Wiskott–Aldrich syndrome protein deficiency in natural killer and dendritic cells affects antitumor immunity

Marco Catucci1, Ivan Zanoni2, Elena Draghici1, Marita Bosticardo1, Maria C. Castiello1,3, Massimo Venturini4, Daniela Cesana1, Eugenio Montini1, Maurilio Ponzoni5, Francesca Granucci2 and Anna Villa1,6

1 TIGET, San Raffaele Scientific Institute, Milan, Italy
2 Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy
3 Vita-Salute San Raffaele University, Milan, Italy
4 Experimental Imaging Center, Department of Radiology, San Raffaele Scientific Institute, Milan, Italy
5 Pathology Unit, San Raffaele Scientific Institute, Milan, Italy
6 IRGB CNR Milan Unit, Milan, Italy

Wiskott–Aldrich syndrome (WAS) is a primary immunodeficiency caused by reduced or absent expression of the WAS protein (WASP). WAS patients are affected by microthrombocytopenia, recurrent infections, eczema, autoimmune diseases, and malignancies. Although immune deficiency has been proposed to play a role in tumor pathogenesis, there is little evidence on the correlation between immune cell defects and tumor susceptibility. Taking advantage of a tumor-prone model, we show that the lack of WASP induces early tumor onset because of defective immune surveillance. Consistently, the B16 melanoma model shows that tumor growth and the number of lung metastases are increased in the absence of WASP. We then investigated the in vivo contribution of Was−/− NK cells and DCs in controlling B16 melanoma development. We found fewer B16 metastases developed in the lungs of Was−/− mice that had received WT NK cells as compared with mice bearing Was−/− NK cells. Furthermore, we demonstrated that Was−/− DCs were less efficient in inducing NK-cell activation in vitro and in vivo. In summary, for the first time, we demonstrate in in vivo models that WASP deficiency affects resistance to tumor and causes impairment in the antitumor capacity of NK cells and DCs.

Keywords: Antitumor immunity • DCs • Immune surveillance • NK cells • Wiskott–Aldrich syndrome

See accompanying Commentary by Catucci et al.

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Introduction

Wiskott–Aldrich syndrome (WAS) is a primary immunodeficiency caused by defective expression of the WAS protein (WASP) in hematopoietic cells. WAS patients are severely affected by microthrombocytopenia, eczema, recurrent infections, autoimmunity, and malignancies [1]. The most frequent malignancy reported is B-cell lymphoma, even though myelodysplasia was also observed in some patients [2, 3]. Immune deficiency has been proposed as a contributing factor in tumor pathogenesis. Indeed, immune surveillance of transformed cells is considered...
an important host protection process to prevent carcinogenesis [4]. Moreover, chronic inflammation caused by autoimmunity or recurrent infections could contribute to tumor susceptibility in WASP patients [2]. However, a recent report has demonstrated that WASP-deficient tumor-associated macrophages are defective in migration capacity and production of EGF, two functions that promote cancer invasion and metastasis [5].

The WAS murine model, Was−/− mice, well recapitulates the immune cell defects observed in WAS patients, although it does not develop overt autoimmune manifestations and neoplasia. To study how the lack of WASP in immune cells contributes to tumor onset, we crossed Was−/− mice with Cdkn2a−/− tumor-prone mice [6], in order to generate Cdkn2a−/−/Was−/− mice. The Cdkn2a gene codes for p16ink4a and p19Arf [7], thus the lack of these two proteins in Cdkn2a−/− mice impairs the suppression of neoplastic growth and induces high susceptibility to develop tumors in several tissues and organs [6]. In the present study, we report that Cdkn2a−/−/Was−/− mice are more susceptible to develop tumors, confirming the hypothesis that WASP deficiency affects immune surveillance. To evaluate tumor control, we challenged Was−/− mice by injecting B16 melanoma cells. We observed higher tumor size and an increased number of lung metastases. It has been established that NK cells play a critical role in resistance to B16 melanoma by triggering an appropriate immune response [8]. NK cells from WAS patients are less efficient in killing tumor cell lines, because of the reduced cytolytic ability, correlated with a decreased accumulation of F-actin at the immune synapse [9,10]. Moreover, Borg et al. [11] showed that NK-cell cytotoxic activity against YAC-1 cells in WAS is also affected because of the defective interaction with WASP-deficient DCs. Indeed, NK-cell activation requires triggering by DCs, which recognize tumor cells and release a peculiar set of cytokines [12]. Herein, we report that WASP deficiency in NK cells affects their ability to suppress B16 metastasis development and, in general, causes a defective response to bacterial stimuli such as LPS. Moreover, we show that Was−/− DCs are less effective in activating NK cells, thus contributing to tumor pathogenesis. In this report, we show the first in vivo evidences that WASP deficiency accelerates tumor onset and affects the antitumor capacity of NK cells and DCs.

Results and discussion

Lack of WASP leads to acceleration of tumor onset

To evaluate the contribution of WASP deficiency to tumor susceptibility, we generated Cdkn2a−/−/Was−/− mice. Similarly to Was−/− mice, Cdkn2a−/−/Was−/− mice were characterized by impaired T-cell proliferation at low doses of anti-CD3, normal T-cell proliferation to mitogen stimulation, and significant decrease in platelet counts with respect to control mice (Supporting Information Fig. 1A–C). However, survival in Cdkn2a−/−/Was−/− mice (N = 27, median survival = 204 days) was significantly reduced with respect to the Cdkn2a−/− mice (N = 23, median survival = 228 days, p = 0.035 log-rank Mantel–Cox test) (Fig. 1A). To assess whether the absence of WASP accelerates the onset of tumor and to avoid the influence of genetic defects in nonhematopoietic cells, we transplanted lethally irradiated CD45.1 C57BL/6 WT mice with total BM cells from CD45.2 WT, Was−/−, Cdkn2a−/−, or Cdkn2a−/−/Was−/− mice. One month after transplant, we analyzed blood cells by flow cytometry and found that all groups of mice were fully engrafted and equally immune reconstituted (Supporting Information Fig. 2A–C). Since hematopoietic tumors lead to the accumulation of immune cells in secondary lymphoid organs, such as spleen, with an increase of organ volume, we evaluated spleen size in living animals by ultrasound imaging 20 weeks after transplant. The mean spleen volume of WT mice transplanted with Cdkn2a−/−/Was−/− BM cells was higher, yet not significantly different (p = 0.06), as compared with the mean spleen volume of WT mice transplanted with Cdkn2a−/− BM cells (Fig. 1B). When compared with WT mice transplanted with WT BM cells, the mean spleen volume of WT mice transplanted with Cdkn2a−/− BM cells was not statistically different, while the mean spleen volume of WT mice transplanted with Cdkn2a−/−/Was−/− BM cells was significantly higher (Fig. 1B). We also found significant increase in spleen volume of untreated Cdkn2a−/−/Was−/− mice as compared to age-matched WT or Was−/− controls (data not shown). Twenty-one weeks after transplant, mice were sacrificed and lung, spleen, liver, and BM were collected for histological analysis of tumor occurrence. As expected, no tumors were found in tissues from mice transplanted with WT or Was−/− BM cells (Supporting Information Table 1). Remarkably, all mice (N = 12) transplanted with Cdkn2a−/−/Was−/− BM cells developed tumors (p < 0.005 as compared with Cdkn2a−/−, Fisher’s two-sided exact test), while only three of nine mice receiving Cdkn2a−/− BM cells showed neoplasia (Supporting Information Table 1). These findings show that immune deficiency due to the lack of WASP leads to acceleration of tumor onset in the Cdkn2a−/−/Was−/− mouse model.

Was−/− NK cells contribute to B16 metastasis development

To evaluate tumor growth in Was−/− mice, we injected s.c. into both flanks B16 melanoma cells and measured nodule size at day +7 and +14. Fourteen days after injection, we found that the volume of tumors that developed in Was−/− mice was significantly higher when compared with the volume of tumors that developed in WT mice (Fig. 1C). At the same time point, we collected the tumors and prepared single-cell suspensions to be analyzed by flow cytometry for the presence of infiltrating leukocytes. Frequencies of total CD3+, CD8+, and CD11c+ cells in tumors from Was−/− mice were similar to those found in WT controls (Fig. 1D). Remarkably, infiltrating NK cells were highly reduced in tumors from Was−/− mice (Fig. 1D). To further evaluate tumor growth control in the absence of WASP, we induced development of lung metastases by i.v. injection of B16 melanoma cells. Fourteen days after B16 cell injection, mice were sacrificed and lungs were collected. We found that Was−/− mice developed a significantly higher number of lung metastases as compared with WT mice (Fig. 1E),
Cellular immune response

NK cells are known to play a key role in resistance to development of B16 lung metastases by activating antitumor immune response via IFN-γ production [8]. To evaluate whether Was<sup>−/−</sup> NK cells contribute to the impairment in tumor growth control, we transferred NK cells isolated from the spleens of WT or Was<sup>−/−</sup> mice into Was<sup>−/−</sup> mice prior to injection of B16 cells. We found that Was<sup>−/−</sup> mice receiving WT NK cells developed fewer lung metastases as compared with mice receiving Was<sup>−/−</sup> NK cells (Fig. 1E), supporting the hypothesis that WASP is required for NK-cell antitumoral function.

**NK-cell activation is impaired both in vitro and in vivo when induced by Was<sup>−/−</sup> DCs**

NK-cell priming by DCs is an essential step in antitumor response and it requires the encounter between the two cell types, which allows a polarized release of cytokines and facilitates the recognition of costimulatory molecules expressed on the plasma membrane [12]. LPS administration is known to induce DC-dependent NK-cell activation [13]; thus, to characterize a possible defect in DC–NK-cell cross-talk, we challenged Was<sup>−/−</sup> mice with LPS and assessed the presence of IFN-γ-expressing NK cells in the spleen. Five hours after i.v. injection of LPS, we found that the frequency of IFN-γ-expressing NK cells in the spleen of Was<sup>−/−</sup> mice was significantly reduced as compared with that in WT mice (Fig. 2A). Since NK-cell activation in the spleen is dependent on the presence of DCs, we analyzed the percentage of splenic DCs in treated mice by flow cytometry without observing any relevant difference between WT and Was<sup>−/−</sup> mice (Fig. 2B). These findings show that NK-cell activation mediated by DCs is strongly impaired in Was<sup>−/−</sup> mice and such defect is not due to reduced DC numbers in the spleens of Was<sup>−/−</sup> mice. In order to discriminate between NK-cell and DC defect, we performed an in vitro functional assay by co-culturing WT or Was<sup>−/−</sup> BM-derived DCs (BMDCs) with splenic WT or Was<sup>−/−</sup> NK cells, in the presence or absence of LPS. After 48 h of co-culture, the amount of IFN-γ released by NK cells in supernatants was measured by ELISA. WT NK cells activated by WT BMDCs produced a significantly higher amount of IFN-γ than WT NK cells activated by Was<sup>−/−</sup> BMDCs, while Was<sup>−/−</sup> NK cells were strongly impaired in releasing IFN-γ, even if co-cultured with WT BMDCs (Fig. 2C). To further confirm Was<sup>−/−</sup> DC defect in inducing NK-cell activation in an in vivo model, we took advantage of the CD11c.DOG transgenic mouse [14], in which the diphtheria toxin receptor is under the control of the CD11c promoter. By injecting diphtheria toxin (DT), we depleted DCs (CD11c<sup>high</sup>) in spleen, LNs, thymus, and BM of CD11c.DOG mice. Twenty-four hours after DT administration, we transferred i.v. Was<sup>−/−</sup> or WT BMDCs to obtain mice in which only DCs were Was<sup>−/−</sup>, while all other cell types were WT. Twenty-four hours after DC...
Figure 2. (A, B) In vivo NK-cell activation. The frequency of (A) IFN-γ-producing NK cells and (B) DCs in the spleens of Was−/− or wt mice 5 h after i.v. injection of 50 μg LPS or PBS is shown. (C) In vitro NK-cell activation. IFN-γ release in supernatants of DC-NK-cell co-culture incubated for 48 h with LPS (1 μg/mL) was determined by ELISA. (D) The frequency of IFN-γ-producing NK cells in the spleens of treated mice and controls, 5 h after LPS injection, was determined by flow cytometry. All data are shown as mean ± SEM of nine mice pooled from three experiments performed. **p < 0.005, ***p < 0.001, Student’s t-test.

transfer, mice were injected i.v. with LPS and the production of IFN-γ by splenic NK cells was evaluated by flow cytometry. We found that the percentage of IFN-γ-producing NK cells in mice that had received Was−/− DCs was significantly reduced, compared with that in mice that had received WT DCs (Fig. 2D), confirming that in vivo NK-cell activation is impaired when induced by Was−/− DCs.

Was−/− DCs are less effective in controlling B16 metastasis formation

By using the CD11c.DOG mouse model described above, we also evaluated the capacity of Was−/− DCs to control tumor growth. Twenty-four hours after i.v. injection of WT or Was−/− BMDCs into DC-depleted CD11c.DOG recipients, B16 melanoma cells were injected i.v. and lung metastases formation was monitored 14 days later. As expected, DC-depleted mice that did not receive BMDCs developed higher number of lung metastases as compared with CD11c.DOG mice not treated with DT, thus confirming the requirement of DCs in controlling metastasis formation (Fig. 3A). Mice receiving WT BMDCs developed lower number of metastases compared with mice receiving Was−/− BMDCs. Interestingly, we found a higher NK-cell frequency in the lungs of mice receiving WT BMDCs compared with that in mice receiving Was−/− BMDCs (Fig. 3B), thus suggesting that Was−/− BMDCs may be defective in releasing chemokines that recruit NK cells or in inducing NK-cell proliferation. It is known that also NKT cells and CD8+ T cells play a role in tumor suppression upon triggering by DCs. IFN-γ production by NKT cells enhances cytotoxic activity of NK cells and CD8+ T cells [15], while CD8+ T cells are able to directly kill tumor cells [16]. We analyzed the percentage of NKT cells (CD3+/NK1.1+) and CD8+ T cells in lungs of treated mice, and no significant differences were observed in the percentage of NKT or CD8+ T cells (Fig. 3C and D). These data strongly suggest that Was−/− DCs are less effective in controlling lung metastasis formation and this defect is correlated to a reduced NK-cell recruitment into tumor-affected organ.

In summary, in the present work, we show that WASP deficiency affects control of antitumor response. By in vivo studies using the Was−/− mouse model, we demonstrated that both NK cells and DCs are defective in controlling lung metastasis growth. In particular, our data suggest that the cross-talk between these two immune cell populations is affected thus resulting in a defective immune surveillance in WAS.

Concluding remarks

We report the first in vivo evidence that the lack of WASP increases tumor susceptibility and accelerates tumor growth. Several studies have demonstrated that the Was−/− mouse closely
Figure 3. (A–D) Analysis of DC-depleted DOG mice, transferred with wt or Was<sup>−/−</sup> BMDCs and injected i.v. with B16 melanoma cells. (A) The number of B16 metastases, (B) the frequency of NK, (C) the frequency of CD8<sup>+</sup>T cells, and (D) the frequency of NKT cells in the lungs of treated mice were analyzed 14 days after injection with B16 cells. Data were normalized to values of DC-depleted mice and are shown as mean ± SEM of nine mice pooled from three experiments performed. **p < 0.005, Student’s t-test.

may affect DC ability to get in contact with tumor antigens as well as their capacity to prime effector cells in response to tumor detection. In NK cells, WASP can be required for polarization and release of cytotoxic vesicles, thus explaining the reduced cytolytic ability of WASP-deficient NK cells [8, 9]. Moreover, it has been shown that NK cell cytotoxic capacity can be affected by defective interaction with WASP-deficient DCs [10]. On this basis, we have evaluated the antitumor capacity of Was<sup>−/−</sup> NK cells and DCs in vivo experimental tumor models.

We generated the Cdkn2a<sup>−/−</sup>/Was<sup>−/−</sup> mouse in order to evaluate whether immune deficiency caused by the lack of WASP increases tumor occurrence in a tumor-prone murine model. As we expected, tumor onset was significantly accelerated in Cdkn2a<sup>−/−</sup>/Was<sup>−/−</sup> mice as compared with the Cdkn2a<sup>−/−</sup> mice, clearly confirming that WASP deficiency impairs tumor growth control. In order to investigate the antitumor capacity of Was<sup>−/−</sup> NK cells and DCs, we injected Was<sup>−/−</sup> mice with B16 melanoma cells. First, we found accelerated growth of subcutaneous melanoma and higher number of experimental metastases in Was<sup>−/−</sup> mice as compared with that in WT controls. Next, we showed that both Was<sup>−/−</sup> NK cells and DCs caused defective antitumor response in vivo. Since it is well known that effective NK-cell activation against tumor requires triggering by DCs [12], we investigated the capacity of Was<sup>−/−</sup> DCs to induce IFN-γ production by NK cells upon in vivo challenge with LPS. We found that Was<sup>−/−</sup> DCs are significantly less effective in activating NK cells both in vitro and in vivo. Therefore, our work provides clear evidence that WASP expression is required for NK cell and DC function against tumor development.

Materials and methods

Mice

C57BL/6 Was<sup>−/−</sup> mice were kindly provided by K.A. Siminovitch [18]. Cdkn2a<sup>−/−</sup>/Was<sup>−/−</sup> mice were generated by crossing Was<sup>−/−</sup> with Cdkn2a<sup>−/−</sup> mice. Newborns were screened to identify double mutant mice. The analysis was performed, using the following primers: Cdkn2a KO forward, 5′-GTG ATC CCT CTA CTT TTT CTT CTG ACT T-3′; Cdkn2a KO reverse, 5′-GAG ACT AGT GAG ACG TGC TAC TTC CA-3′; Cdkn2a WT forward, 5′-GTG ATC CCT CTA CTT TTT CTG ACT T-3′; Cdkn2a WT reverse, 5′-CTAAGGCTCTTTACTATTGTCT-3′; Was KO forward, 5′-ACTGAAGGCTCTTTACTATTGTCT-3′; Was KO reverse, 5′-CTACTAGGCTCTTTACTATTGTCT-3′; Was WT forward, 5′-CAATGGATTGCAGTGGTGTATAGTGTC-3′; Was WT reverse, 5′-CAATGGATTGCAGTGGTGTATAGTGTC-3′.
Sigma-Aldrich, St. Louis, MO, USA). Mice were housed under specific pathogen-free conditions and treated according to protocols approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute (IACUC 406).

**Animal follow-up**

Tumor-prone mice and controls were weekly evaluated to monitor their general health status. For blood cell analysis, samples were collected in 4.5% EDTA and analyzed with a KX21N counter (Sysmex, Kobe, Japan). For histopathology evaluation of tumor occurrence, organs (lung, spleen, liver, and BM) were collected, fixed with formalin, and embedded in paraffin. Tissue sections (4 μm thick) were stained with H&E.

**B16 tumors**

B16 mouse melanoma cells were kindly provided by Dr. V. Russo (San Raffaele Scientific Institute, Milan, Italy). For the induction of intradermal tumor, 5 × 10⁴ B16 cells were resuspended in 100 μL D-PBS (EuroClone, Pero, Italy) and injected s.c. into both flanks of mice. For the induction of tumor metastases, 1 × 10⁵ B16 melanoma cells were resuspended in 100 μL D-PBS and injected i.v. Length (a) and width (b) of tumors growing into dermal tissues were measured at the indicated time points. Tumor volume was calculated according to the formula: \( V = \frac{a^2b}{2} \) [19]. Single-cell suspensions of tumor tissues were analyzed for the presence of leukocyte infiltration by flow cytometry. For the evaluation of the number of lung metastases, lungs were removed from mice 14 days after the injection of B16 cells and metastasis were counted.

**BM transplantation**

Total BM cells were purified from CD45.2 WT, Was⁻/⁻, Cdkn2a⁻/⁻, or Cdkn2a⁻/⁻/Was⁻/⁻ mice. One million total BM cells were transferred i.v. into CD45.1 WT recipient mice lethally irradiated (910 rad). One month after BM transplantation, blood cells were collected to evaluate donor cell engraftment by flow cytometry and cell count by hemogram analysis.

**Ultrasound**

Spleen size was measured in anesthetized mice by a high-resolution ultrasound (VisualSonics Vevo2100 High Resolution Ultrasound System, Toronto, Canada). We measured the axial, transverse, and longitudinal diameters in order to calculate spleen volume.

**Cells**

For NK cell isolation, spleens of 8-week-old CD45.1 Was⁻/⁻ or WT mice were collected and single-cell suspensions were prepared. After red blood cell elimination, NK cells were isolated by positive selection using biotin-conjugated anti-CD49b (DX5) (BD Pharmingen, San Diego, CA, USA) and streptavidin-conjugated magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). NK-cell purity assessed by flow cytometry was higher than 90%. For NK-cell transfer, 2 × 10⁶ NK cells were resuspended in 100 μL D-PBS (EuroClone, Pero, Italy) and injected i.v. BMDCs were differentiated in vitro using culture medium containing granulocyte macrophage colony-stimulating factor (GM-CSF, 80 ng/mL). After 12 days of culture, expression of CD11c was higher than 90% and BMDCs obtained were used for experiments. For BMDC transfer, 1 × 10⁷ WT or 1.5 × 10⁷ Was⁻/⁻ BMDCs were resuspended in 100 μL D-PBS and injected i.v. Transfer of different amounts of WT or Was⁻/⁻ BMDCs allowed to overcome migratory Was⁻/⁻ BMDC defect, thus obtaining a similar percentage of BMDCs that entered into the spleen and lung of host mice 24 h after i.v. injection (data not shown).

**T-cell proliferation**

Splenic T cells were isolated from 8-week-old mice by means of anti-CD90 conjugated magnetic beads (Miltenyi Biotech) and stimulated for 48 h with plate-bound anti-CD3 mAbs at the indicated concentrations (clone 17A2, BD Pharmingen) or Concanavalin A (ConA, 5 μg/mL) [20]. Proliferation was measured by ³H-thymidine pulsing and liquid scintillation counting.

**In vivo NK-cell activation**

Eight-week-old Was⁻/⁻ or WT mice were injected i.v. with 50 μg LPS (Enzo Life Sciences, Lausen, Switzerland). After 5 h, mice were sacrificed and spleens were collected. Single-cell suspensions of spleens were incubated with phorbol myristate acetate (PMA, 50 ng/mL; AppliChem, Darmstadt, Germany), ionomycin (100 ng/mL; Sigma-Aldrich), and brefeldin A (10 μg/mL, Sigma-Aldrich) in complete medium at 37°C. After 3 h, cells were collected and stained for flow cytometric analysis. For intracytoplasmic detection of IFN-γ, cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Pharmingen).

**In vitro NK-cell activation**

Two hundred thousand NK cells and 1 × 10⁵ BMDCs were cocultured at 37°C with LPS 1 μg/mL in a 96-well plate in IMDM supplemented with 10% FBS, glutamine, and penicillin/streptomycin. After 48 h of co-culture, supernatants were harvested and IFN-γ released in supernatants was evaluated by Bio-Plex Technology (Bio-Rad, Hercules, CA, USA), following manufacturer’s instructions.
Flow cytometry

Single-cell suspensions were incubated with anti-CD3 (17A2), anti-CD8a (53–6.7), anti-CD11c (HL3), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD49b (DX5), anti-I-A[b] (AF6-120.1), anti-IFN-γ (XMG1.2), all purchased from BD Pharmingen. Cells were acquired on a FACS CANTO (BD Pharmingen) and analyzed with FCS Express software (version 2.3; De Novo Software).

Statistical analysis

Unless otherwise indicated, experimental groups were compared with a two-tailed Student’s t-test. p values < 0.05 were considered significant.

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Abbreviations: BMDC: BM-derived DC  DT: diphtheria toxin  WAS: Wiskott-Aldrich syndrome  WASP: WAS protein

Full correspondence: Dr. Anna Villa, TIGET, San Raffaele Scientific Institute, Via Olgettina, 58, Milan, 20132, Italy
Fax: +39-02-26434668
e-mail: villa.anna@hsr.it
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