Role for coronin 1 in mouse NK cell function

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Short title: Coronin 1 and mouse NK cells

Key words: Coronin 1; NK cells; leukocyte activation

Abbreviations: VSV, vesicular stomatitis virus; ICS, intercellular cytokine staining

Abstract

Coronin 1, a member of the evolutionary conserved WD repeat protein family is expressed in all leukocytes, but a role for coronin 1 in natural killer (NK) cell homeostasis and function remains unclear. Here, we have analyzed the number and functionality of NK cells in the presence and absence of coronin 1. In coronin 1-deficient mice, absolute NK cell numbers and phenotype were comparable to wild type mice in blood, spleen and liver. Following *in vitro* stimulation of the activating NK cell receptors NK1.1, NKp46, Ly49D and NKG2D, coronin 1-deficient NK cells were functional with respect to interferon- γ production, degranulation and intracellular Ca^{2+} mobilization. Also, both wild type as well as coronin 1-deficient NK cells showed comparable cytotoxic activity. Furthermore, activation and functionality of NK cells following Vesicular Stomatitis Virus (VSV) infection was similar between wild type and coronin 1-deficient mice. Taken together these data suggest that coronin 1 is dispensable for mouse NK cell homeostasis and function.

Introduction

Natural killer (NK) cells play an important role for early control of viral infections as well as for anti-tumor defense. Hence, NK cell deficiency is associated with poor control of common herpes- and papilloma virus infections and increased occurrence of tumors [1-7]. NK cells sense their environment for low expression of major histocompatibility (MHC) class I molecules that are often down-regulated on virus infected or tumor cells and allows to escape CD8⁺ T cell-mediated immune control [8,9]. Upon viral infection, pro-inflammatory cytokines such as type I interferons are released by virus-infected cells as well as dendritic cells which result in NK cell activation and proliferation [2]. NK cell activation is tightly controlled by an arsenal of activating and inhibitory receptors [10-12]. Moreover, direct cell-to-cell contact is needed to promote efficient NK cell activation and cytotoxic function [10,13]. Hence, whether NK cells become activated depends on the balance and strength of the activating and inhibitory signals.

Coronin 1 is a member of the highly conserved WD-repeat containing protein family of coronins. Coronin 1 is expressed in all leukocytes as well as a subset of neurons [14-17]. In leukocytes, coronin 1 regulates the survival of pathogenic mycobacteria within macrophages as well as naïve T cell homeostasis [14,18-20]. Whereas initially, coronin 1 was proposed to regulate T cell survival via regulation of F-actin dynamics, more recent work suggests that induction of F-actin alteration fails to induce apoptosis in T cells [20,21]. Instead, the capacity of coronin 1 to modulate mycobacterial survival and regulate T cell homeostasis has been linked to its function in modulating signal transduction including Ca²⁺/calcineurin and phosphatidylinositol 3-kinase (PI3K)-dependent processes [22-25]. A role for coronin 1 in the activation of signal transduction in leukocytes is furthermore consistent with the recently described role of neuronally expressed coronin 1 in the modulation of the cAMP/protein kinase A and Ca²⁺/calcineurin pathways in neurons as well as the role for the coronin 1

homologue expressed in the slime mold *Dictyostelium discoideum*, coronin A, in the initiation of cAMP-dependent development [15,26,27].

In B cells, dendritic cells and mast cells coronin 1 appears to be dispensable for their functionality [28-30]. Whether or not coronin 1 expressed in mouse NK cells performs any function in NK cell homeostasis and/or functionality is unclear. In this study, we report that coronin 1-deficient NK cells can be activated and are functional upon stimulation by antibodies directed against activating NK cell receptors. Moreover, NK cells from wild type and coronin 1-deficient mice displayed comparable cytotoxicity. In addition, NK cells from acutely VSV-infected coronin 1-deficient or wild type mice produced comparable amounts of interferon- γ . Taken together the results presented here suggest that coronin 1 is dispensable for NK cell survival, signaling and activation.

Material and methods

Mice

C57BL/6 mice and the previously generated transgenic coronin 1-deficient mice [23] were bred in-house and kept under specific pathogen free (SPF) conditions. All animal experiments were conducted according to the regulations of the Cantonal Veterinary Office (Zurich/Basel, Switzerland). Six to twelve weeks old mice were used for all experiments.

Antibodies

Monoclonal antibodies for flow cytometry assays were purchased from BioLegend (Switzerland) if not indicated otherwise. The following antibodies were used: anti-NK1.1-Alexa Fluor 647 (clone PK136), anti-CD3ε-Pacific blue (clone 145-2C11), anti-interferon-γ-PE (clone XMG1.2), anti-CD107-Brilliant-Violet 421 (clone 1D4B), anti-CD122-PE (clone 5H4), PE Annexin V apoptosis detection kit (BD Pharmingen™, Cat. No. 559763). Antibodies for stimulation, blocking and Ca²⁺-flux analysis were purchased from BioLegend (Switzerland): LEAF purified and biotinylated anti-Ly49D (Clone 4E5), LEAF purified anti-NKp46 (Clone 29A1.4), LEAF purified and biotinylated anti-NK1.1 (Clone PK136), LEAF purified anti-NKG2D (Clone A10) and LEAF purified anti-CD16/32 (Clone 93).

Virus

Virus was provided by Prof A. Oxenius (ETH Zurich, Switzerland). VSV (serotype Indiana) was propagated on BHK-21 cells as previously described [31]. 5 x 10⁵ PFU – 5 x 10⁶ PFU of virus was used to infect mice intravenously (i.v.) via the tail vein.

Lymphocytes isolation from the liver and spleen

Cell isolation was essentially performed as described [32]; in brief, mice were euthanized with high percentage of CO₂. After perfusion via the right heart ventricle (with 5-10 ml ice

cold PBS) to remove all contaminating blood from the organs, the liver was harvested, and dissected into small pieces. Digestion was performed with 3 ml RPMI (Gibco) supplemented with 2.4 mg/ml Collagenase (Gibco, Cat. No. 17100-017), 0.2 mg/ml DNase I (Roche, Cat. No. 1 284 932) and 10% FCS for 20 minutes at 37°C, 5% CO₂. Tissue samples were then gently disintegrated by pulling them through an 18G needle and additionally incubated in 2 ml of digestion medium for 20 min. Subsequently, cell suspensions were smashed through a 70 µm cell strainer (BD, Falcon) and washed twice with PBS. Samples were resuspended in 90% isotonic Percoll and overlaid with 37% Percoll and layered with PBS containing 30% Percoll (Amersham Biosciences Europe GmbH; Cat. No. 17-0891-02) and centrifuged (800 *xg*) for 30 min at 4°C. Lymphocytes were collected from the interphase and washed twice with RPMI, counted and resuspended in RPMI containing 10% FCS, 100 units penicillin-streptomycin and 2 mM L-glutamine for further analysis. Single cell suspensions of spleen were produced by gently smashing the organ through a grid of stainless steel. Depending on the experiment, erythrocytes were lysed by incubation in 1 ml ACK buffer (155 mM NH₄Cl, 10 mM KHCO₂, 0.1 mM EDTA, pH 7.4) for 3 min at 4°C. Viable cells were counted by trypan blue exclusion using a Neubauer counting chamber.

NK cell enrichment from spleen

Splenocytes were used to sort untouched NK cells using a NK cell enrichment kit (Stemcell technologies, EasySep™ Mouse NK Cell Enrichment Kit, Cat. No. 19755). NK cells were enriched according to the manufacturer's protocol and viable cells were counted by trypan blue exclusion using a Neubauer counting chamber.

NK cell stimulation

Maxi-sorp 96 well plates (Nunc; Cat. No. 439454) were coated at 4°C overnight with anti-NK1.1 (5 µg/ml), anti-NKp46 (5 µg/ml), anti-Ly49D (5 µg/ml) or anti-NKG2D (10 µg/ml)

antibodies in 200 μ l carbonate buffer (Na_2CO_3 (29 mM), NaHCO_3 (71 mM), pH 9.5). The next day, plates were washed twice with PBS and $0.5\text{--}1 \times 10^6$ splenocytes in 200 μ l RPMI, 10% FCS, 10 mM HEPES and 50 μ M β -mercaptoethanol were added to the wells in the presence of brefeldin A or 1 μ g anti-CD107a-Brilliant Violet 421. Cells were cultured at 37°C for 4.5 hours prior staining for NK1.1, CD3 ϵ and interferon- γ .

Immunofluorescence staining and analysis

Unspecific binding of fluorochrome-conjugated antibodies to Fc receptors was blocked by incubation anti-CD16/32 for 5 min at 4°C prior surface staining. Fluorochrome-conjugated monoclonal antibodies were added to the samples for 20 min at 4°C. After a washing step, cells were re-suspended in 200 μ l MACS buffer for analysis (PBS, 5 mM EDTA, 2% FCS). Samples were analyzed with a 6 or 8 color BD FACS Canto II Flow Cytometer using FACS Diva software. The data were analyzed with Flow-Jo version 7.5.2 (Tree Star Inc., Ashland, USA).

Immunofluorescence microscopy

Enriched NK cells (3×10^5) from spleen were allowed to adhere to poly-L-lysine coated, 10-well Teflon-surrounded glass slides (Polysciences) for 60 min at 37°C, 5% CO_2 . Slides were washed three times with PBS and fixed for 15 min with 3% paraformaldehyde at room temperature and then blocked and permeabilized for 60 min in PBS, 5% FBS, 0.1% TritonX-100. The cells were then stained for 120 min at 37°C with antibodies to mouse-anti-mouse actin (Chemicon) and rabbit-anti-mouse coronin-1 (1002) [33]. Cells were subsequently washed with PBS and stained with secondary antibodies (goat anti-mouse–Alexa Fluor 637, goat anti-rabbit–Alexa Fluor 568; 1:200 each) for 60 min at 37°C. Slides were washed five times with PBS and mounted with ProLong® Gold Antifade Reagent (Invitrogen, Cat. No.

P36934). Slides were analyzed using a LSM510 Meta confocal laser scanning microscope (Zeiss) and the corresponding software.

SDS-PAGE and Western Blotting

Enriched NK cells (1×10^6) from spleen were lysed in 200 μ l lysis buffer (50 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% Triton X-100, 1 mM PMSF, 1X Protein inhibitor cocktail (Roche, Complete Protease Inhibitor Cocktail) for 15 min on ice. Nuclei and unbroken cells were sedimented by centrifugation for 10 min at 15'000 rpm, 4°C. 80 μ l supernatant was mixed with 20 μ l 5X sample buffer (250 mM TrisHCl (pH6.8), 10% SDS, 30% Glycerol, 5% β -mercaptoethanol, 0.02% bromophenol blue) and boiled for 5 min at 95°C. 20 μ l of the samples were then separated on a 10% SDS-PAGE gel and transferred onto nitrocellulose membrane (Amersham Biosciences) and blotted against anti-actin (Chemi-Con) and anti-coronin 1 (1002) serum [33].

Scanning electronic microscopy

Enriched NK cells from spleen were expanded with IL-15 (10 ng/ml) for 7 days by replacing the medium every second day. NK cells were seeded on poly-L-lysine treated 12 mm coverslips at a density of 5×10^4 cells and let adhere for 45 min at 37 °C, 5% CO₂. Cells were washed with ice-cold PBS and fixed with 2.5% glutaraldehyde (Sigma). Cells were subsequently processed for scanning electron microscopic analysis using the critical point drying technique [34] followed by analysis using a Phillips XL 30 ESEM.

Intracellular cytokine staining

Cells (lymphocytes, splenocytes, at $5 \times 10^5 - 2 \times 10^6$) were resuspended in 200 μ l RPMI supplemented with 10% FCS. RPMI/10% FCS (200 μ l) containing brefeldin A (20 μ g/ml) if not indicated else, was added to the cells. Depending on the experiment 1 μ g of fluorescent

dye-conjugated anti-mouse CD107a antibody was added to the stimulation. Cells were incubated for 4.5 – 6 hours at 37°C, 5% CO₂. Cells were washed with FACS-buffer (PBS, 2% FCS, 5mM EDTA, 0.05% NaN₃) and treated prior surface staining for 5 min at 4°C with a blocking anti-mouse CD16/32 antibody. Cells were subsequently stained for surface marker for 20-30 min at 4°C and fixed with 2% PFA in FACS buffer. For interferon-γ staining cells were permeabilized with 0.5% saponin in FACS buffer for 10 min at room temperature in the dark prior to intracellular staining (30 min at 4°C). After washing, samples were analyzed using a 6-8 color BD FACS Canto II Flow Cytometer and Flowjo version 7.5.2.

Ca²⁺ mobilization measurements in NK cells

Enriched NK cells from spleen were loaded in RPMI 1640 (Invitrogen) without phenol red supplemented with 2mM glutamine and 10% FBS for 30 minutes at 37 °C with 4 μM Fluo-4 and 10 μM FuraRed (both Invitrogen) in the presence of Pluronic F-127 (Invitrogen). Cells were washed with medium, resuspended in 300 μL medium, incubated for 5 minutes at 37 °C and analyzed by flow cytometry (FACS Canto II, BD Biosciences). After 30 seconds baseline recording, 5 μl of biotinylated anti-NK1.1 mAB (0.5 mg/ml in PBS, clone PK136) or anti-Ly49D mAB (0.5 mg/ml in PBS, clone 4E5) was added and 60 seconds later 5 μl of streptavidin was added (1 mg/ml in PBS; Sigma). Streptavidin only served as a negative control. Data were analyzed with FlowJo software. NK cells were gated on forward/side scatter plots. The ratio between the mean fluorescence intensity of Fluo-4 (PE channel) and FuraRed (PerCP channel) was calculated and plotted as a function of time. The 30 second baseline before antibody addition was used to allow normalization between the recordings and maximum increase of intracellular Ca²⁺ was plotted.

⁵¹Cr – cytotoxic assay

Freshly isolated splenocytes or purified NK cells from spleen were either directly used (according to the indicated E:T ratio) for the cytotoxic assay or were treated prior to the assay for 20-22 hours with IL-15 (10 ng/ml) or medium alone in a round bottom 96-well cell culture microtiterplate. YAC-1 cells were cultured in RPMI supplemented with 10% FCS and 200 mM L-glutamine. YAC-1 cells (2×10^7) were loaded with 300 μCi $\text{Na}_2^{51}\text{CrO}_4$ (1 mCi/ml in PBS, Hartmann Analytic) for 90 min on a 37°C warm thermoshaker. YAC-1 cells were subsequently washed twice with PBS and resuspended at a concentration of 5×10^5 cells/ml RPMI, 10% FCS and used as target cells. YAC-1 cell suspension (5×10^4 cells; 100 μl) was then added to the pre-titrated splenocytes to obtain the indicated effector:target ratio. The cell mixture was then incubated for 4.5 hours at 37°C, 5% CO_2 . Plates were centrifuged for 5 min at 500 $\times g$ and 40 μl of supernatant was then transferred and mixed with 200 μl liquid scintillator solution (Microscint 40, Perkin Elmer, Cat. No. 601 36 41). CPM was then counted in a Packard TopCount Microplate scintillation counter (Model C9906, Packard). Specific cell lysis was calculated as follow: (experimental release – spontaneous release) / (maximum release – spontaneous release). Spontaneous release was between 11 and 16% of maximum release.

NK cell survival

Freshly isolated splenocytes or purified NK cells were cultured in the presence of IL-15 (10 ng/ml) or medium alone (RPMI; 10% FCS, 200 mM L-Glutamine) at 37°C, 5% CO_2 for 4 and 24 hours. At the indicated time points, cells were harvested, stained for CD3 ϵ , NK1.1, and 7-AAD. Cells were counted by adding counting beads prior to FACS analysis purchased from Invitrogen (CountBright™ Absolute Counting Beads, Cat. No. C36950). Cells were then analyzed using a BD FACScanto II Flow Cytometer using FACS Diva software. The data were analyzed with Flowjo version 7.5.2.

Statistical Analysis

Non-parametric two-tailed Mann-Whitney t-test was used for group comparisons using Graph Pad Prism (GraphPad Software, La Jolla, CA). The p-values are indicated in the figure legends.

Results

NK cell numbers and phenotype in the presence and absence of coronin 1

To analyze the consequences of coronin 1 deletion for the NK cell population, we first compared the frequencies and absolute numbers of NK-cells in blood, spleen and liver of coronin 1-deficient and wild type mice by flow cytometry after staining for CD3 and the NK cell marker NK1.1 (Figure 1). We found that coronin 1-deficient mice showed significantly increased NK cell frequencies in all organs tested compared to wild-type mice (Figure 1A, B). However, absolute NK cell numbers were similar in spleen and blood. In contrast, a twofold increased NK cell number were observed in livers of coronin 1-deficient mice (Figure 1C), possibly as a result of T cell lymphopenia [18-22].

Next, we enriched NK cells from the spleen of wild-type and coronin 1-deficient mice to analyze the morphology of NK cells and the distribution and expression of coronin 1 and CD122, the β -chain of the IL-15 (and IL-2) receptor, given the importance of IL-15 for NK cell survival and function [35] (Figure 2). The efficiency of NK cell enrichment by negative selection was found to be similar for both wild-type and coronin 1-deficient NK cells (Figure 2A, please note that coronin 1-deficient mice express enhanced green fluorescent protein (EGFP) driven by the coronin 1 promoter). Analysis of coronin 1 expression by immunoblotting of NK cell lysates obtained from either wild type or coronin 1-deficient NK cells revealed that coronin 1 is expressed in wild-type NK cells, but is absent in NK cells of coronin 1-deficient mice (Figure 2B).

To analyze the effect of coronin 1 deletion on the morphological appearance of NK cells, NK cells were purified from either wild type or coronin 1-deficient mice, mixed in a 1:1 ratio, fixed and stained using anti-coronin 1 and anti-actin antibodies. As shown in Figure 2C, wild type (GFP-negative) and coronin 1-deficient (GFP-positive) NK cells were indistinguishable

as observed by bright field microscopy or actin staining. Moreover, CD122 expression was similar between wild-type and coronin 1-deficient NK cells, both regarding the frequency of CD122 expressing NK cells (Figure 2D) as well as the mean fluorescence intensity (MFI) of CD122 (Figure 2E). From these results we conclude that NK cell numbers, their morphology and CD122 expression are not affected by the absence of coronin 1.

NK cell receptor stimulation and NK cell activation in the presence and absence of coronin 1

NK cells have an arsenal of activating (including NK1.1, NKp46, Ly49D, NKG2D) and inhibitory (such as Ly49a, c, e, f, g, i) receptors which signal through non-covalently bound adaptor molecules such as DAP10, DAP12, CD3 ζ and Fc ϵ R γ . To analyze an involvement of coronin 1 in activating NK cell receptor signaling, NK cells were stimulated by plate bound antibodies directed against NK1.1 (DAP12), NKp46 (DAP12), Ly49D (CD3 ζ and Fc ϵ R γ) and NKG2D (DAP10) (Figure 3). Thereafter, as a readout of activation, interferon- γ or CD107a expression (as a surrogate for degranulation) was analyzed by flow cytometry (Figure 3). The frequencies of IFN γ - or CD107a-positive NK cells were similar in coronin 1 and wild type mice after stimulation via NK1.1, NKp46 and NKG2D (Figure 3B-E), whereas an increased frequency of interferon- γ producing NK cells after stimulation via Ly49D in coronin 1-deficient mice was found (Figure 3B).

Since stimulation of ex vivo isolated NK cells via NKG2D did not induce any significant IFN γ -production or degranulation (Fig. 3C and E, left panels), splenocytes were pre-activated *in vitro* with IL-2 for 20h and then stimulated with plate-bound anti-NKG2D (Fig. 3C and E, right panels). While IFN γ -production was similar between wild type and coronin 1-deficient NK cells, we found a slight reduction of CD107a-internalization in coronin 1-deficient NK cells. In accordance with these data, calcium mobilization was similar in both wild type as well as coronin 1-deficient NK cells after stimulation with anti-NK1.1 or Ly49D

(supplementary Figure 1). While the reason for the elevated IFN- γ production upon Ly49D stimulation remain to be analyzed, coronin 1-deficiency appears to have no significant influence on the activation of NK cells by plate bound antibodies directed against NK1.1, NKp46 and NKG2D.

Cytotoxic activity of NK cells against YAC-1 cells in the presence and absence of coronin 1

We reasoned that the capacity of coronin 1-deficient NK cells to produce interferon- γ and to degranulate does not necessary reflect their cytotoxic potential. Therefore, we investigated whether coronin 1-deficient NK cells were able to induce cytotoxicity in the MHC class I negative tumor cell line YAC-1 by a classical ^{51}Cr -release assay. As shown in Figure 4, splenocytes from wild-type and coronin 1-deficient mice were equally effective to lyse YAC-1 cells. In addition, co-cultivation of YAC-1 cells with either wild type or coronin 1-deficient NK cells led to comparable interferon- γ production (data not shown). From these results we conclude that NK cell cytotoxicity was not impaired in the absence of coronin 1.

Ex vivo survival of wild-type and coronin 1-deficient NK cells

In naïve T cells, coronin 1 is essential to provide pro-survival signals to allow T cell survival in peripheral immune organs [18-22]. To analyze a role for coronin 1 in *ex vivo* survival of NK cells, NK cells were purified and expanded for 21 days in the presence of interleukin-15. As shown in Figure 5A, both wild type as well as coronin 1-deficient NK cells expanded similarly *in vitro*. To analyze the morphology of wild type and coronin 1-deficient NK cells, we expanded enriched NK cells *in vitro* in the presence of interleukin-15 for seven days. Analysis by scanning electron microscopy failed to reveal any morphological differences between wild type and coronin 1-deficient NK cells (Figure 5B). To investigate whether in

vitro-activated coronin 1-deficient NK cells were functional, NK cells were cultured in the presence or absence of interleukin-15 for 20 – 22 hours and their ability to induce target cell lysis was then analyzed in a standard 4.5 hours Cr-51 release assay using YAC-1 as target cells. NK cells from both wild type and coronin 1-deficient mice, cultured in medium only, were not viable at this time point and failed to induce cytotoxicity (Fig. 5C, *mock*), NK cells from both wild type and coronin 1-deficient mice induced a similar and strong cytotoxicity when cultured in the presence of interleukin-15 (Fig. 5C, *IL-15*). Together these data suggest that coronin 1 is dispensable for IL-15-dependent NK cell survival, cytotoxic activity and proliferation *in vitro*.

NK cell immune response to VSV in wild type and coronin 1-deficient mice

To analyze a role for coronin 1 in NK cell responses upon a viral infection *in vivo*, wild type and coronin 1-deficient mice were infected with Vesicular Stomatitis Virus (VSV) intravenously. Forty-eight hours post infection, NK cell number and functionality from spleen and liver was analyzed by flow cytometry (Figure 6A). VSV-infection resulted in a slight decrease of total NK cells numbers in the spleen but not in the liver of both wild type and coronin 1-deficient mice (Figure 6B). However, the frequency and total number of IFN γ -producing activated NK-cells was similarly increased after VSV infection in both wild type and coronin 1-deficient mice (Figure 6C,D). These data suggest that coronin 1 deletion does not affect interferon- γ production of NK cells after VSV infection.

Discussion

Coronin 1 is a member of the WD repeat protein family of coronins that is highly expressed in leukocytes. Whereas coronin 1 is essential for naïve T cell homeostasis in mice and human [17-20,22] the role for coronin 1 in other leukocytes remains unclear [17,23,28-30,36,37]. Here, we have investigated the number, phenotype and functionality of NK cells. Although NK cell frequencies were increased in blood, spleen and liver of coronin 1-deficient mice, this was most likely due to T cell lymphopenia since total NK cell numbers (blood, spleen) or about twofold increased (liver) in the absence of coronin 1. These findings are consistent with normal NK cell numbers present in the blood of human individuals lacking coronin 1 expression [35,38]. Thus, as is the case for B cells, macrophages, neutrophils and mast cells, coronin 1 appears to be largely dispensable for mouse NK homeostasis and function.

Activating signaling pathways in NK cells have strong similarities with T cell receptor signaling [10,12,39]. The different activating receptors (e.g. NK1.1, NKp46, Ly49D and NKG2D) transmit their signal via their specific ITAM containing adaptor molecules (DAP12, DAP10, CD3 ζ and Fc ϵ R γ). These adaptor molecules recruit Src kinases (e.g. Syk, Fyn, LCK) and initiate, upon activation, a downstream phosphorylation cascade, which depends on PLC γ and PI3K, resulting in intracellular Ca²⁺ release, calcineurin activation and transcription factor translocation to the nucleus [4,10,12,40,41]. Given these parallels between NK cell and T cell signaling as well as the importance of coronin 1 in activating the Ca²⁺/calcineurin pathway in T cells [17,18,22], we investigated whether coronin 1 is involved in NK cell survival and/or functionality. As shown here, NK cells lacking coronin 1 became similarly activated compared to wild type cells after stimulation with antibodies directed against several activating receptors that use different signaling adaptor molecules including DAP 10, DAP 12 or Fc ϵ R γ [10,13]. Both interferon- γ production and degranulation were efficiently triggered in coronin 1-deficient NK cells, demonstrating that activating NK cell receptor signaling is

not impaired in the absence of coronin 1. In addition, NK cells from wild-type and coronin 1-deficient mice were able to lyse YAC-1 tumor cells to a similar extent suggesting a full induction of the cytotoxic machinery in a coronin 1-independent manner.

In naïve T cells, coronin 1 deletion results in their apoptosis due to a lack of an appropriate survival signal through the T cell receptor [21]. In NK cells, as in other leukocytes such as macrophages, neutrophils and mast cells [22,23,30,36], coronin 1 appears to be dispensable for cell survival *in vivo* and *in vitro* as judged by a similar capacity for expansion in the presence of interleukin-15. Furthermore, we could not observe any difference in the morphology of NK cells from wild type and coronin 1-deficient mice *ex vivo* or after *in vitro* expansion in interleukin-15-containing medium. These results suggest that IL-15-driven expansion and differentiation of NK cells is independent of coronin 1. In accordance with the results described here, a recent study reported that in human NK cells the absence of coronin 1 results in normal interferon- γ production, activation receptor signaling and Ca^{2+} mobilization are not altered [42]. These authors do however describe a requirement for coronin 1 in cytolytic activity of human NK cells in contrast to the absence of a role for coronin 1 in cytotoxicity described here. Whether or not this discrepancy is due to species-specific differences or the exact cytotoxicity assay employed remains to be analyzed.

Although the here reported results are consistent with the absence of a role for coronin 1 during VSV infection in mice, we cannot exclude that coronin 1 may play some role in particular NK cell subtypes. Also, as we have focused on the *in vitro* analysis on activating NK cell receptors, a potential role for coronin 1 in expression, signal transduction or functionality of inhibitory NK cell receptors remains to be analyzed. Furthermore, although the *in vivo* data on NK cell activity exclude a fundamental role of coronin 1 for NK-cell activity after VSV-infection, a role for coronin 1 in other viral infections, which require NK-cell activity for early viral containment, remains to be analyzed. Finally, besides coronin 1

there are several other coronin homologous that are expressed in leukocytes, and therefore it cannot be excluded that one or more of these coronin molecules are functionally redundant with coronin 1.

In conclusion, the data presented here suggest that NK functionality is largely independent on the presence of coronin 1. Whether or not in NK cells, a function for coronin 1 is compensated for by the presence of other members of the coronin protein family or has become redundant because of compensatory (co) stimulatory mechanisms, as is for example the case for B cell activation [28] may be the subject of further studies.

Acknowledgement

We would like to thank Somdeb BoseDasGupta and the Center for Microscopy, University of Basel for electron microscopy, the members of the research group of Prof. Annette Oxenius (ETH Zurich, Switzerland) for invaluable discussions and sharing of reagents, Lea Häberli for expert technical assistance and PD Dr. Annelies Zinkernagel and Prof. Dr. med. Nicolas Müller for providing laboratory space and general support. Michael Stiess is a recipient of an HFSP Fellowship as well as an EMBO Long Term Fellowship. This work was further supported by the Canton of Basel, by the Swiss National Science Foundation to UK (grant PP0033-110737) and JP (31003A_146463), the Gebert Rueff Foundation, the Heuberg Foundation (Zürich), by the Aetas Foundation (Geneva) and by the Promedica Foundation (Chur; all from Switzerland) and by the FWF Austrian Science Fund (“Lise-Meitner” M1636-B23 to KS). A patent related to this work have been filed (WO2007110385A2) by the University of Basel.

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Figure Legends

Figure 1: Frequency and number of NK cells in coronin 1-deficient mice

Lymphocytes isolated from spleen, liver and blood were stained for NK1.1 and CD3 ϵ and analyzed by flow cytometry. A. Representative FACS dot-plots of NK cell frequencies in spleen, liver and blood of wild type and coronin 1-deficient mice. B. Frequencies of NK cells in spleen, liver and blood. C. Absolute NK cell numbers in spleen, liver and blood. Absolute numbers were calculated from the lymphocyte counts per organ determined using a Neubauer counting chamber and the NK frequencies obtained by flow cytometry. Statistical analyses were performed using the non-parametric two tailed Mann-Whitney test (** $p < 0.01$; * $p < 0.05$). Each symbol represents data of an individual mouse, mean values and SEM are indicated.

Figure 2: Phenotype of wild-type and coronin 1-deficient NK cells

NK cells from spleen of wild-type and coronin 1-deficient mice were enriched by negative selection and analyzed for expression of actin, coronin 1 and CD122 (IL2R β) by flow cytometry. Note that coronin 1-deficient mice express eGFP driven by the coronin 1 promoter. A. FACS dot-plots of pre-enriched and enriched NK cells (by negative selection) stained with anti- NK1.1 and analyzed for Alexa Fluor 647 and FITC. B. Lysates of enriched NK cells were separated on 10% SDS gel and blotted against actin and coronin 1. C. Enriched NK cells (~65-70% purity) from wild-type and coronin 1-deficient mice mixed 1:1 were adhered on poly-L-lysine-treated cover slips, stained for actin (Alexa Fluor 637, *blue*) and coronin 1 (Alexa Fluor 568, *red*) and analyzed by confocal microscopy. D,E. CD122 expression on NK1.1+ cells from spleens (gated on CD3 ϵ negative lymphocytes).

Representative FACS dot blots are shown. The bar graph depicts the mean fluorescence intensity (MFI) of CD122 expression on NK cells.

Figure 3: Stimulation of activating NK cell receptors of wild-type and coronin 1-deficient NK cells

Splenocytes from wild type or coronin 1-deficient mice were stimulated with plate-bound antibodies. A. Representative FACS gating strategy. B-E: IFN γ production (B,C) and CD107a surface exposure (D,E) of wild type and coronin 1-deficient NK-cells was analyzed by flow cytometry after 4.5 hours stimulation by plate bound antibodies against the activating NK cell receptors NK1.1, NKp46, Ly49D and NKG2D. For NKG2D, splenocytes were left untreated (0 hours) or additionally cultured for 20 hours with IL-2 (10 ng/ml; IL2 + 20 hours) prior to stimulation with plate-bound anti-NKG2D (10 μ g/ml) coated plate. Statistical analyses were performed using the non-parametric two tailed Mann-Whitney test (* $p < 0.03$). Error bars represents the SEM.

Figure 4: Cytotoxic activities of coronin 1-deficient NK cells against YAC-1 cells

Splenocytes from spleens of wild-type or coronin 1-deficient mice were co-cultured with 5×10^4 Cr-51 radio-labeled YAC-1 cells at the indicated effector:target ratio. Cytotoxicity was measured in a 4.5 hours Cr-51-release assay. The graph depicts the mean and SEM of four independent experiments with two mice per group.

Figure 5 Effect of IL-15 on coronin 1-deficient NK cells

NK cells from wild-type and coronin 1-deficient mice treated with IL-15 for the indicated time were analyzed for expansion, expansion and morphology. A. NK cells were enriched from spleen and cultured ($1-2 \times 10^5$) for 21 days in medium supplemented with recombinant IL-15 (10 ng/ml). At the indicated time points, cells were harvested and counted by flow cytometry using counting beads. B. Enriched NK cells were expanded for seven days with IL-15 (10 ng/ml) and analyzed by scanning electron microscopy. NK cell purity (measured by NK 1.1 and CD3) at day 7 was up to 99%. C. Splenocytes were isolated and the frequencies of NK cells in the spleen of wild-type and coronin 1-deficient mice were measured by FACS analysis. Equal numbers of wild type and coronin 1-deficient NK cells (as determined based on the frequency and the indicated effector:target (E:T) ratio) were stimulated with IL-15 (10 ng/ml) or medium alone (mock) for 20-22 hours. Cr-51 radio-labeled YAC-1 cells (5×10^4) were then added to the splenocytes and cytotoxicity was measured in a 4.5 hours Cr-51-release assay (pooled data from two independent experiments).

Figure 6: NK cell activation after VSV-INDG infection in vivo

Coronin 1-deficient and wild-type mice were infected with 5×10^5 - 5×10^6 PFU VSV-INDG i.v. or injected with medium alone (mock). After 48 hours, splenocytes and intrahepatic liver lymphocytes (IHL) were isolated and cultured for 4 hours in the presence of brefeldin A. Cells were subsequently stained for NK1.1, CD3 ϵ and intracellular interferon- γ and analyzed by flow cytometry. A. Representative FACS dot-plot of splenocytes and IHL from VSV-infected or medium-treated mice are shown. B. Total NK cell numbers in the spleen and liver. C. Frequency of interferon- γ producing NK cells in the spleen and liver. D. Absolute number of interferon- γ producing NK cells after VSV infection. Absolute NK cell numbers were

calculated from FACS data. Symbols indicate individual mice (gray symbols, 5×10^6 PFU, black/white 5×10^5 PFU). Horizontal bars indicate the mean of 2-4 mice per group.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

B

C

Figure 6