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研究課題名（和文）Role of MEIS1 in the immune evasion of myeloid leukemic cells

研究課題名（英文）Role of MEIS1 in the immune evasion of myeloid leukemic cells

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研究成果の概要（和文）：In acute myeloid leukemia, the transcription factor MEIS1 is critical for in vivo invasion and propagation of HOXA9-transformed leukemic cells. This project led to the discovery of an unknown function of MEIS1 : MEIS1 confers immune evasion capability to HOXA9-transformed leukemic cells.

研究成果の概要（英文）：In acute myeloid leukemia, the transcription factor MEIS1 is critical for in vivo invasion and propagation of HOXA9-transformed leukemic cells. We previously showed that MEIS1 overexpression was critical for bone marrow engraftment of leukemic cells. However, we found that restoring engraftment capacity was not sufficient for leukemia onset to occur. Indeed, we found that leukemic cells are under immune attack in vivo but have the ability to escape this immune assault. We therefore hypothesized that MEIS1 was critical for immune evasion.
This project led to the discovery that : (1) HOXA9-transformed cells are detected by FcεRI+ cells followed by eradication by T lymphocytes. (2) MEIS1 overexpression confers immune evasion capability to HOXA9-transformed leukemic cells by making cells insensitive to FcεRI+ cells.

研究分野： Leukemogenesis

キーワード： Acute Myeloid Leukemia　Immune evasion　Meis1　Oncology

１．研究開始当初の背景

Acute Myeloid Leukemia (AML) is the most frequent type of leukemia and accounts for ~90% of all acute leukemia in adults. The five-year survival rate of patients with AML is the poorest of all leukemia with only 40%, which highlights the urgent necessity to improve therapy, and thereby implying a better comprehension of the molecular mechanisms governing the onset and progression of the disease.

In past years, much effort has been made to characterize the molecular etiology of acute leukemia. It was found that nonrandom chromosomal translocations are hallmark mutations associated with human leukemia. One such translocation involves MLL (Mixed Lineage Leukemia), a gene that has been shown to be fused with over 60 different partner genes and which is associated with unfavorable survival.

The MLL gene encodes a histone methyl-transferase that regulates gene transcription. In acute leukemia, the resulting fusion genes encode constitutively active forms of MLL. Efforts have therefore been pursued to discover the critical genes regulated by MLL chimeric proteins and responsible for the transformation of hematopoietic progenitors into leukemic cells. Two genes upregulated directly by MLL were found to be sufficient for transformation : *Hoxa9* and *Meis1*, as their co-expression is sufficient to transform cells into acute myeloid leukemia.

HOXA9 is a DNA-binding homeobox protein and MEIS1 is a TALE-class homeodomain protein that acts as a DNA-binding cofactor of HOXA9. Within the hematopoietic system, HOXA9 and MEIS1 are normally expressed in stem cells and immature progenitor compartments, but they are downregulated during myeloid differentiation. **In Acute Myeloid Leukemia, downregulation of HOXA9 and MEIS1 does not occur due to the constitutive activity of MLL**. This leads to a differentiation block and the subsequent expansion of immature myeloid progenitors unable to generate mature effector cells, thereby leading to AML. Therefore, aberrant overexpression of HOXA9 and MEIS1 is now considered as a hallmark of MLL-rearranged leukemia.

Because HOXA9 and MEIS1 are transcription factors and uneasy to target for therapy, one important question was raised : what are the critical target genes of HOXA9 and MEIS1 responsible for the onset and progression of AML ? Interestingly, while overexpression of HOXA9 is sufficient to induce transformation of cells *in vitro*, HOXA9-overexpressing cells are unable to induce AML *in vivo*. Indeed, for *in vivo* propagation, co-expression of HOXA9 with MEIS1 is critically required, demonstrating that **essential target genes are exclusively upregulated in the presence of MEIS1**. This observation also suggests that the *in vivo* bone marrow environment is naturally non-adequate to HOXA9-transformed leukemic cells and that mere transformation of cells does not confer the ability to induce leukemia. Instead, in order to expand *in vivo*, HOXA9-transformed cells need an additional set of genes that are regulated by MEIS1.

２．研究の目的

The aim of this project is therefore to identify the critical MEIS target genes necessary for *in vivo* expansion of leukemic cells. Adequately, we sought to identify genes exclusively upregulated in the presence of MEIS1 through microarray analysis, by comparing the RNA content of stem cells overexpressing both HOXA9 and MEIS1 with stem cells overexpressing HOXA9 only. Our analysis revealed one important gene regulated by MEIS1 : ***Sytl1***.

Sytl1 is a synaptotagmin-like protein that promotes intracytoplasmic transportation and export of molecules and/or vesicles through interaction with Rab27a/b.

Interestingly, we found that overexpression of Sytl1 in HOXA9-overexpressing leukemic cells was sufficient to bypass MEIS1 absence and restored the ability of leukemic cells to engraft bone marrow *in vivo*. This result suggests that leukemic cells need to communicate with the *in vivo* environment through Sytl1-mediated export of molecules (secreted or located at the plasma membrane).

Surprisingly, however, although development of HOXA9-Sytl1 overexpressing cells followed the same pattern of expansion than HOXA9-MEIS1-overexpressing cells during the first two weeks following inoculation, HOXA9-Sytl1 cells gradually disappeared from mice thereafter and AML did not occur. This result suggested that Sytl1 is required for the initial engraftment of leukemic cells that allows short term expansion of cells (up to two weeks), but not for long term expansion.

These preliminary results demonstrated that one of the fundamental *in vivo* roles of MEIS1 is to provide the ability for cells to interact with the bone marrow environment, through Sytl1 expression. However, since in MEIS1-deficient cells, Sytl1 overexpression restores engraftment and expansion only temporarily (two weeks), this study also demonstrated that additional genes under the control of MEIS1 are required for long term expansion of leukemic cells. In other words, this study unveiled a previously unknown biphasic mode of expansion where Sytl1 is requested at the early phase while other MEIS1 target genes are critical for the long-lasting phase.

The purpose of this project is therefore to identify the critical MEIS1 target genes necessary for long-term expansion of leukemic cells, but also to understand the cellular mechanisms governed by these genes.
Since HOXA9-Sytl1-overexpressing cells initially expand *in vivo* before gradually disappearing, we hypothesized that these cells are eradicated by the immune system.
In other words, we hypothesized that MEIS1 may also control genes involved in the immune evasion of myeloid leukemic cells.

３．研究の方法

<u>1) Are myeloid leukemic cells under immune attack *in vivo* ?</u>
HOXA9-MEIS1-overexpressing cells will be inoculated into immune competent (C57Bl/6 mice) and immune deficient background (NOD/SCID and/or RAG2$^{-/-}$ mice).
We performed this experiments and, as expected, leukemia onset occurred more rapidly in immune deficient background(s) such as NOD/SCID and RAG2$^{-/-}$ mice.
This result demonstrated that HOXA9-MEIS1-overexpressing cells are under immune attack in C57Bl/6 mice, but have the ability to escape this immune assault.

<u>2) Is MEIS1 responsible for the immune evasion of leukemic cells ?</u>
As mentioned previously, after an initial expansion HOXA9-Sytl1-overexpressing cells gradually disappeared from C57Bl/6.
However, when these cells were inoculated into an immune deficient background (such as NOD-SCID mice), HOXA9-Sytl1-overexpressing cells expanded and leukemia occurred. **This result demonstrated that the immune system is responsible for the eradication of HOXA9-Sytl1-overexpressing cells in C57Bl/6 mice.** Therefore, unlike HOXA9-MEIS1-overexpressing cells, HOXA9-Sytl1-overexpressing cells are immunologically visible and their presence triggers an immune response that is fully detrimental for their survival. In other words, Sytl1-overexpression is sufficient to bypass MEIS1 role for bone marrow engraftment, but not for immune evasion.
Therefore, in addition of providing the ability to interact with the bone marrow environment, we uncovered a supplementary function for MEIS1, which is to confer the ability for leukemic cells to escape the immune system attack. **Consequently, MEIS1 is responsible for the immune evasion of leukemic cells.**

<u>3) What is the identity of the immune cells responsible for the attack ?</u>
In order to clarify the precise nature of this immune response, mice deficient in different compartments of the immune system will be inoculated with HOXA9-Sytl1-overexpressing cells and their expansion will be time traced.

At first, the involvement of the lymphocyte compartment (T and B cells) will be investigated through the inoculation of RAG2$^{-/-}$ mice, since T and B cells are absent from these mice. If HOXA9-Sytl1-overexpressing cells can survive in RAG2$^{-/-}$ mice, a more narrow investigation will be undertaken through the usage of CD3$^{-/-}$ (T cell deficient) and μMT (B cell deficient) mice. Concerning T cells, a more specific examination using CD4$^{-/-}$ and CD8$^{-/-}$ mice will also be conducted.
The role of Natural Killer (NK) cells will also be investigated by inoculation of HOXA9-Sytl1-overexpressing cells into NK cells-depleted C57Bl/6 mice through injection of a depleting antibody (anti-NK1.1 Ab). In addition, sensitivity of leukemic cells to NK cells killing will be verified by *in vitro* co-incubation.
Finally, involvement of dendritic cells and macrophages will also be verified by usage of CD11c-DTR and CD11b-DTR mice respectively, injected with Diphteria Toxin for depletion.

Overall, the survival of HOXA9-Sytl1-overexpressing cells into a specifically depleted immune compartment should indicate the identity of the immune cells responsible for the eradication of

leukemic cells. Predictably, cytotoxic CD8 T cells and/or NK cells may be involved in this immune reaction since these cells have been reported to be able to kill various human AML cells, although their role in the elimination of HOXA9-MEIS1- or HOXA9-Sytl1-overexpressing cells has never been investigated.

Furthermore, in parallel to *in vivo* experiments, *in vitro* co-culture of leukemic cells with splenic cells will be performed using wt or immune deficient spleens, and proliferation of leukemic cells will be time-traced.

<u>4) How does MEIS1 confer an immune shield to leukemic cells ?</u>
Unlike HOXA9-Sytl1-overexpressing cells, HOXA9-MEIS1-overexpressing cells are not deeply affected by the immune system attack and can expand in an immune competent background. **This suggests that MEIS1 affords immune escape capability, a critical protection for leukemic cells during *in vivo* propagation**.

Two scenario can be envisaged :
a) HOXA9-MEIS1-overexpressing cells are resistant to programmed cell death

In this scenario, the role of MEIS1 would be to confer death resistance. Indeed, killer cells such as CD8 T cells and NK cells can induce death of target cells through two different routes : the Fas death-receptor pathway and the perforin-granzyme pathway.
In order to determine which pathway is employed to eliminate HOXA9-Sytl1-overexpressing cells,

HOXA9 and Sytl1 will be overexpressed in bone marrow cells from $Fas^{-/-}$ mice, and the resulting $Fas^{-/-}$-HOXA9-Sytl1-overexpressing cells will be inoculated into C57Bl/6 mice to determine whether the absence of Fas confers protection. If so, *in vitro* and *ex vivo* HOXA9-MEIS1- and HOXA9-Sytl1-overexpressing cells will be stimulated with FasL to determine whether MEIS1 overexpression inhibits Fas-mediated cell death.
If not, $Fas^{-/-}$-HOXA9-MEIS1-overexpressing cells will be created and inoculated into allogeneic BALB/c mice, with $Fas^{-/-}$-HOXA9-Sytl1-overexpressing cells as control cells. In case of specific survival of $Fas^{-/-}$-HOXA9-MEIS1-overexpressing cells, but not $Fas^{-/-}$-HOXA9-Sytl1-overexpressing cells, this result will demonstrate that MEIS1 is specifically required for perforin-granzyme-mediated cell death protection.

b) HOXA9-MEIS1-overexpressing cells immunosuppress the immune system

In this interesting scenario, MEIS1 would be responsible for taking control of the immune system by inducing immune tolerance.
It has been reported that some human AML cells have the ability to downmodulate the immune system through the production or recruitment of immunosuppressive cells. For instance, some AML cells have the capacity to induce the differentiation of CD4 T cells into Tregs (regulatory T cells) *in vitro*. This ability is achieved through the production of an enzyme called Indoleamine 2,3-dioxygenase 1 (IDO1) in response to IFN-γ stimulation.
Therefore, first, HOXA9-MEIS1- and HOXA9-Sytl1-overexpressing cells will be IFN-γ-stimulated and investigated for IDO1 expression by immunoblot. In the case IDO1 would be specifically expressed by HOXA9-MEIS1-overexpressing cells, MEIS1 binding to *IDO1* promoter will be investigated by luciferase assay and ChIPseq analysis.
Second, in order to determine whether HOXA9-MEIS1-overexpressing cells have the ability to induce Tregs differentiation, these cells will be co-incubated with naive CD4 T cells (CD3-stimulated or not) and the differentiation of Tregs ($CD4^{+}CD25^{+}Foxp3^{+}$ cells) will be followed, in the presence of IFN-γ or not. The ability for HOXA9-Sytl1-overexpressing cells to induce or not the same phenomenon will also be investigated to demonstrate the specific role of MEIS1 in this process.
Alternatively, other immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs), which have a wider spectrum of immunosuppression than Tregs, have been reported to be recruited into the bone marrow of AML patients. Therefore, the presence of MDSCs in leukemic bone marrows will also be investigated by magnetic isolation.

<u>5) What are the critical MEIS1-target genes responsible for immune evasion ?</u>
In addition to the experiments mentioned above, we will seek to identify the MEIS1-target genes responsible for the process of immune evasion achieved by HOXA9-MEIS1-overexpressing cells.
We previously performed microarray analysis comparing the RNA content of HOXA9-MEIS1- with

HOXA9-Sytl1-overexpressing cells using *in vitro* cultured cells. However, it is likely that, following *in vivo* inoculation, new genes under the control of MEIS1 are expressed specifically and required to escape the immune attack. Therefore, we will perform a new microarray analysis using HOXA9-MEIS1- and HOXA9-Sytl1-overexpressing cells that have been inoculated in mice for 1-2 weeks, and compare the genes differentially expressed between the two cells, but also with the genes expressed when cells are cultured *in vitro*. This experiment should provide a good indication of the genes specifically expressed by HOXA9-MEIS1-overexpressing cells when inoculated *in vivo*. We expect genes involved in immune suppression to be highlighted.

6) Validation of MEIS-1 target genes

First of all, in order to determine whether these genes are direct target of MEIS1 or not, luciferase assays and ChIPseq analysis will be performed.

Second, in order to validate their role in immune evasion and leukemogenesis, overexpression of these genes into HOXA9-Sytl1-overexpressing cells will be performed and cells will be inoculated into C57Bl/6 mice, expecting the restoration of their survival and expansion. Also, HOXA9 and MEIS1 will be overexpressed into bone marrow cells from mice deficient for these genes (when available) to annihilate the immune evasion capacity of HOXA9-MEIS1-overexpressing cells.

Finally, investigation of the role of these genes in the immune evasion of other types of cancer will also be investigated.

４．研究成果

Our results obtained with HOXA9-Sytl1-overexpressing cells turned out to be artifactual due to *Meis1* gene contamination in our model system.

Indeed, HOXA9-Sytl1-overexpressing cells are actually unable to expand even in RAG2$^{-/-}$ mice.

However, the protein Syk was recently shown to be an indirect target of MEIS1 and overexpression of Syk can bypass MEIS1 absence.

Mohr et al. (2017), "***Hoxa9 and Meis1 cooperatively induce addiction to Syk signaling by suppressing miR-146a in acute myeloid leukemia***", Cancer Cell 31, 549-562.

Indeed, when HOXA9-Syk-overexpressing cells were inoculated into lethally irradiated C57Bl/6 mice, leukemia occurred at the same rate and speed than HOXA9-MEIS1-overexpressing cells.

We therefore cloned Syk gene and transfected HOXA9-overexpressing cells with Syk, followed by inoculation into non-irradiated C57Bl/6 mice, which are therefore not damaged for the immune system. We found that HOXA9-Syk-overexpressing cells were unable to survive into non-irradiated C57Bl/6 mice, while these cells could expand into non-irradiated NOD/SCID and RAG2$^{-/-}$ mice.

These results therefore demonstrated that the protein Syk can rescue HOXA9-transformed cells for bone marrow engraftment, but not for immune escape. Consequently, MEIS1 is responsible for the immune evasion of leukemic cells through a Syk-independent mechanism.

In addition, the fact that HOXA9-Syk-overexpressing cells were able to expand in RAG2$^{-/-}$ mice suggested that the immune reaction responsible for leukemic cell elimination was T and/or B cells-mediated. Inoculation of HOXA9-Syk-overexpressing cells into CD3$^{-/-}$ and μMT$^{-/-}$ mice showed that the immune response was T cells-mediated since HOXA9-Syk-overexpressing cells only expanded in CD3$^{-/-}$ mice.

Moreover, inoculation of HOXA9-Syk-overexpressing cells into mice did not lead to serum IgG production, giving an unlikely role for B cells.

Furthermore, *in vivo* depletion of NK cells did not rescue HOXA9-Syk-overexpressing cells, confirming the non-involvement of NK cells in leukemic cells eradication.

Therefore, T cells are the immune cells responsible for leukemic cells eradication.

In vitro cell death assays showed no difference in doxorubicin or Fas-mediated cell death sensitivity between HOXA9-MEIS-overexpressing and HOXA9-Syk-overexpressing cells, suggesting that the escape of HOXA9-MEIS1-overexpressing cells is not due to resistance to T cell-mediated cell killing.

Interestingly, *in vitro* co-culture of leukemic cells with C57Bl/6 splenocytes showed a severe proliferation inhibition on HOXA9-only and HOXA9-Syk-overexpressing cells, followed by cell death, while HOXA9-MEIS1-overexpressing cells were

unaffected.
This result demonstrates that MEIS1 protects leukemic cells from proliferation inhibition mediated by splenocytes.

Surprisingly, however, this proliferation inhibition activity generated by splenocytes was still achieved when using $RAG2^{-/-}$ spleens, demonstrating that the cells of origin for this phenomenon were not T/B lymphocytes.

We eventually found that $Fc\varepsilon RI^{+}$ cells (basophils and/or mast cells) were the cells responsible for inhibiting the proliferation of HOXA9-only and HOXA9-Syk-overexpressing cells.
Indeed, depletion of $Fc\varepsilon RI^{+}$ cells from splenocytes led to the abrogation of proliferation inhibition on leukemic cells.

Interestingly, we found that proliferation inhibition by $Fc\varepsilon RI^{+}$ cells did not require cell contact and was achieved by secreted factors since supernatant transfer was sufficient to block proliferation of HOXA9-only and HOXA9-Syk-overexpressing cells, while HOXA9-MEIS1-overexpressing cells were unaffected. Analysis of supernatant content is currently under investigation.

Finally, *in vivo* depletion of $Fc\varepsilon RI^{+}$ cells in C57Bl/6 mice rescued HOXA9-Syk-overexpressing cells, which led to leukemia onset in a similar pattern than $RAG2^{-/-}$ mice.

Overall, these results demonstrate a $Fc\varepsilon RI^{+}$ cells / T cells collaborative immune axis that detects and eliminate leukemic cells deprived of MEIS1. MEIS1 overexpression protects leukemic cells from this immune attack by conferring leukemic cells insensitivity to $Fc\varepsilon RI^{+}$ cells activity.
The search for MEIS1 target genes responsible for conferring this capacity is under process. The product of these genes could become targets for therapy in order to annihilate immune evasion of leukemic cells.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　０件）

〔学会発表〕（計　３件）

Poster：
The 5th JCA-AACR Special Joint Conference – July 2016
Title of presentation：
"*A New Role for MEIS1 in the Immune Evasion of Myeloid Leukemic Cells*".
Arnaud COUZINET, Takashi YOKOYAMA, and Takuro NAKAMURA

Poster：
The 75th Annual Meeting of the Japanese Cancer Association (JCA) – Oct 2016
Title of presentation：
"*A New Role for MEIS1 in the Immune Evasion of Myeloid Leukemic Cells*".
Arnaud COUZINET, Takashi YOKOYAMA, and Takuro NAKAMURA

Poster：
The 76th Annual Meeting of the Japanese Cancer Association (JCA) – Oct 2017
Title of presentation：
"*A New Role for MEIS1 in the Immune Evasion of Myeloid Leukemic Cells*".
Arnaud COUZINET, Takashi YOKOYAMA, and Takuro NAKAMURA

〔図書〕（計　０件）

〔産業財産権〕

○出願状況（計　０件）

○取得状況（計　０件）

〔その他〕
ホームページ等
http://www.jfcr.or.jp/english/laboratory/department/carcinogenesis/index.html

６．研究組織

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