Expression of membrane-bound IL-15 by bone marrow fibroblast-like stromal cells in aplastic anemia

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Keywords: aplastic anemia, bone marrow, bone marrow fibroblast-like stromal cells, bone marrow stromal cells, IL-15

Abstract

In order to explore the relationship between IL-15 and aplastic anemia (AA), bone marrow (BM) fibroblast-like stromal cells (BMFSCs) were obtained from BM samples of 23 AA patients by density centrifugation and primary culturing in vitro. Indirect immunofluorescence labeling as well as flow cytometry and confocal laser scanning microscopy analysis were used to determine the expression of membrane-bound IL-15 (mIL-15) on the surface of BMFSCs derived from AA patients (AA-BMFSCs). The effects of IFN-γ and cyclosporin A (CsA) on the expression of mIL-15 were also investigated. [³H]thymidine incorporation test as well as specific antibody inhibition and Transwell separation experiment was adopted to functionally evaluate the expression of mIL-15 on the surface of AA-BMFSCs. mIL-15 was found to be over-expressed on the surface of AA-BMFSCs. IFN-γ further significantly up-regulated its expression, which, however, was inhibited by CsA. Interestingly, a tight correlation was found between the expression of mIL-15 and IL-15Rα on the surface of AA-BMFSCs. AA-BMFSCs had the capability to stimulate the proliferation of T lymphocytes, which was partly or completely inhibited by using neutralizing anti-IL-15Rα antibody, neutralizing anti-IL-15 antibody, blocking anti-IL-2/15Rc mAb or Transwell chambers with a 0.3-µm pore size membrane to block the direct cell-to-cell contact between AA-BMFSCs and T cells. Apparently, BMFSCs as the most important component of BM hematopoietic microenvironment usually over-express mIL-15 in AA patients. Therefore, AA-BMFSCs may indirectly participate in the T cell-mediated destruction of hematopoietic progenitors in AA by recruiting T cells to BM and stimulating them in situ.

Introduction

IL-15, an important T cell growth factor, was originally discovered as a T cell stimulatory activity that mimics several biological effects of IL-2. Like IL-2, IL-15 induces T cell proliferation and chemotaxis, stimulates IFN-γ production and generates cytotoxic effector cells (1–5). However, both IL-2 and IL-15, which belong to the 4α-helix bundle cytokine family and share two receptor subunits (IL-2Rβ chain and common γ chain), also provide distinct and contrasting contributions to T cell-mediated immune responses. Due to its pivotal role in activation-induced cell death (AICD), IL-2 controls peripheral tolerance by the elimination of self-reactive T cells (6, 7). In contrast, IL-15 manifests anti-apoptotic actions and inhibits IL-2-mediated AICD and stimulates persistence of memory phenotype CD8+ T cells (7–9). This carries the risk to the organism of the survival of autoreactive T cells that could lead to the development of autoimmune diseases (AIDs) (10). Indeed, laboratory and clinical evidence accumulated in recent 5 years has confirmed that IL-15 over-expression is associated with an array of inflammatory AIDs, such as inflammatory bowel disease, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis (RA) and pulmonary sarcoidosis (11, 12).

Aplastic anemia (AA) is an uncommon but serious disorder characterized by pancytopenia resulting from non-function of the bone marrow (BM). Substantial clinical and experimental evidence indicates that T cell-mediated suppression of
hematopoiesis is the most important pathogenic mechanism of BM failure in AA (13, 14). To date, AA is regarded as a special BM failure disease which is usually mediated by abnormally activated T cells, sharing pathophysio logical mechanisms with other organ-specific AIDs, in which T<sub>0</sub>1/TC1 cells affect organ-specific destruction (14). In AA, CD34<sup>+</sup> progenitors express abnormally high level of Fas, suggesting that Fas-mediated cell death of hematopoietic progenitor and stem cells plays a key role in the formation of severe pancytopenia (15).

In AA patients, apart from stem cells, BM stromal cells (BMSCs) also show a certain degree of dysfunction. BMSCs comprise a multifunctional tissue consisting of heterogeneous cell populations that provide a specialized microenvironment (‘stem cell niches’) in BM for controlling the process of hematopoiesis (16). Although BMSCs from AA patients (AA-BMSCs) were found to be able to support the generation of granulocyte macrophage colony-forming units from normal hematopoietic cells (17), some cytokines, such as granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor and macrophage inflammatory protein-1α, were found to be significantly higher in the supernatants from AA-BMSCs cultured in vitro (18), indicating that abnormality exists within the microenvironment of hematopoiesis. In addition, BMSCs have been proven to be potential IL-15-expressing cells (19).

Thus, we hypothesize that in AA patients there may be an abnormal expression of IL-15 by AA-BMSCs, which may contribute to T cell recruitment and expand in BM. To test this hypothesis, we selected BM fibroblast-like stromal cells (BMFSCs), a major and representative cell population of BMSCs (20), as targeting cells. Since we previously showed that there was no soluble IL-15 (sIL-15) in the supernatants from AA-BMSCs and IL-15 has recently been found to be expressed in membrane-bound form (21–23), in the present study, we focused on analyzing membrane-bound IL-15 (mIL-15) expressed on the surface of BMFSCs from AA patients (AA-BMFSCs).

**Methods**

**Patients and controls**

Twenty-three AA patients with a median age of 27 years (range 11–65 years of age) were included in the study (Table 1). The diagnosis of AA was established by BM biopsy and peripheral blood (PB) cell counts according to the criteria of the International Aplastic Anemia and Agranulocytosis Study (24). BM was aspirated from all patients at diagnosis. Control marrow specimens were obtained from 10 BM transplant donors with median age of 30 years (range 18–53 years). Informed consent was obtained from all patients and healthy donors.

**Primary BMFSC cultures**

BM specimens were obtained from different sites and depths along the posterior iliac crest, to minimize contamination with PB cells. The aspirates were diluted immediately with 15 ml RPMI 1640 containing heparin. Erythrocytes were lysed from buffy coats in hemolytic buffer (155 mmol l<sup>–1</sup> NH<sub>4</sub>Cl, 100 mmol l<sup>–1</sup> KHCO<sub>3</sub>, 1 mmol l<sup>–1</sup> EDTA) for 2 min at 37°C. Low-density BM mononuclear cells (BMMNCs) (<1.077 g ml<sup>–1</sup>) were separated and collected by Ficoll–Hypaque (Shanghai Second Chemistry Factory, Shanghai, China) density gradient centrifugation and used to establish BMFSCs cultures according to the method described by Khaldoyanidi and his colleagues (20) with a slight modification. BMMNCs were plated at 1 × 10<sup>6</sup> cells ml<sup>–1</sup> in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 12.5% FCS, 12.5% horse serum, 2 mmol l<sup>–1</sup> L-glutamine, 100 U ml<sup>–1</sup> penicillin, 100 μg ml<sup>–1</sup> streptomycin, 1 mmol l<sup>–1</sup> pyruvate sodium (Sigma, St Louis, MO, USA), 10<sup>–6</sup> mol l<sup>–1</sup> hydrocortisone sodium succinate (Sigma) and 10<sup>–4</sup> mol l<sup>–1</sup> β-mercaptoethanol (Sigma). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 24 h. Thereafter, non-adherent cells were washed out and the remaining cells were further cultured under the same conditions. Cultures were re-fed once a week by exchanging half of the supernatant with an equal volume of fresh medium. After 4 weeks, the cells reaching confluence were used for experiments.

**Cell morphology and cytochemical stains**

BMFSCs were seeded directly on glass coverslips in 6- or 24-well plates (Corning-Costar, Cambridge, MA, USA) and grew to confluence. After being rinsed twice with PBS and dried on air, the BMFSCs layers were stained with Wright’s stain, alkaline phosphatase and non-specific esterase. Alkaline phosphatase was demonstrated by Gomori method (25). The non-specific esterase activity was demonstrated using alpha naphthyl acetate as a substrate (26).

**Flow cytometric analysis**

BMFSCs were trypsinized and washed before two-step immunofluorescence labeling. For the analysis of IL-15, cells were first incubated with primary mouse anti-human IL-15 mAb (Clone number: E-4, Catalog number: sc-8437, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at 4°C. Then the cells were washed and further incubated with

Table 1. Characteristics of patients under study

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100 mmol l<sup>–1</sup> KHCO<sub>3</sub>, 1 mmol l<sup>–1</sup> EDTA) for 2 min at 37°C. Low-density BM mononuclear cells (BMMNCs) (<1.077 g ml<sup>–1</sup>) were separated and collected by Ficoll–Hypaque (Shanghai Second Chemistry Factory, Shanghai, China) density gradient centrifugation and used to establish BMFSCs cultures according to the method described by Khaldoyanidi and his colleagues (20) with a slight modification. BMMNCs were plated at 1 × 10<sup>6</sup> cells ml<sup>–1</sup> in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 12.5% FCS, 12.5% horse serum, 2 mmol l<sup>–1</sup> L-glutamine, 100 U ml<sup>–1</sup> penicillin, 100 μg ml<sup>–1</sup> streptomycin, 1 mmol l<sup>–1</sup> pyruvate sodium (Sigma, St Louis, MO, USA), 10<sup>–6</sup> mol l<sup>–1</sup> hydrocortisone sodium succinate (Sigma) and 10<sup>–4</sup> mol l<sup>–1</sup> β-mercaptoethanol (Sigma). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 24 h. Thereafter, non-adherent cells were washed out and the remaining cells were further cultured under the same conditions. Cultures were re-fed once a week by exchanging half of the supernatant with an equal volume of fresh medium. After 4 weeks, the cells reaching confluence were used for experiments.
The data were presented as stimulation index (SI) values calculated by using the following formula: SI = proliferation of T cells co-cultured with BMFSCs/proliferation of T cells alone. The experiments were performed at least three times for each described point.

Transwell blocking experiments
Twenty-four-well Transwell chambers with a 0.3-μm pore size membrane (Corning-Costar) were used to separate physically T cells from BMFSCs in the blocking experiments. In brief, BMFSCs were allowed to grow to confluence on the bottom of the lower Transwell chamber and treated with mitomycin-c (Sigma) at 30 μg ml⁻¹ for 30 min at 37°C. After being washed twice, the lower Transwell chamber was filled with 600 μl of RPMI 1640 (GIBCO) containing 1 μg ml⁻¹ Con A (Sigma), and 100 μl of T cell suspension (1 × 10⁶ cells ml⁻¹) was added to the upper Transwell chamber. After 56 h incubation at 37°C, T cells in upper chamber were harvested, transferred to 96-well plates (Corning-Costar) and pulsed with [³H]TdR for further 16 h.

Statistics
Statistical analysis was performed on SPSS software (Chicago, IL, USA). Statistical significance was determined using the non-parametric Mann–Whitney U test or Student’s t test. A P value of <0.05 was considered significant. Correlations were analyzed with the Spearman test.

Results

Morphological and cytochemical characteristics of AA-BMFSCs
AA-BMFSCs were composed of small spindle-shaped and stellate cells. They had more condensed nuclei and less extensive cytoplasm on Wright stain and were separated from one another. They did not form a confluent layer or a syncytium of cells. Cultures consist of alkaline phosphatase-negative and non-specific esterase-positive cells (Fig. 1).

AA-BMFSCs abnormally expressed mIL-15
By using anti-IL-15 mAb in an indirect fluorescence flow cytometric (FCM) assay, AA-BMFSCs showed different degrees of specific immunoreactivity, which, however, was not seen with BMFSCs derived from normal donors (normal

![](image-url)

Fig. 1. Morphological and cytochemical characteristics of AA-BMFSCs. Cultures were established in DMEM medium (for details see Methods). Cells were stained with benzidine–hematoxylin, alkaline phosphatase (ALP) and non-specific esterase (NSE). (a) Cells’ morphology after Wright staining. (b) Cells are negative for ALP staining. (c) Cells are positive for NSE staining.
Amounts of mIL-15, quantified as MFI ratio, in 23 AA patients were estimated as 5.62 ± 2.14, which were significantly above the values of 1.01 ± 0.30 obtained for ten healthy donors (P = 0.001) (Fig. 3). Although we detected surface expression of mIL-15, ELISA did not detect any sIL-15 in the culture supernatants. This result suggested that surface expression was not accompanied by secretion of sIL-15 and that AA-BMFSCs expressed abnormally high levels of mIL-15.

**IFN-γ significantly up-regulated expression of mIL-15 on AA-BMFSCs**

To determine whether IFN-γ was able to modulate mIL-15 expression on the surface of AA-BMFSCs, the cells were stimulated for 24 h with 500 U ml⁻¹ IFN-γ, harvested and stained for indirect immunofluorescence FCM analyses. As shown in Fig. 4, IFN-γ significantly enhanced the expression of mIL-15 on the surface of AA-BMFSCs. The values of MFI ratio for mIL-15 on IFN-γ-treated AA-BMFSCs were 22.17 ± 4.31, significantly higher than those on untreated AA-BMFSCs (P = 0.001) (Fig. 4). The increased expression of mIL-15 on AA-BMFSCs could also be visualized by using confocal laser scanning microscopic (CLSM) assay (Fig. 5). Interestingly, when the supernatants of IFN-γ-treated AA-BMFSCs were examined for sIL-15 by ELISA, no IL-15 could be detected. Apparently, IFN-γ could strongly and significantly up-regulate the constitutive expression of mIL-15, but not of sIL-15, by AA-BMFSCs.

**Cyclosporin A significantly inhibited the expression of mIL-15 up-regulated by IFN-γ**

To further investigate whether cyclosporin A (CsA), a frequently used immunosuppressant in the treatment of AA, could counteract the up-regulating effects of IFN-γ on mIL-15 expression, AA-BMFSCs were co-cultured with IFN-γ (500 U ml⁻¹) and CsA (1 µg ml⁻¹) for 24 h and harvested and determined by indirect immunofluorescence FCM analyses. The levels of mIL-15, quantified as MFI ratio, on the surface of AA-BMFSCs treated with IFN-γ and CsA were 10.87 ± 3.37, significantly lower than those on AA-BMFSCs treated only with IFN-γ (P = 0.001) (Fig. 4). The decreased expression of mIL-15 on AA-BMFSCs could also be visualized by using CLSM (Fig. 5). Except one patient whose BMFSCs poorly responded to CsA treatment, all patients responded significantly to CsA, including two patients whose BMFSCs were very sensitive to CsA treatment. Although CsA could significantly inhibit the expression of mIL-15 up-regulated by IFN-γ, most AA-BMFSCs maintained their mIL-15 expression at a relatively high level in co-cultures with IFN-γ and CsA.

The expression of mIL-15 was highly correlated with that of IL-15Rα-chain. In addition to investigate the expression of mIL-15 on AA-BMFSCs, we also detected the expression of IL-15Rα, a private binding molecule specific for IL-15, on AA-BMFSCs. The results showed that AA-BMFSCs highly expressed IL-15Rα on their surface (Fig. 6A) and that the influence of IFN-γ and CsA on the expression of IL-15Rα was comparable to that of mIL-15 (Fig. 6B). By comparing the expression of mIL-15 and IL-15Rα on the surface of
AA-BMFSCs, we found that the expression of mIL-15 was highly correlated with that of IL-15Rα (Fig. 6B). mIL-15 on the surface of AA-BMFSCs exerted biological activity
Because AA-BMFSCs express mIL-15 on their surface, we wanted to know whether mIL-15 was biologically active since IL-15 is a potent growth factor for T cell proliferation. Purified human T cells were co-cultured with mitomycin-treated AA-BMFSCs. As shown in Fig. 7, the proliferation of T cells was significantly enhanced by AA-BMFSCs as compared with T cells alone. The addition of neutralizing anti-IL-15 or blocking anti-IL-2/15Ry c antibodies to the cultures significantly inhibited T cell proliferation. Simultaneously adding the above two antibodies resulted in synergistic responses. These data suggest that IL-15 is a major mediator in T cells activation induced by AA-BMFSCs. Furthermore, the pre-incubation of AA-BMFSCs with neutralizing anti-IL-15Rα antibody resulted in significant suppression of T cell proliferation in the co-culture system, suggesting that IL-15Rα expressed on AA-BMFSCs play some role in T cell proliferation. When Transwell chambers with a 0.3-μm pore size membrane were used to separate the cell-to-cell contact between AA-BMFSCs and T cells, T cell proliferation was nearly completely abrogated, suggesting that it was mIL-15, and not sIL-15, which stimulated T cells to proliferate. Unexpectedly, there was a significant reduction in T cell proliferation when T cells were co-cultured with BMFSCs derived from normal donors.

Discussion
IL-15 is a 14–15 kDa member of the 4α-helix bundle family of cytokines. In contrast to the expression of IL-2, which is mainly controlled at the transcriptional level, the regulation of IL-15 is much more complex and takes place predominantly...
at the level of translation and translocation. The expression of IL-15 is controlled by a broad array of negative regulatory mechanisms, suggesting that IL-15 is a critical regulatory protein whose uncontrolled expression might be dangerous to cells or organisms (28). The studies on IL-15 indicate that IL-15 is an inflammatory cytokine capable of inhibiting AICD on T cells and its indiscriminate expression could be associated with serious disorders, especially T cell-mediated, organ-specific AIDs (7–12).

Recently, Oppenheimer-Marks and co-workers (29) demonstrated for RA that IL-15 was produced by endothelial cells in rheumatoid tissues and that this cytokine markedly increased transendothelial migration of T cells. Furthermore, they showed that IL-15 led to T cell accumulation in RA synovial tissues engrafted into severe combined deficiency mice in vivo. In a parallel murine model the intra-articular injection of IL-15 induced a local tissue inflammatory infiltrate of T lymphocytes. These data suggest that IL-15 can recruit and activate T cells into the synovial membrane, possibly contributing to the pathogenesis of RA. In AA, a site-directed infiltration and a local proliferation of T cells in the BM has recently been demonstrated by a quantitative immunohistochemical analysis of BM biopsies (30). To define the relationship between IL-15 and AA, we selected BMFSCs, the most important component of BM microenvironment where immune-mediated destruction of hematopoietic stem cells occurs in AA, to systematically analyze their IL-15 expression. Our results demonstrate that there exists an abnormal expression of bioactive mIL-15 on the surface of AA-BMFSCs. Accordingly, one may speculate that AA-BMFSCs abnormally expressing mIL-15 will also be able to recruit and activate T cells into the BM hematopoietic microenvironment and play a partial role in the course of BM infiltration by T lymphocytes, ultimately resulting in severe pancytopenia.

IFN-γ, a typical cytokine of Th1 cells, has been demonstrated to have an important inhibitory activity capable of directly suppressing hematopoietic cell proliferation in vitro. Studies on AA patients demonstrated that T cells infiltrating BM expressed cytosolic IFN-γ, and secreting this cytokine directly into BM microenvironments was hypothesized as a key step of hematopoietic suppression in vivo. Therefore, IFN-γ is considered to be a marker of immune activity in AA (14, 31). Recently, IFN-γ was detected to be a potent stimulus for IL-15 expression on human monocytes (21). Therefore, we investigated next whether IFN-γ was also able to modulate mIL-15 expression on the surface of AA-BMFSCs. The results demonstrated that the expression of mIL-15 on the surface...
of AA-BMFSCs was further up-regulated by treatment with IFN-γ. Since it has been confirmed that IL-15 was able to stimulate T cells to up-regulate IFN-γ (32, 33), we reason that there may exist a positive feedback loop constituted by IL-15 and IFN-γ between AA-BMFSCs and T cells.

CsA is a potent immunosuppressant first used clinically in transplantation, and it is being used more frequently in the treatment of AID, including AA. It suppresses the production of cytokines that originate from T cells, either by inhibiting nuclear factor of activated T cells or by interfering with the inducible degradation of nuclear factor-κB inhibitors. Expression of many cytokine genes in T cells is inhibited by CsA (34, 35). Recently, it was found that CsA was able to inhibit tumor necrosis factor-α (TNF-α) production by B cells, suggesting that the action of CsA is not restricted to T cells and depends on cell types and stimuli (36). Considering that immunosuppressive therapy is usually employed in AA patients and that addition of CsA to horse anti-thymocyte globulin (ATG) or rabbit anti-lymphocyte globulin (ALG) can improve response and survival rates of AA patients (14, 35), we further investigated whether CsA could inhibit the up-regulating effects of IFN-γ on the expression of IL-15. The results showed that the expression of mIL-15 on the surface of AA-BMFSCs was significantly decreased when CsA was added to the AA-BMFSCs cultured in the presence of IFN-γ. This is consistent with Cho and his co-workers’ finding, who showed that CsA decreased the levels of IL-15 and TNF-α that were spontaneously secreted from fibroblast-like synoviocytes isolated from synovial tissue of RA patients (37). Considering the long-accepted fact that CsA can inhibit the expression of IFN-γ by T cells, it is evident that CsA can disrupt the positive feedback loop described above, in not only the half-loop of IL-15 but also the half-loop of IFN-γ. Possibly, that is why adding CsA to ATG or ALG can improve AA patients’ response and survival rates (14).

The phenomenon that the expression of mIL-15 on the surface of AA-BMFSCs was highly correlated with that of IL-15Rα on T cells and the fact that there was no detectable mIL-15 on AA-BMFSCs after acid stripping (data not shown) suggest that mIL-15 on the surface of AA-BMFSCs may most possibly be IL-15Rα-bound IL-15 which, as demonstrated by Dubois (23), has a higher and wider biological activity than does sIL-15. Combining with the results of Transwell blocking experiments which showed that direct cell contact is mandatory for AA-BMFSCs to stimulate T cells, it becomes a plausible hypothesis that IL-15Rα which has a very high affinity for IL-15 ($K_D = 10 \text{ pM}$), by extremely rapid capturing and then anchoring secreted sIL-15 on AA-BMFSCs, favors and restricts the persistent T cells activation and inflammation in BM microenvironment.

The relationship between IL-15 and AA prompts us to speculate that maybe it is a prospective therapeutical strategy to use IL-15 inhibitors, such as anti-IL-15 mAb and sIL-15Rα, in the treatment of AA. This treatment could help to delete activated T cells by AICD and allow naive T cells to keep their capability to perform immune response and surveillance, in order to avoid the clonal hematologic diseases arising from AA after the current immunosuppressive therapies, such as paroxysmal nocturnal hemoglobinuria, myelodysplasia and acute leukemia. This tempting hypothesis needs our further exploration.

**Acknowledgements**

This study was supported by grants from National Basic Research Priorities Program Foundation (number 2001CB510003), Basic Figure 7. T cell proliferation stimulated by mIL-15 on the surface of AA-BMFSCs. T cells were incubated with medium, normal BMFSCs and AA-BMFSCs, or the last plus anti-IL-15 and/or anti-IL-2/15Rγγ antibodies. In the case of IL-15Rα, BMFSCs were pre-incubated with 10 μg ml⁻¹ neutralizing anti-IL-15Rα antibody before the addition of T cells to the co-culture (for details see Methods). Proliferative activity was assessed by a [³H]TdR incorporation assay. Results are expressed as SI (for details see Methods). Significance was analyzed with Student t test. Asterisk indicates significant enhancement compared with ‘T cells alone culture’ group (Student’s t test, $P < 0.05$). Hash sign indicates significant inhibition compared with ‘T cells alone culture’ group (Student’s t test, $P < 0.05$). Section sign indicates significant inhibition compared with ‘T cells + AA-BMFSCs’ group (Student’s t test, $P < 0.05$).
IL-15 and aplastic anemia

References


