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TITLE: The Role of Necroptosis in the Pathophysiology of Bone Marrow Failure

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We proposed that: 1) persistent spontaneous necroptosis in a very small number of hematopoietic cells might be the cause of BMF syndromes; 2) necroptotic cells release molecules which trigger the onset of T cell-dependent adaptive immune reactions causing BMF syndromes; 3) immune cells suppress BM hematopoiesis by producing inflammatory cytokines, including IFN-gamma and TNF-alpha. The dynamic alteration of IFN-gamma and TNF-alpha levels might determine disease progression in BMF syndromes. To address such hypotheses, we propose to use the “chronic BMF” model of our C"^Tak^{fs} mice to study: 1) the role of necroptosis in the pathogenesis of BMF in C"^Tak^{fs} mice by examining whether the inhibition of necroptosis can prevent BMF in such mice; 2) how IFN-gamma and TNF-alpha-induced signals are communicated in hematopoietic regulation and contribute to the BMF phenotype in C"^Tak^{fs} mice; 3) whether immune-mediated hematopoietic destruction contributes to the pathogenesis of BMF in C"^Tak^{fs} mice.

Current conclusion: CD4^{+} activated T cells, CD4^{+} memory T cells, and CD4^{+} Th1 T cells are significantly increased in C"^Tak^{fs} mice. Such cells might be the cause of BMF in C"^Tak^{fs} mice, because depletion of CD4^{+} T cells by anti-CD4 antibody treatment can almost completely restore normal hematopoiesis in C"^Tak^{fs} mice.
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Introduction:

Prior exposure to certain chemicals or infections may contribute to the development of some bone marrow failure (BMF) syndromes. Cryptic clonal genetic lesions from somatic mutations have been detected in BMF patients’ cells. In most cases, such mutant cells in BMF represent only a very small percentage of a patient’s bone marrow (BM) cells. Many BMF syndromes can evolve into clonal disorders and can progress to leukemia. An inflammatory mechanism of hematopoietic repression based on autoimmune conditions has been proposed to explain the pathogenic physiology of most if not all BMF syndromes. However, whether and how chemicals/infections and the small percentage of mutant cells actually induce these immune-inflammatory reactions remain unknown. The cellular and molecular effectors of the immune/inflammatory reactions which mediate the repression of normal hematopoiesis and promote the clonal evolution of the mutant cells have still not been completely identified due to a lack of appropriate and effective animal models.

We believe that our Mx1Cre$^{+}$Tak1$^{fx/fx}$ mice (C$^{+}$Tak$^{fx}$ hereafter) provide an excellent model system to address such questions. Without induction, the spontaneous deletion of the Tak1 gene in 2-3% of hematopoietic cells is sufficient to induce BMF in mice within 6 months. The significant increase in active T cells and inflammatory cytokines in C$^{+}$Tak$^{fx}$ mice is reminiscent of the clinical features presenting in human BMF syndromes. In addition, we found that inactivation of the TNF signal in C$^{+}$Tak$^{fx}$ mice significantly enhances the development of BMF.

Many Tak1-null cells die of necroptosis. We propose that: 1) The antigens released by necroptotic cells can stimulate autoimmune responses and inflammatory reactions. Chronic necroptotic death of a small number of hematopoietic cells in the BM might be the cause of BMF by induction of autoimmune-mediated hematopoietic destruction; 2) The hematopoietic suppression in BMF is primarily induced by IFN-$\gamma$, while TNF might antagonize IFN’s function by promoting the clonal evolution of malignant cells.

Specific aims

Aim 1: Study the role of necroptosis in the pathogenesis of BMF in C$^{+}$Tak$^{fx}$ mice by examining whether the inhibition of necroptosis (by Rip3 knockout, Rip3$^{-/-}$ hereafter) can prevent BMF in such mice.

Aim 2: Investigate how IFN and TNF-induced signals are integrated in the regulation of HSC self-renewal and contribute to BMF in C$^{+}$Tak$^{fx}$ mice. We will examine the effects of the inactivation of IFN signaling on hematopoiesis in C$^{+}$Tak$^{fx}$ mice as well as in Tak1/Tnfr compound-knockout mice.

Aim 3: Investigate whether immune-mediated hematopoietic destruction contributes to the pathogenesis of BMF in C$^{+}$Tak$^{fx}$ mice. We will examine the degree to which BMF can be prevented by anti-T lymphocyte immunosuppressive treatment.
Body:

**Aim 1:** Study the role of necroptosis in the pathogenesis of BMF in $C^+Tak^{fx}$ mice by examining whether the inhibition of necroptosis by $Rip3^{-/-}$ can prevent BMF in such mice.

Experiments designed for this Aim are delayed because our $Rip3^{-/-}$ mice were infected by norovirus. By collaborating with the transgenic animal facility at Northwestern University, we were able to clear the norovirus from the $Rip3^{-/-}$ mouse line by redervation. We crossed the $Rip3^{-/-}$ mice with $C^+Tak^{fx}$ mice. Currently, we have obtained the first batch of double-mutant mice. We are in the process of expanding the mice for analysis. We predict that we should be able to complete the experiments designed for this Aim by early next year.

**Aim 2:** Investigate how IFN and TNF-induced signals are integrated in the regulation of HSC self-renewal and contribute to BMF in $C^+Tak^{fx}$ mice. We will examine the effects of the inactivation of IFN signaling on hematopoiesis in $C^+Tak^{fx}$ mice as well as in $Tak1/Tnfr$ compound-knockout mice.

We have purchased $Ifn^{-/-}$ mice from the Jax laboratory. We crossed the $Ifn^{-/-}$ mice with $Tak1/Tnfr$ compound-knockout mice. Currently, we have obtained several mice which show deletion of all 3 genes. We are in the process of analyzing these mice. We predict that we should be able to complete the experiments designed for this Aim by early next year.

**Aim 3:** Investigate whether immune-mediated hematopoietic destruction contributes to the pathogenesis of BMF in $C^+Tak^{fx}$ mice. We will examine the degree to which BMF can be prevented by anti-T lymphocyte immunosuppressive therapy.

Significant progress has been made in this specific Aim. To determine the potential autoimmune-related BMF in $Tak1/Tnfr$ compound-knockout mice, we further examined the changes of immune cells in $Tak1/Tnfr$ compound-knockout BMF mice. We found a significant increase in CD44$^{\text{high}}$CD62L$^{\text{low}}$ memory CD4$^+$ lymphocytes (Fig. 1), CD69$^+$CD25$^+$ activating CD4$^+$ T cells (Fig. 2), Th1 and IL-10$^+$ T lymphocytes (Fig. 3) but a reduction of repressive Th2 cells (Fig. 3) in $Tak1/Tnfr$ compound-knockout mice compared to wild type (WT) littermate controls. However, Th17 and CD4$^+$CD25$^+$Foxo3$^+$ Treg cells are comparable between $Tak1/Tnfr$ compound-knockout mice and WT control mice (data not shown).

To test whether this increase in CD4$^+$ T lymphocytes is the cause of BMF in $Tak1/Tnfr$ compound-knockout mice, following the development of BMF, we injected the mice with 250µg of anti-CD4 antibody every week for a total of 4 weeks. By examining the percentage of CD4$^+$ cells after the mice were euthanized, we found that a significant reduction of CD4$^+$ T cells in peripheral blood, spleens and bone marrow of the anti-CD4 injected mice compared to isotype antibody-injected controls and WT mice, suggesting the success of CD4 T cell depletion by such antibody injection (Fig. 4). By carefully analyzing the animals, we demonstrated that anti-CD4 antibody injection can almost completely cure the BMF in $Tak1/Tnfr$ compound-knockout BMF
mice (Fig. 5), as shown by restoration of body weight and hematopoietic cells. Currently we are in the process of evaluating the role of CD8+ cells in the pathogenesis of BMF in Tak1/Tnfr compound-knockout mice by depleting CD8+ T cells using anti-CD8 antibody injection. We also isolated CD4+ T cells from Tak1/Tnfr compound-knockout mice and injected these cells into WT mice to determine whether we can induce BMF using such activated CD4+ cells.

Figure 2. Increase of CD69+CD25-activated CD4+ T cells in lymph nodes and bone marrow of Tak1/Tnfr compound-knockout mice.

Figure 3. Increased Th1 and IL-10+ T lymphocytes but reduced Th2 in Tak1/Tnfr compound-knockout mice
Figure 4. Successful depletion of CD4+ T lymphocytes in Tak1/Tnfr compound-knockout mice. After obvious BMF developed, Tak1/Tnfr compound-knockout mice were injected with 250µg of anti-CD4 antibody weekly for a total 4 injections. CD4+ and CD8+ cells were analyzed at the end of 4th week of injections.

Figure 5. Restoration of normal hematopoiesis in Tak1/Tnfr compound-knockout mice by anti-CD4 treatment. After obvious BMF developed, Tak1/Tnfr compound-knockout mice were injected with 250µg of anti-CD4 antibody weekly for a total 4 injections. Mice were sacrificed and analyzed at the end of 4th week of injections.
Research Accomplishments:

Aim 1:

We were able to obtain the first batch *Rip3*−/− *C*TakFx compound-mutant mice. We are in the process of expanding these mice and preparing them for analysis.

Aim 2:

We obtained the several *Ifnr*−/− *C*TakFx*Tnfr*−/− compound-mutant mice. We are in the process of expanding these mice and preparing them for analysis.

Aim 3:

We further verified the immune parameters in the *C*TakFx*Tnfr*−/− compound-mutant mice and found that activated and memory Th1 cells are significantly increased in such mice. By depleting CD4+ T lymphocytes in *C*TakFx*Tnfr*−/− compound-mutant mice after the development of BMF, we were able to nearly completely restore normal hematopoiesis in these mice. Our studies demonstrated that the BMF in *C*TakFx*Tnfr*−/− compound-mutant mice is due to the increased CD4+ Th1 cells. We are in the process of evaluating the role of CD8+ cells in such BMF.

Reportable Outcomes:

Increased CD4+ T cells (Th1, activated and/or memory T cells) are the cause of BMF in *C*TakFx*Tnfr*−/− compound-mutant mice.

Conclusion:

The progression of the project is moving smoothly. As of now, the only technical problem we have encountered is the noroviral infection of *Rip3*−/− mice, which has been rectified. We predict that we can complete all of the designed experiments within the originally-proposed time frame.

Increased CD4+ T cells (Th1, activated and/or memory T cells) are the cause of BMF in *C*TakFx*Tnfr*−/− compound-mutant mice.