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Lethal NK-mediated inflammation induced by IL-12 in the absence of polymorphic and nonpolymorphic MHC class I molecules

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Abstract

Interleukin-12 is a potent activator and initiator of type-1 T cell development, and can be used as an adjuvant to bias for the development of vaccine-induced Th1 immune responses. During vaccination of MHC class I deficient beta-2 microglobulin knockout mice ($\beta 2M^{-/-}$) with an IL-12/ IL-4 Th1 biasing procedure, all of the mice died. None of the IL-12/ IL-4 treated wild type mice developed any noticeable complications. We hypothesized that NK cells may be activated by IL-12 treatment in these $\beta 2M^{-/-}$ mice, leading to necrosis and eventual death. IL-12/ IL-4 treatment of $\beta 2M^{-/-}$ mice resulted in increased NK cell numbers and activation status (IFN- γ ⁺, CD69⁺). Finally, in vivo depletion of NK cells reversed the pathology induced by IL-12/ IL-4 treatment in $\beta 2M$ deficient mice. These results indicate that IL-12 combined with IL-4 irreversibly activates NK cells leading to a disseminated inflammatory pathology and death in $\beta 2M^{-/-}$ mice.

Keywords

Interleukin 12 (IL-12); Polymorphic and nonpolymorphic MHC; Beta-2 microglobulin ($\beta 2M$); Natural killer cells (NK); Inflammation

1. Introduction

Interleukin-12 is the key component driving type-1 immune responses essential for clearance of many bacterial, viral, and parasitological infections and diseases. First discovered as an NK cell stimulatory factor (NKSF) synthesized by stimulated EBV-transformed B-cells, IL-12 was shown to activate NK cells to develop lytic potential for both NK-sensitive and normally resistant target cells, as well as drive IFN- γ production in both T cells and NK cells [1]. Subsequent studies revealed the role of IL-12 as a potent

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immunomodulator [2, 3]. Since IL-12 is the major component driving type-1 immune responses, it has been explored as both a therapeutic tool and as a biasing adjuvant in candidate vaccines. IL-12 in combination with pathogen-specific antigens in a variety of animal models efficiently invoked antigen specific type-1 immune responses leading to enhanced protection against pathogens including *T. cruzi*, *Leishmania*, and Herpes simplex virus [2, 4, 5]. In murine studies, IL-12 has been used successfully to treat tumors including carcinomas, sarcomas, and melanomas [6, 7]. Clinical trials using IL-12 have not been as successful – in fact early phase-II clinical trials involving renal cell carcinoma patients shocked the research community with severe toxic effects resulting in hospitalization of 12 of the 17 subjects and 2 deaths [8, 9]. These toxic effects were most likely due to repeated doses of large amounts of IL-12. However, since IL-12 has such great potential as a cancer therapeutic and as a vaccine adjuvant, and because it has caused severe side effects and deaths in models ranging from mice to humans, further exploration of IL-12-mediated pathology is necessary.

2. Methods

2.1 Mice and immunization regimen

Six week old Taconic wild type B6, MHC-II deficient Ab^{-/-}, and MHC-I deficient beta-2 microglobulin deficient mice ($\beta_2M^{-/-}$) were immunized with rmIL-12 (1 μ g s.c.), IL-4 monoclonal antibody 11B11 (0.5mg i.p), and *Trypanosoma cruzi* lysate (25 μ g s.c.) as shown in Fig. 1A. In some cases, mice were treated twice weekly with 0.3 mg of the NK 1.1-specific monoclonal antibody PK136 (ATCC HB-191) to deplete NK cells.

2.2 Pathologic studies

Liver, lung, heart, kidney, gastrointestinal tract, pancreas, and mesentery (abdominal and retroperitoneal fat) tissue sections were stained with hematoxylin & eosin, and analyzed by a veterinary pathologist in a blinded fashion.

2.3 NK cell flow cytometry

Spleen cells from IL-12/ IL-4 treated wild type and $\beta_2M^{-/-}$ mice were incubated at 37°C for 3–4 hours in complete medium containing monensin (BD GolgiStop 0.67 μ l/ml). Cells were surface stained with NK1.1-APC, CD3-PerCP and CD69-FITC (BD Biosciences). Intracellular cytokine staining was performed using a CytoPerm/Cytofix kit and IFN- γ -PE (BD Biosciences). Data were acquired using a Becton Dickinson FACSCalibur flow cytometer and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

In order to analyze NK cell sensitivity to IL-12 $\pm$ IL-4, naïve spleen cells from wild type and $\beta_2M^{-/-}$ mice (4×10^6 /ml) were incubated in flat bottom 24 well plates for 24 hours with varying amounts of IL-12 (0–25 ng/ml) in the presence or absence of IL-4 (10 μ g/ml). Brefeldin A (BD GolgiPlug, 1 μ l/ml) and monensin (BD GolgiStop 0.67 μ l/ml) were added for the final 6 hours of incubation, then cells were stained for surface markers and intracellular IFN- γ as described above.

2.4 NK cell activity assays

To determine NK cell function, some mice were i.p. injected with 0.2 mg polyinosinic-polycytidylic acid (poly-I:C) and spleen cells isolated the following day. YAC-1 target cells were pulsed with 50 μ Ci of Na₂⁵¹CrO₄ for 1 hour, washed 3 times, and placed in 96 well round bottom plates (1×10^4 cells per well). Spleen cells were added to these wells with 1,000 U/ml rmIL-2 (Roche) at various effector:target ratios. After 4–6 hours cell supernatants were collected using a Skatron supernatant collection system and gamma irradiation measured on an ICN Isomedic 4/600 HE gamma counter (Costa Mesa, CA).

Percent lysis of YAC-1 cells was calculated using media and 2% Triton-X100 as spontaneous and maximum release controls, respectively. Percent lysis = [(sample release cpm – spontaneous release cpm) ÷ (maximum release cpm – spontaneous release cpm)] × 100.

3. Results

3.1 Immunization with type 1 biasing agents induces pathology and mortality in $\beta_2M^{-/-}$ mice

In order to study the role of different subsets of T cells in *T. cruzi* protective immunity, we vaccinated wild type B6, MHC class II deficient Ab^{-/-} mice, and MHC class I deficient beta-2 microglobulin knockout mice ($\beta_2M^{-/-}$) with our classic Th1 biasing procedure, as detailed in Fig 1A. None of the wild type Th1-biased mice developed noticeable complications, however, all of $\beta_2M^{-/-}$ mice became sick and died (Fig 1B; $P < 0.005$). Ab^{-/-} MHC II deficient mice were unaffected by this immunization regimen (not shown). IL-12 combined with IL-4 resulted in more severe toxicity in $\beta_2M^{-/-}$ mice than treatment with IL-12 alone, and furthermore, administration of antigen is not necessary nor does it have an effect on IL-12/IL-4 induced toxicity (Fig 1C). Necropsy of these mice revealed markedly dilated small intestines, enlarged gall bladders, and multiple white foci in the abdominal fat and pancreas and perirenal fat (Figs. 1D–1G). Upon histopathologic evaluation, treated $\beta_2M^{-/-}$ mice displayed severe subserosal fat necrosis and inflammation in the gastrointestinal tracts, spleen and other abdominal and retroperitoneal organs. In the pancreas, necrosis extended into the parenchyma resulting in multifocal to diffuse necrotizing pancreatitis. None of the IL-12 + IL-4 treated wild type B6 mice studied showed any abnormalities.

3.2 NK cells activated by IL-12/IL-4 treatment cause severe pathology and death in β_2M knockout mice

On days 7 and 14 of the IL-12/IL-4 dosing schedule, spleen cells from representative mice were analyzed by flow cytometry to determine NK cell frequency and activation status (CD69 and intracellular IFN- γ expression). Wild type mice displayed consistent NK cell frequencies (Fig 2A) and activation states (Fig 2B) during the treatment cycles. However, treated $\beta_2M^{-/-}$ mice had markedly increased numbers of NK cells present in the spleen 7 days into the treatment schedule (0.47% on day 0, 6.05% on day 7). Numbers of NK cells in the spleens of $\beta_2M^{-/-}$ mice normalized by day 14 (1.35%, data not shown). Although a transient peak in NK cell numbers from treated $\beta_2M^{-/-}$ mice occurred by day 7, these cells did not seem to be activated as determined by IFN- γ and CD69 expression (NK1.1 gated population contained only 0.2% IFN- γ^+ , CD69 $^+$). However, by day 14 nearly 13% of NK cells were IFN- γ^+ , CD69 $^+$, a 65 fold increase. This effect was not seen in treated wild type mice.

Additionally, we demonstrate that NK cells obtained from naïve $\beta_2M^{-/-}$ mice are much more sensitive to IL-12 than NK cells from wild type mice. Naïve spleen cells were stimulated overnight with IL-12 ± IL-4 prior to surface staining, intracellular cytokine staining and flow cytometric analysis. NK cells from wild type and $\beta_2M^{-/-}$ mice did not spontaneously produce IFN- γ after overnight culture (Fig. 2C). However, addition of IL-12 to these cultures resulted in IFN- γ production in both wild type and $\beta_2M^{-/-}$ NK cells (>10% NK1.1 $^+$ IFN- γ^+). More importantly, a higher frequency of NK cells obtained from $\beta_2M^{-/-}$ mice produced IFN- γ after IL-12 stimulation than NK cells from wild type mice (1.3 to 2 fold increased; Figs. 2C&D.) Furthermore, IL-12 combined with IL-4 seemed to have a synergistic effect on $\beta_2M^{-/-}$ NK cells, but not in wild type B6 NK cells.

The NK1.1 specific monoclonal antibody PK136 has been used previously to eliminate NK cells in vivo. As seen in Fig 2E, spleen cells from normal B6 mice, but not from NK cell depleted B6 mice were able to lyse NK-sensitive YAC-1 cells. In order to determine the role of NK cells in the IL-12/ IL-4 pathology seen in Fig. 1, $\gamma M^{-/-}$ mice were treated with NK1.1 (or isotype control) in addition to IL-12/ IL-4 administration. As expected, $\gamma M^{-/-}$ mice treated with IL-12/ IL-4 died during treatment. However, addition of NK1.1 to the treatment regimen prevented mortality (Fig. 2F, $P < 0.05$ by 2-tailed Fisher exact test).

4. Acknowledgements

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5. Discussion

Natural killer cells target other cells for destruction by recognizing deficiencies in MHC class I expression. More specifically, mature NK cells express activating receptors that are triggered by ubiquitous cell surface ligands, but normally receive deactivating signals after recognition of self surface MHC class I. It would be expected then that NK cells present in animal models lacking proper class I MHC expression would be overly activated and destroy endogenous cells, however, the opposite holds true; NK cells from mice lacking MHC class I ($\gamma M^{-/-}$) and even NK cells from mice deficient in expression of the transporter associated with antigen processing (TAP) genes are not overtly activated, and are much more likely to remain inactive during normal NK cell stimulation events [10, 11]. The process by which NK cells develop normal function in MHC class I sufficient mice is termed NK cell licensing, whereby during maturation in the presence of self MHC class I, NK cells become competent for functional activity mediated through the balance (steady resting state) and unbalanced predominant positive signals. These licensed NK cells recognize stressed cells with reduced MHC class I expression ('missing self') and secrete cytokines and other products resulting in lysis of the target cells. In environments lacking MHC class I, or in NK cells lacking inhibitory receptor expression, NK cells are unresponsive to activation receptor-mediated stimulation and are defined as unlicensed NK cells. Cross-linking of NK1.1 surface molecules in NK cells from wild type mice (licensed) but not from γM knockout mice (unlicensed) results in high level IFN- γ expression [12]. However, NK cells obtained from mice lacking MHC class I expression are capable of producing as much IFN- γ as NK cells from wild type mice after polyclonal stimulation with PMA + ionomycin [12]. In addition, pre-stimulation with IL-2 or poly-I:C lessens the differences detected in NK cells obtained from MHC-competent (licensed) and MHC-deficient (unlicensed) host [12]. These combined results indicate that unlicensed NK cells present in MHC class I deficient hosts are able to develop effector molecules in the presence of other immune modulators. In our model system, we believe that in the absence of MHC class I expression, IL-12 in combination with IL-4 overrides normal inhibitory stimuli leading to uncontrolled NK lytic activity.

IL-12 has been studied for possible inclusion in vaccine constructs (to drive type 1 CD4⁺ and CD8⁺ T cell responses) as well as in tumor treatment (to drive NK cell-mediated clearance of cancer cells). However, several occurrences of IL-12 mediated toxicity have been reported, most notably in a phase II clinical trial in which multiple doses of rhIL-12 (5 times per week) were administered to renal cell carcinoma volunteers, resulting in the hospitalization of 12/17 patients and 2 deaths [9]. In phase I trials, a priming dose of IL-12 administered to patients prior to multiple-successive day treatment dosing seemed to protect volunteers from experiencing acute IL-12-induced toxicity such as those observed in the phase II trial in which patients were not given a priming IL-12 dose [13]. The side effects

observed in the phase II clinical trial described above may have been due to IL-12's shifting of the TH1/TH2 balance in CD4⁺ T cells, and/or in causing excessive activation of NK cells. In fact, there were lower amounts of IFN- γ in serum from patients in the phase I trial (IL-12 priming dose prior to daily dosing) compared to serum from patients in the phase II trial (no priming dose) [13].

In wild-type animals in which NK cells are licensed through MHC class I interaction with inhibitory receptors, IL-12 had no effect on the health/survival of treated mice. However, $\beta_2\text{M}$ knockout mice (lacking both MHC class I surface expression and licensed NK cells) became sick and died during the IL-12 treatment regimen. Second, IL-12 induced pathology in $\beta_2\text{M}$ knockout mice appeared to worsen upon addition of IL-4 neutralizing antibody to the IL-12 treatment regimen. IL-4 has been shown to block IL-2-induced NK cell development, activation and proliferation [14, 15]. Thus, neutralization of IL-4 may result in enhanced NK cell proliferation and activation, resulting in the enhanced clinical pathology associated with IL-12 + IL-4 treatment. Thus, our observation of worsening IL-12 mediated NK cell activity and pathology in the absence of MHC class I upon addition of -IL-4 deserves further exploration and evaluation of the relevance for the previously observed toxic effects associated with IL-12 adjuvant chemotherapy in human cancer patients.

Acknowledgments

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Abbreviations

IL-12	Interleukin 12
$\beta_2\text{M}$	Beta-2 microglobulin
NK	Natural killer cells
i.p.	Intraperitoneal
s.c.	Subcutaneous

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Highlights

- In mice and humans, repeated high doses of IL-12 induces severe side effects
- In the absence of MHC I, IL-12 plus IL-4 induces NK cell expansion and activation
- Irreversibly activated NK cells induce severe pathology (and death) in $2m^{-/-}$ mice

Figure 1. Type 1 immune bias is associated with severe toxicity in $\beta_2M^{-/-}$ mice

Wild type B6 and β_2M deficient mice were treated with two cycles of IL-12, IL-4, and antigen as shown in panel A. All of the treated wild type mice remained healthy throughout the treatment schedule, however, all $\beta_2M^{-/-}$ MHC class I deficient mice died during treatment (B, N=13 mice per group, $P < 0.005$ by Fisher 2 tailed test). In panel C, $\beta_2M^{-/-}$ mice were treated with various components of the IL-12/IL-4/T. cruzi antigen regimen. Only a small number of mice died after treatment with IL-12 alone while all mice which received IL-12 + IL-4, regardless of antigen inclusion, died during the treatment regimen (N=3 mice per group). Shown in panels D and E are H&E-stained pancreas sections from representative IL-12/IL-4 treated wild type B6 (D) and $\beta_2M^{-/-}$ mice (E). Islets are labeled

as “T” in the images, while arrows point to areas of acinar cell necrosis. Note histologically normal pancreatic acinar cells surrounding islets in sections from IL-12/ IL-4 treated wild type mice, and acinar cell necrosis present in pancreas sections from IL-12/ IL-4 treated $\alpha_2M^{-/-}$ mice. Shown in panels F and G are H&E-stained sections of mesenteric fat from representative IL-12/ IL-4 treated wild type B6 (F) and $\alpha_2M^{-/-}$ mice (G). Arrows in panel F point to small vessels, and areas of extensive fat necrosis are labeled by “N” in panel G. Note histologically normal adipocytes surrounding small vessels in IL-12/ IL-4 treated wild type tissues, while extensive fat necrosis and scattered inflammatory cell infiltration within adjacent viable fat are present in sections obtained from IL-12/ IL-4 treated $\alpha_2M^{-/-}$ mice.

Figure 2. NK cells are responsible for IL-12/ IL-4 associated toxicity in $\beta_2M^{-/-}$ mice
Spleen cells from IL-12 + IL-4 treated wild type B6 and $\beta_2M^{-/-}$ mice were analyzed by flow cytometry for NK, CD69, and IFN- γ expression. NK cells expanded >10 fold by day 7 of the treatment cycle in $\beta_2M^{-/-}$ mice (A). More strikingly, by day 14, >13 % of NK cells were activated (IFN- γ ⁺CD69⁺) in treated $\beta_2M^{-/-}$ MHC class I deficient mice, compared with only <1% in treated B6 and control $\beta_2M^{-/-}$ MHC class I deficient mice (B). In panels C and D, naïve spleen cells from wild type B6 and $\beta_2M^{-/-}$ mice were stimulated overnight with 0 – 25 ng/ml rmIL-12 with or without 10 μ g/ml IL-4 prior to determination of NK cell intracellular IFN- γ expression (C, frequencies of NK cells expressing IFN- γ ; D, normalization of NK1.1⁺/IFN- γ ⁺ frequencies to frequencies observed in IL-12 treated wild

type splenocytes. In panel E, wild type B6 mice were treated twice weekly with NK1.1 (PK136), injected with 0.2 mg poly-I:C on day 16, then harvested one day later to analyze lytic activity against NK-sensitive YAC-1 target cells. In F, $\alpha 2M^{-/-}$ mice were treated with IL-12 and IL-4 as shown in Fig 1A, and given 0.3 mg of NK1.1 (or isotype control) twice weekly. As expected, all mice receiving IL-12 and IL-4 (no PK136) died, however, mice depleted of NK cells (PK136 treated in addition to IL-12 + IL-4) remained healthy throughout the treatment regimen. (N=4 mice per group, $P < 0.05$ by Fisher 2 tailed Exact Test).