial and continued eculizumab treatment.

The review of safety parameters in this study shows that eculizumab, administered per the specified induction and maintenance dose, appears safe and well tolerated. Most AEs were mild (88%). There were no major adverse vascular events, thrombotic events, infusion reactions or episodes of anaphylaxis reported during the study. No patient discontinued participation in the study due to an AE or died. The safety profile of eculizumab reported in the Japanese trial was also consistent with that reported in the multinational phase III trials of eculizumab. The most frequent treatment-emergent AEs in trial were headache (52%), nasopharyngitis (41%), and nausea (21%), similar to the most common AEs in the SHEPHERD multinational study (headache 52.9%; nasopharyngitis 32%; upper respiratory tract infection 29.9%; nausea 20.6%) [4]. Furthermore, the observation is that most headaches were mild and the kinetics of the reported headaches in the Japanese trial was consistent with the observations in the SHEPHERD multinational study. Specifically, in SHEPHERD, 94% of patients who experienced headache did so within the first 48 h of drug administration and most were restricted to the first 2 weeks of therapy. A rapid increase in levels of nitric oxide has been shown to result in the transient induction of headache through vasodilatation [8], suggesting that headaches observed with eculizumab in the AEGIS and multinational PNH study populations may be related to the initial, rapid therapeutic reductions in intravascular hemolysis, cell-free Hgb, and nitric oxide consumption.

Thrombosis is the leading cause of death due to PNH in the Caucasian population, accounting for 40–67% of deaths [21]. However, past studies have suggested that TE is less prominent in Japanese patients with PNH [27, 28]. In the current study, we note that 5/29 or 17% of patients entering the Japanese PNH trial had a history of TE. This incidence is similar to the 19% observed in the randomized, double blind placebo PNH multinational phase III trial [22]. Previous studies have also demonstrated the ongoing TE risk in PNH patients, despite the absence of clinical evidence of TE [16]. Western patients with PNH have been shown to have subclinical TE (as detected by MRI) elevated levels of prothrombotic (e.g. D-dimer) and pro-inflammatory (e.g. IL-6) markers even without evidence of previous clinical thrombosis [13, 16, 40]. Consistent with these previous studies, we observed that 38% (11/29) of Japanese PNH patients had D-dimer levels above the ULN at baseline. Indeed, 52% (15/29) of Japanese patients were at demonstrably elevated risk for TE as indicated by either an elevated D-dimer measurement and/or history of documented thrombosis.

TE appears to be mediated by terminal complement activation in Japanese PNH patients. The observations that a significant proportion of Japanese PNH patients have elevated D-dimer levels at baseline and that eculizumab treatment normalizes this measure of hemostatic activation further confirm both the elevated risk for TE and the beneficial effect of eculizumab treatment in Japanese PNH patients. Indeed, the substantial reduction of TEs during eculizumab treatment in the multinational PNH study (over 281 patient years) empirically demonstrates that terminal complement activation plays a prominent role in the pathogenesis of thrombosis. There are strong similarities between Western and Japanese PNH patients. It has been clearly demonstrated that PNH evolves from the same somatic genetic mutations in the PIGA gene in both Western and Japanese populations [38, 41]. Additionally, the impact of blocking terminal complement-mediated hemolysis on significant morbidities and potentially life-threatening complications in Japanese patients is similar to that observed in the multinational trials, demonstrating that the physiology of hemolysis does not differ between the Japanese and Western population. It is possible that previously reported minor differences in patient presentation or symptoms may be related to different patterns of diagnosis of PNH or cultural sensitivities to various morbidities, low sample sizes due to rarity of the disease, or unappreciated comorbid factors in the two regions. In the future, participation in a PNH registry may be able to address cultural differences in regard to clinical manifestations in PNH patients.

Chronic eculizumab treatment provided clinically meaningful benefit to PNH patients in this study by reducing the primary manifestation of PNH, chronic intravascular hemolysis, with consequent significant improvements in fatigue, dyspnea, overall QoL, kidney disease, hemostatic activation and measures of thrombotic risk, anemia and transfusion requirements. These results demonstrate that eculizumab treatment provides significant clinical benefit to Japanese patients with PNH and the substantial reductions in morbidities and complications are consistent with the clinical benefits observed in the previous multinational studies.

References

1. Aaronson NK, Ahmedzai S, Bergman B, Bullinger M, Cull A, Duez NJ, Filiberti A, Flechtner H, Fleishman SB, de Haes JC. The European Organization for Research and Treatment of Cancer QLQ-C30: a quality-of-life instrument for use in

international clinical trials in oncology. J Natl Cancer Inst. 1993;85:365–76.
2. Audebert HJ, Planck J, Eisenburg M, Schrezenmeier H, Haberl RL. Cerebral ischemic infarction in paroxysmal nocturnal hemoglobinuria report of 2 cases and updated review of 7 previously published patients. J Neurol. 2005;252:1379–86.
3. Bessler M, Mason PJ, Hillmen P, Miyata T, Yamada N, Takeda J, Luzzatto L, Kinoshita T. Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene. EMBO J. 1994;13:110–7.
4. Brodsky RA, Young NS, Antonioli E, Risitano AM, Schrezenmeier H, Schubert J, Gaya A, Coyle L, de Castro CC, Fu CL, Maciejewski JP, Bessler M, Kroon HA, Rother RP, Hillmen P. Multicenter phase 3 study of the complement inhibitor eculizumab for the treatment of patients with paroxysmal nocturnal hemoglobinuria. Blood. 2008;111:1840–7.
5. Cella D. Manual of the Functional Assessment of Chronic Illness Therapy (FACIT) measurement system. 4th ed. Evanston, IL: Center on Outcomes, Research and Education (CORE), Evanston Northwestern Healthcare and Northwestern University; 1997.
6. Cella D, et al. The FACIT-Fatigue Scale: description, reliability and validity. Evanston, IL: Center on Outcomes, Research and Education; 2003. p. 1–18.
7. Cella D, Lai JS, Chang CH, Peterman A, Slavin M. Fatigue in cancer patients compared with fatigue in the general United States population. Cancer. 2002;94:528–38.
8. Christiansen I, Iversen HK, Olesen J. Headache characteristics during the development of tolerance to nitrates: pathophysiological implications. Cephalalgia. 2000;20:437–44.
9. Clark DA, Butler SA, Braren V, Hartmann RC, Jenkins DE Jr. The kidneys in paroxysmal nocturnal hemoglobinuria. Blood. 1981;57:83–9.
10. de Latour RP, Mary JY, Salanoubat C, Terriou L, Etienne G, Mohty M, Roth S, de Guibert S, Maury S, Cahn JY, Socie G. Paroxysmal nocturnal hemoglobinuria: natural history of disease subcategories. Blood. 2008;112:3099–106.
11. Delles C, Klingbeil AU, Schneider MP, Handrock R, Schaufele T, Schmieder RE. The role of nitric oxide in the regulation of glomerular haemodynamics in humans. Nephrol Dial Transpl. 2004;19:1392–7.
12. Gabbai FB. Effects of nitric oxide synthase blockers on renal function. Nephrol Dial Transpl. 2001;16(Suppl 1):10–3.
13. Helley D, de Latour RP, Porcher R, Arrais C, Fauroux I, Matheron J, Duval A, Shved JF, Fischer AM, Socie G. Eculizumab inhibits clot generation and endothelial dysfunction in patients with paroxysmal nocturnal haemoglobinuria. Haematologica. 2010;95:574–81.
14. Hill A, Muus P, Duhrsen U, Socie G, Risitano A, De Paz R, Van den Neste E, Zanella A, Lai J, Hillmen P, Rother R, Cella D. Improvement in fatigue with eculizumab treatment of patients with PNH occurs independent of changes in anemia. EHA. 2008.
15. Hill A, Rother RP, Wang X, Morris SM, Quinn-Senger K, Richards SJ, Bessler M, Kelly R, Hillmen P, Gladwin M. Effect of eculizumab on haemolysis-associated nitric oxide depletion, dyspnoea, and measures of pulmonary hypertension in patients with paroxysmal nocturnal haemoglobinuria. Br J Haematol. 2010;149:414–25.
16. Hill A, Reid SA, Rother RP, Gladwin MT, Collinson PO, Gaze DC, Lowe A, Guthrie A, Sivananthan MU, Hillmen P. High definition contrast-enhanced mr imaging in paroxysmal nocturnal hemoglobinuria (PNH) suggests a high frequency of subclinical thrombosis. Blood. 2006;108(11) (Abstract 979).
17. Hill A, Hillmen P, Richards SJ, Elebute D, Marsh JC, Chan J, Mojcik CF, Rother RP. Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria. Blood. 2005;106:2559–65.
18. Hill A, Richards SJ, Hillmen P. Recent developments in the understanding and management of paroxysmal nocturnal haemoglobinuria. Br J Haematol. 2007;137:181–92.
19. Hillmen P, Elebute M, Kelly R, Urbano-Ispizua A, Rother R, Khursigara G, Fu CL, Browne P, Rosse W. Long-term effect of the complement inhibitor eculizumab on kidney function in patients with paroxysmal nocturnal hemoglobinuria. Am J Hematol. 2010;85(8):553–9.
20. Hillmen P, Lewis SM, Bessler M, Luzzatto L, Dacie JV. Natural history of paroxysmal nocturnal hemoglobinuria. N Engl J Med. 1995;333:1253–8.
21. Hillmen P, Muus P, Duhrsen U, Risitano AM, Schubert J, Luzzatto L, Schrezenmeier H, Szer J, Brodsky RA, Hill A, Socie G, Bessler M, Rollins SA, Bell L, Rother RP, Young NS. Effect of the complement inhibitor eculizumab on thromboembolism in patients with paroxysmal nocturnal hemoglobinuria. Blood. 2007;110:4123–8.
22. Hillmen P, Young NS, Schubert J, Brodsky RA, Socie G, Muus P, Roth A, Szer J, Elebute MO, Nakamura R, Browne P, Risitano AM, Hill A, Schrezenmeier H, Fu CL, Maciejewski J, Rollins SA, Mojcik CF, Rother RP, Luzzatto L. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. N Engl J Med. 2006;355:1233–43.
23. Kanakura Y, Ohyashiki K, Shichishima T, Okamoto S, Ando K, Ninomiya H, Kawaguchi T, Nakao S, Nakakuma H, Nishimura J, Kinoshita T, Bedrosian C, Valentine ME, Ozawa K, Omine M. Chronic renal insufficiency in japanese patients with paroxysmal nocturnal hemoglobinuria (PNH): improvement with eculizumab treatment in the long-term follow-up of the AEGIS Study. Blood. 2009;114 (Abstract 1980).
24. Muus P, Risitano A, Castro-Malaspina H, Jones C, Fuller S, Socie G. Clinical impact of unregulated terminal complement activity in never-transfused patients with paroxysmal nocturnal hemoglobinuria. Blood. 2009;114 (Abstract).
25. Nath KA, Vercellotti GM, Grande JP, Miyoshi H, Paya CV, Manivel JC, Haggard JJ, Croatt AJ, Payne WD, Alam J. Heme protein-induced chronic renal inflammation: suppressive effect of induced heme oxygenase-1. Kidney Int. 2001;59:106–17.
26. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. 2002.
27. Nishimura J, Kanakura Y, Ware RE, Shichishima T, Nakakuma H, Ninomiya H, Decastro CM, Hall S, Kanamaru A, Sullivan KM, Mizoguchi H, Omine M, Kinoshita T, Rosse WF. Clinical course and flow cytometric analysis of paroxysmal nocturnal hemoglobinuria in the United States and Japan. Medicine (Baltimore). 2004;83:193–207.
28. Parker C, Omine M, Richards S, Nishimura J, Bessler M, Ware R, Hillmen P, Luzzatto L, Young N, Kinoshita T, Rosse W, Socie G. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. Blood. 2005;106:3699–709.
29. Parker CJ. The pathophysiology of paroxysmal nocturnal hemoglobinuria. Exp Hematol. 2007;35:523–33.
30. Rinder CS, Rinder HM, Smith BR, Fitch JC, Smith MJ, Tracey JB, Matis LA, Squinto SP, Rollins SA. Blockade of C5a and C5b-9 generation inhibits leukocyte and platelet activation during extracorporeal circulation. J Clin Invest. 1995;96:1564–72.
31. Rosse W. Paroxysmal nocturnal hemoglobinuria. In: Hoffman R, editor. Hematology: basic principles and practice. 3rd ed. Philadelphia: Churchill Livingstone, Inc. 2000.
32. Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. JAMA. 2005;293:1653–62.
33. Rother RP, Rollins SA, Mojcik CF, Brodsky RA, Bell L. Discovery and development of the complement inhibitor eculizumab

for the treatment of paroxysmal nocturnal hemoglobinuria. Nat Biotechnol. 2007;25:1256–64.

34. Schlaich MP, Schmitt D, Ott C, Schmidt BM, Schmieder RE. Basal nitric oxide synthase activity is a major determinant of glomerular haemodynamics in humans. J Hypertens. 2008;26:110–6.
35. Schneider R, Raff U, Vornberger N, Schmidt M, Freund R, Reber M, Schramm L, Gambaryan S, Wanner C, Schmidt HH, Galle J. L-Arginine counteracts nitric oxide deficiency and improves the recovery phase of ischemic acute renal failure in rats. Kidney Int. 2003;64:216–25.
36. Socie G, Mary JY, de Gramont A, Rio B, Leporrier M, Rose C, Heudier P, Rochant H, Cahn JY, Gluckman E. Paroxysmal nocturnal haemoglobinuria: long-term follow-up and prognostic factors. French Society of Haematology. Lancet. 1996;348: 573–7.
37. Socie G, Hillmen P, Muus P, Schubert J, Duhrsen U, Risitano AM, Rother RP, Brodsky RA, Szer J. Sustained improvements in transfusion requirements, fatigue and thrombosis with eculizumab treatment in paroxysmal nocturnal hemoglobinuria. ASH Annu Meet Abstr. 2007;110:3672.
38. Takeda J, Miyata T, Kawagoe K, Iida Y, Endo Y, Fujita T, Takahashi M, Kitani T, Kinoshita T. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. Cell. 1993;73:703–11.
39. Thomas TC, Rollins SA, Rother RP, Giannoni MA, Hartman SL, Elliott EA, Nye SH, Matis LA, Squinto SP, Evans MJ. Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv. Mol Immunol. 1996;33:1389–401.
40. Weitz IC, Ghods M, Rochanda L, Prazavi P, Zwicker J, Furie B, Liebman H. Eculizumab therapy results in rapid and sustained decreases in markers of thrombin generation and inflammation in patients with PNH. ASH Annu Meet Abstr. 2008;112:407.
41. Yamada N, Miyata T, Maeda K, Kitani T, Takeda J, Kinoshita T. Somatic mutations of the PIG-A gene found in Japanese patients with paroxysmal nocturnal hemoglobinuria. Blood. 1995;85: 885–92.