Reduced perforin expression in systemic juvenile idiopathic arthritis is restored by autologous stem-cell transplantation

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Objectives. Familial haemophagocytic lymphohistiocytosis (FHL) is a disorder characterized by deficient cytotoxic T-cell function and activated macrophages, owing to a defect in the perforin gene and absent perforin expression. Because symptoms of patients with systemic juvenile idiopathic arthritis (sJIA) are sometimes clinically very similar to those with FHL, we studied whether perforin expression in sJIA patients would be reduced also.

Methods. We determined the perforin expression levels on two subsets of CD8+ cells (CD8+CD28−CD45RA− and CD8+CD28−CD45RA+) and natural killer (NK) cells from patients with sJIA under conventional treatment as well as before and after autologous stem-cell transplantation (ASCT).

Results. CD45RA− cytotoxic effector cells of sJIA patients (n=13) express significantly lower levels of perforin than polyarticular juvenile idiopathic arthritis (pJIA, n=9) patients [sJIA mean fluorescence intensity (MFI) 34.6; pJIA MFI 98.0] or control donors (MFI 124.6, n=5). A similar pattern was seen in the CD45RA+ subset. Also NK cells from sJIA patients expressed significantly less intracellular perforin (sJIA MFI 398.4; controls MFI 972.4). In four patients with sJIA who were treated with ASCT, a clear increase in perforin expression was found at 12 months after ASCT in both cytotoxic effector cell subsets (CD45RA− subset before ASCT MFI 13.2; 12 months after ASCT MFI 172.3).

Conclusion. We conclude that perforin expression can be severely reduced in sJIA. This finding may implicate defective cytotoxicity and haemophagocytosis and could thus explain why sJIA may be complicated by macrophage activation syndrome. ASCT leads to a reconstitution of the (T cell) immune system with a normal expression of perforin.

Key words: Perforin expression, Juvenile idiopathic arthritis, Stem-cell transplantation.

Systemic-onset juvenile idiopathic arthritis (sJIA) is a form of JIA which is characterized by arthritis and spiking fever, exanthema, lymphadenopathy, hepatosplenomegaly and serositis [1]. sJIA often begins with only systemic features, later followed by a chronic arthritis. The arthritis is severe, mostly polyarticular and the disease is often associated with a poor quality of life and an estimated mortality risk of 1 to 3% [1].

Because of the chronic arthritis, systemic JIA is classified as a special onset form of JIA. This classification is questionable. The pathogenesis of systemic JIA could be different from the other onset forms, which is
underlined not only by the characteristic clinical presentation and course of the disease but also by the occurrence of the macrophage-activating syndrome (MAS). MAS, a serious complication of systemic JIA, is characterized by fever, progression of hepatosplenomegaly, sudden pancytopenia, coagulopathy and neurological abnormalities [2, 3]. MAS occurs mainly in systemic JIA, but the complication is not always recognized as such, because of the similarity of symptoms. The different pattern of the fever, the pancytopenia and the coagulation abnormalities are characteristic for MAS. MACs can be considered as a reactive haemophagocytic lymphohistiocytic disorder.

Other diseases or syndromes that are characterized by haemophagocytosis are familial haemophagocytic lymphohistiocytosis (FHL) and the virus-associated haemophagocytic syndrome (VAHS) [4–6].

FHL is a rare, autosomal recessive disease with a rapid fatal outcome, which occurs in previously healthy infants or young children. The disease presents itself with fever, hepatosplenomegaly, pancytopenia, coagulation disorders and neurological abnormalities. Accumulation of activated macrophages and CD8+ DR+ lymphocytes characterizes this disease. A hallmark of the diagnosis is the haemophagocytosis in the bone marrow, spleen, liver, lymph nodes or central nervous system. The function of the T cells and natural killer (NK) cells has often been reported to be defective [7]. Antigens on a target cell (such as an infected T cell) are presented to cytotoxic T cells that will lyse the target cell by a perforin-dependent process. When these activated cytotoxic T cells are not down-regulated, they will continue to activate macrophages, thus causing the characteristic clinical and laboratory features.

Linkage analysis has shown that FHL is heterogeneous and linked to 9q 21.3–22, 10q 21–22 and other loci [8, 9]. Lymphocytes of these patients have a defective cytotoxic activity and perforin is not, or hardly, demonstrable on these lymphocytes. Because of the clinical similarity between FHL and systemic JIA we investigated the expression of perforin in systemic JIA and showed defective perforin expression on CD8 T cells and NK cells of systemic JIA patients.

Patients and methods

Study population

Twenty-two patients under conventional treatment (10 females, 12 males, ages 2 to 15 yr) were included [sJIA n = 13; polyarticular JIA (pJIA) n = 9]. The diagnosis was made using the recently revised classification criteria for JIA [10]. The duration of the disease varied between 1 month and 10 yr (mean 5 yr). Medication used at the time of blood sampling was nonsteroidal anti-inflammatory drugs (NSAIDs, n = 19), corticosteroids (n = 7, all sJIA), methotrexate (n = 16), cyclosporin A (n = 2) and sulphasalazine (n = 2). No patients were on etanercept. To exclude a possible drug-induced effect, three patients were tested early in the course of their disease before corticosteroids or methotrexate were prescribed. In addition, four patients with JIA were included and tested before and after autologous stem-cell transplantation that was performed for severe and drug-resistant sJIA with polyarticular course, as described elsewhere [11].

Laboratory methods

Peripheral blood mononuclear cells from heparinized blood were isolated by Ficoll-Paque and stored in liquid nitrogen until later use. Perforin and granzyme expression levels in cytotoxic effector cells and in NK cells were determined by three- or four-colour immunofluorescence. For analysis of cytotoxic effector cells, samples of 10⁶ mononuclear cells had their surfaces stained with PerCP-conjugated CD8 (Becton Dickinson, San Jose CA, USA), allopurinol-conjugated CD28, and phycoerythrin-conjugated CD45RA (Coulter Clone, Miami, FL, USA). For NK cells, surface staining was performed with phycoerythrin-conjugated CD16 and CD56 antibodies (Becton Dickinson). Cells were then fixed for 30 min with 2% buffered formaldehyde and washed with phosphate-buffered saline (PBS). Cells were then permeabilized with 0.3% saponin in PBS for 10 min, preincubated with 2.5% v/v normal human serum (NHS) in PBS and stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-human perforin antibody (Ancell, Bayport, MN, USA) or with biotinylated granzyme A antibody (a gift of Dr E. Hack, Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam). In the latter case, this was followed by an incubation with streptavidin-CyChrome (BD Pharmingen, San Diego, CA, USA). Finally, cells were washed and fixed in 2% formaldehyde. Cells were run on a fluorescence activated cell sorter (FACS) Calibur (Becton Dickinson) equipped with an argon laser (488 nm excitation light) and a red diode laser (635 nm excitation light). Data were analysed using Cellquest software (Becton Dickinson) and expressed as mean fluorescence intensity (MFI). Statistical analysis was performed using a two-sided Student’s t-test.

Results

CD8+ cells were analysed for the expression of molecules involved in cellular cytotoxicity, i.e. perforin and granzyme A. Perforin is preferentially expressed in cytotoxic effector cells (CD8+CD28−CD45RA− and CD8+CD28−CD45RA+) and in NK cells, so perforin and granzyme were determined in these phenotypically distinct subsets. In CD8 cells obtained from nine out of 12 patients with sJIA, the expression of perforin was severely impaired, while granzyme expression was normal (Fig. 1). As shown in Table 1, CD8+CD45RA− cells of sJIA patients express significantly lower (P < 0.05) levels of perforin than pJIA patients (MFI in sJIA 34.6; in pJIA 87.6; in healthy control donors 120.7). Also CD8+CD45RA+ cells and NK cells from sJIA patients expressed significantly less (P < 0.01) intracellular perforin than healthy controls or patients with other forms of JIA (Table 1). Although mean perforin expression levels were decreased in sJIA, considerable variation did exist between individual patients. We therefore looked for a potential correlation between parameters of disease activity and expression of perforin. There is no clear correlation between the level of perforin expression and disease duration or medication use, since three newly diagnosed patients who had not yet been treated with corticosteroids or methotrexate also showed decreased perforin expression (Table 2). In addition, three patients
with active systemic disease had a normal perforin expression (MFI over 100) in the cytotoxic T-cell subsets. The normal perforin expression in these three patients was maintained over time. However, the number of patients studied is too low to conclude whether there is an association between disease activity and perforin expression.

In four patients with sJIA who were treated with autologous stem-cell transplantation (ASCT), perforin expression was analysed before and 12 months after transplantation. ASCT is only performed in very severe and drug-resistant cases of sJIA or pJIA. ASCT was found to induce disease remission in the majority of patients with drug-resistant sJIA, as described earlier [11, 12]. In all four patients a clear increase in perforin expression was found in both cytotoxic effector subsets (Table 1 and Fig. 1).

We conclude that perforin expression can be severely reduced in active systemic JIA. If indeed low perforin expression does induce haemophagocytosis in FHL, our finding may explain why sJIA can be complicated by macrophage activation syndrome. ASCT leads to a reconstitution of the (T cell) immune system and perforin expression returns to normal.

**Discussion**

Systemic JIA is a special onset form of JIA. It occurs early in childhood and equally affects boys and girls. Unlike rheumatoid arthritis occurring in adults, there is no clear HLA association and autoantibodies (rheumatoid factor, antinuclear antibodies) are lacking. It is the occurrence of systemic features that sets sJIA apart from other onset forms of JIA. The pathogenesis of sJIA is unknown. Cytokines like tumour necrosis factor (TNF)-α and interleukin (IL)-6 promoters are risk factors, whereas the macrophage-activating syndrome highlights the role of macrophages in sJIA [3, 13, 14]. MAS is a serious complication of sJIA with a high mortality rate. The disease may be induced by medication and infections, and it might occur after ASCT, but MAS is hardly ever seen in other onset forms of JIA [2, 11, 15].

**TABLE 1. Perforin expression in cytotoxic T cells and NK cells of patients with juvenile idiopathic arthritis**

<table>
<thead>
<tr>
<th>Patient group</th>
<th>n</th>
<th>CD8⁺CD28⁺CD45RA⁻</th>
<th>CD8⁺CD28⁻CD45RA⁻</th>
<th>CD16⁺CD56⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>sJIA</td>
<td>13</td>
<td>34.6 ± 2.9</td>
<td>21.6 ± 2.7</td>
<td>398.4 ± 2.8</td>
</tr>
<tr>
<td>sJIA before ASCT</td>
<td>4</td>
<td>11.1 ± 2.0</td>
<td>8.2 ± 1.5</td>
<td>n.t.</td>
</tr>
<tr>
<td>sJIA after ASCT</td>
<td>4</td>
<td>149.6 ± 1.7</td>
<td>62.6 ± 3.6</td>
<td>n.t.</td>
</tr>
<tr>
<td>Polyarticular JIA</td>
<td>9</td>
<td>87.6 ± 2.3</td>
<td>43.0 ± 1.9</td>
<td>512.9 ± 2.8</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>5</td>
<td>120.7 ± 1.4</td>
<td>169.9 ± 1.8</td>
<td>972.4 ± 1.3</td>
</tr>
</tbody>
</table>

n.t., not taken.

**FIG. 1.** Expression of perforin and granzyme in CD8 cells of systemic JIA patients. Peripheral blood mononuclear cells of a patient with systemic JIA before (left panel) or 1 yr after autologous stem-cell transplantation (right panel) had their cell surfaces stained with PerCP-conjugated CD8, allophycocyanin-conjugated CD28 and phycoerythrin-conjugated CD45RA. Cells were then fixed, permeabilized and their cytoplasm was stained with either FITC-conjugated anti-perforin (shaded histograms) or FITC-conjugated anti-granzyme A (open histograms). Data shown are from CD8⁺CD28⁺CD45RA⁻ gated cells.
As far as FHL is concerned, the absence or the functional defect of perforins on CD8+ cytotoxic T cells and NK cells may be explained by mutations in the perforin gene [7]. A possible explanation for the macrophage activation in FHL is the accumulation of activated cytotoxic T cells during infection, which in turn is caused by the absence or a functional defect of perforin [8]. The activated, perforin-deficient, cytotoxic T cells are still able to induce macrophage activation. In other words, due to the accumulation of activated cytotoxic T cells in FHL, macrophages are massively activated. This accumulation of macrophages and CD8+ cells is also seen in perforin/Fas ligand double-deficient mice [16]. These mice are unable to lyse activated macrophages, and therefore are unable to mediate negative feedback regulation by elimination of antigen-presenting cells, thereby preventing further T-cell activation.

FHL is a very serious disease which children do not survive without allogeneic bone marrow transplantation. In this respect FHL is a far more serious disease than sJIA. In analogy to FHL, we examined whether defective perforin expression could play a role in the pathogenesis of sJIA. JIA is a disease with a complex genetic trait in contrast to diseases based on monogenetic defects such as perforin-deficient FHL [17]. Since expression of perforin normalizes in sJIA after ASCT when the disease is in remission, a mutation of the gene encoding for perforin is highly unlikely. As a consequence other factors regulating the expression of perforin must play a role. The observed reduced perforin expression is specific and not due to an underlying defect of cytotoxic cell lysis since expression of granzyme was normal. At least two separate mechanisms control transgenic perforin gene expression, located in the flanking and promoter regions [18]. In addition, transcription of the perforin gene can be suppressed by Ets transcription factors [19]. During the process of differentiation into cytotoxic T effector cells, expression of perforin is induced. This process is regulated by signals derived from IL-2 receptor triggering including STAT5 [20–22].

We currently do not know if the reduced expression of perforin in sJIA patients causes an impaired cell lysis and killing of target cells. It seems logical to speculate that the degree of impairment depends on the quantity of perforin expressed. Alternative killing mechanisms such as Fas-mediated apoptosis may be up-regulated in cases of reduced perforin expression. In mice, targeted disruption of the perforin gene strongly reduces, but does not totally abolish, cytolytic T cell and NK cell activity. Alternative, perforin-independent cytolytic mechanisms are thought to compensate for defective perforin-mediated lysis [23].

Given the perforin deficiency in FHL and our findings in sJIA, it is tempting to speculate about the role of perforin in autoimmunity. There is evidence that perforin expression is important in preventing both humoral and cellular autoimmunity. Perforin-deficient CD8+ and NK cells may perpetuate a chronic proinflammatory response, as seen in sJIA. In a mouse model of graft-vs-host disease, infusion of perforin-deficient T cells ultimately leads to chronic disease with increased B cells, augmented autoantibody production and lupus-like nephritis [24]. In lupus-prone mice, perforin deficiency exacerbates autoimmunity [25]. In addition, perforin deficiency in mice leads to a decreased ability to clear viral infections and diminished tumour surveillance [23, 26, 27]. It is possible that the perforin pathway plays a role in the predisposition to or occurrence of MAS in sJIA patients. In our study, one patient had previously developed an episode of MAS. Cells were obtained some 3 months later and showed low perforin expression in both CD8 subsets and NK cells, but not lower than in other sJIA patients. We showed low perforin expression in both CD8 subsets and NK cells, but not lower than in other sJIA patients. We would expect lower perforin levels in active MAS. Given the rarity of MAS, the pancytopenia and the sudden occurrence it will be difficult to obtain cells just before or during active MAS.

In conclusion, our data indicate that 12 months after successful ASCT in sJIA, perforin expression in CD8 T cells with the phenotypic characteristics of cytotoxic effector cells and in NK cells is restored completely. We are currently setting up experiments for in vitro up-regulation of perforin in sJIA T cells. Identification of the pathways and mediators that regulate perforin expression may provide new directions of study for therapeutic intervention of systemic JIA.

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References