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Abstract (246 words)

Background: Rubella virus-induced granulomas have been described in patients with various inborn errors of immunity. Most defects impair T-cell immunity, suggesting a critical role of T cells in rubella elimination. However, the molecular mechanism of virus control remains elusive.

Objective: To understand the defective effector mechanism allowing rubella vaccine virus persistence in granulomas.

Methods: Starting from an index case with Griscelli syndrome type 2 and rubella skin granulomas, we combined an international survey with a literature search to identify patients with cytotoxicity defects and granuloma. We performed rubella virus immunohistochemistry and PCR and T-cell migration assays.

Results: We identified 21 patients with various genetically confirmed cytotoxicity defects, who presented with skin and visceral granulomas. Rubella virus was demonstrated in all 12 accessible biopsies. Granuloma onset was typically before age 2 years and lesions persisted from months to years. Granulomas were particularly frequent in MUNC13-4 and RAB27A deficiency, where 50% of patients at risk were affected. Although these proteins have also been implicated in lymphocyte migration, 3D migration assays revealed no evidence of impaired migration of patient T cells. Notably, patients showed no evidence of reduced control of concomitantly given measles, mumps or varicella live-attenuated vaccine or severe infections with other viruses.

Conclusions: We identify lymphocyte cytotoxicity as a key effector mechanism for control of rubella vaccine virus, without evidence for its need in control of live measles, mumps or varicella vaccines. Rubella vaccine-induced granulomas are a novel phenotype with incomplete penetrance of genetic disorders of cytotoxicity.

Clinical Implications (28 words):

Lymphocyte cytotoxicity is important for control of rubella vaccine virus persistence. Patients with genetic defects of cytotoxicity including albinism syndromes should not be vaccinated with rubella live vaccine.

Capsule Summary (35 words).

Lymphocyte cytotoxicity is important for control of rubella vaccine without evidence for its need to control concomitantly given live vaccines. Rubella vaccine-induced granulomas are a novel phenotype with incomplete penetrance of disorders of cytotoxicity.

Key words (up to 10):

Cytotoxicity, rubella virus, live vaccine, granuloma, primary immunodeficiency, hemophagocytic lymphohistiocytosis, Griscelli syndrome type 2

Abbreviations.

AT – Ataxia telangiectasia

CHS – Chédiak-Higashi syndrome

CMV - Cytomegalovirus

CNS – central nervous system

CTL – cytotoxic T lymphocyte

DTH – delayed type hypersensitivity

EBV – Epstein-Barr virus

fHLH – familial hemophagocytic lymphohistiocytosis

HLH - hemophagocytic lymphohistiocytosis

HSCT- hematopoietic stem cell transplantation

IEI - inborn errors of immunity

GS2 – Griscelli syndrome type 2

LCMV - lymphocytic choriomeningitis virus

MMR(V) – measles, mumps, rubella, (varicella) live-attenuated vaccine

PBMCs - Peripheral blood mononuclear cells

PCR – polymerase chain reaction

PID – primary immunodeficiency

RV – rubella virus

RV-C - rubella virus capsid

VZV- varicella-zoster virus

Introduction

Perforin-mediated target cell lysis is a key effector mechanism of CD8+ T cells and NK cells. A key function ascribed to cytotoxicity is the control of viral infections by elimination of virus-infected cells (1). This was first documented in perforin-deficient mice that fail to control infection with lymphocytic choriomeningitis virus (LCMV) (2). Patients with genetically impaired perforin-mediated cytotoxicity (including patients with familial hemophagocytic lymphohistiocytosis FHL2-5, Griscelli syndrome type 2, GS2, and Chédiak-Higashi syndrome, CHS) are predisposed to develop the hyper-inflammatory syndrome of familial hemophagocytic lymphohistiocytosis (fHLH) (3). Disease onset can be associated with viral infections (4) and in particular uncontrolled infection with Epstein-Barr virus (EBV) is observed in some fHLH patients. However, in the context of acute fHLH it is difficult to separate whether disease severity reflects lack of antiviral function or rather impaired control of immune stimulation by failure to eliminate antigen-presenting cells (5). Notably, in the majority of infants with the most severe forms of the disease, no viral trigger can be identified (6). There are no reports on live vaccine persistence in fHLH patients. Moreover, uncontrolled viral infection is not a general phenotype of fHLH patients, even when they are not transplanted in the first 2-3 years of life (7). Thus, from a biological point of view, the role of cytotoxicity in the control of many human viruses is not so evident.

Granulomas are organized structures formed by macrophages and other immune cells that participate in antimicrobial defense by preventing spread of a persistent pathogen (8). This also includes viral infections such as measles and EBV (9). Recently, elegant work has demonstrated persistence of rubella virus (RV) vaccine strain RA27/3 in skin and visceral granulomas of patients with inborn errors of immunity (IEI) over decades (10–12). Most commonly, these RV associated granulomas were detected in patients with Ataxia-telangiectasia (AT) and other DNA repair disorders, followed by patients

with various combined immunodeficiencies (13). The T-cell lymphopenia in most of these patients in combination with evidence of cytotoxic T lymphocyte (CTL) epitope escape mutations in the viral genome (14) suggests a key role for CTL in limiting the observed RV persistence in M2 macrophages (12). Since CD8 T cells predominate around the granulomas, it has been suggested that beyond an initial numeric T-cell deficiency allowing prolonged RV replication, functional impairments such as T-cell exhaustion contribute to RV persistence (13). However, the molecular mechanisms that are essential for rubella vaccine virus control remain incompletely understood.

Here we describe a cohort of patients with hereditary cytotoxicity defects, who presented with rubella virus-containing skin and visceral granulomas. There was no evidence that these patients lacked control of concomitantly given measles, mumps or varicella virus or had generally increased susceptibility to viral infections apart from EBV in the context of HLH. Clinically, these observations identify rubella-induced granulomas as a novel phenotype with incomplete penetrance of patients with genetic defects of cytotoxicity. From a biological point of view, they support the concept that lymphocyte cytotoxicity is not needed as effector mechanism against viruses in general, but rather has a selective role against some specific viruses.

Materials and Methods

Patient Recruitment

Patients with cutaneous granulomas and cytotoxicity defects ("granuloma cohort") were identified via literature search (PubMed search terms: haemophagocytic lymphohistiocytosis / HLH / disease name / gene name AND granuloma / skin / cutaneous) and the databases from the German HLH Reference Centers Freiburg and Hamburg. Additional cases were identified through our international network of colleagues from the European Society for Immunodeficiencies (ESID), the Inborn Errors Working Party and the Histiocyte Society. Patients were included based on the following criteria: (i) diagnosis of FHL, CHS or GS2 (clinical in 1 GS2 patient, genetic all other patients) (ii) description of skin lesions consistent with granulomas in the patient files (described as: papular, pustular, maculopapular, granulomatous), and – available in all but 1 patient (iii) photographs of the skin lesions, histology, rubella immunohistochemistry or PCR consistent with the diagnosis.

An additional, partly overlapping "at risk cohort" comprises patients documented in the German HLH Study between 01/1997 and 06/2020 with genetically confirmed cytotoxicity defects. This study captures more than 80% of such patients manifesting in Germany. We included all patients with a minimum age of 18 months before onset of HLH and/or HSCT to restrict the analysis to patients with likely vaccine exposure. Patients who were known not to be vaccinated against rubella or had a negative serology for MMR were excluded. For patients with unknown vaccination status, vaccination against rubella was presumed based on the high MMR vaccination rate in Germany (15).

The research protocol was approved by the Ethics committee, University of Freiburg (EK No. 159/19). All patients in the German HLH study gave informed consent.

Immunohistochemistry

For detection of the rubella virus in patient biopsies, tissue sections were cut from formalin-fixed, paraffin-embedded tissue samples and used for subsequent immunohistochemical analysis. The presence of the viral capsid was visualized by chromogenic or fluorescent detection using the mouse monoclonal anti-rubella virus capsid antibody (clone 9B11, 1:500, Abcam). Tissue sections from sarcoidosis patients were used as negative controls. Staining results were interpreted by an experienced pathologist, based on staining intensity, tissue location of the RV-positive signal and the infected cell type. Further details are provided in the supplementary material.

Molecular Analysis

Frozen skin samples were used to extract RNA for subsequent real-time RT-PCR for rubella virus according to standards of practice.

Cell Culture

Peripheral blood mononuclear cells (PBMCs) from patients and healthy controls were isolated from whole blood using ficoll density gradient centrifugation. To generate CD8+ T-cell lines, PBMCs were stimulated with PHA (1.25 µg/ml, Remel), IL-2 (1:100, cell culture supernatant from an IL-2 producing cell line) and gamma-irradiated feeder cells (61.8 Gy, pool of allogeneic PBMCs). Cells were sorted for CD8+ T cells after the first round of stimulation. Further details are provided in the supplementary material.

Flow Cytometry Based Assays

NK cell and CTL degranulation assays were performed as previously described (16). CXCR3 expression in resting or CXCL11 (100 nM, PeproTech) stimulated CD8+ T-cell lines was analyzed by flow cytometry using an anti-CXCR3 antibody (clone # 49801, R&D Systems). Details of the protocol are provided in the supplementary material.

T-cell Migration Assays

The chemotactic response of CD8+ T-lines to CXCL10 (PeproTech) or CXCL12 (PeproTech) was analyzed using Transwell® permeable supports for 24 well plates (polycarbonate membrane, 5 µm pores, Corning). Migration in micro-channels was performed as described in previously (17). To assess T-cell migration in complex 3D environment, collagen experiments were performed as previously described (18). Details of the T-cell migration protocols are provided in the supplementary material.

Results

A patient with Griscelli syndrome type 2 and rubella granulomas

A 2-year-old girl from a consanguineous family was referred due to persistence of a generalized, papulo-pustular rash over 6 months. Upon admission, the non-itching, papular lesions were found on arms, legs and face (Figure S1A). The patient had silver-blond hair (Figure S1A), but her medical history did not reveal relevant infections or hospitalizations. Based on characteristic pigment distribution in the hair shaft (Figure S1B) and a defect in NK and T-cell degranulation (Figure S1C), she was given the clinical diagnosis of GS2, which was confirmed by identification of a homozygous splice site mutation in *RAB27A* (c.240-2A>T). A skin biopsy showed necrotizing epithelioid and giant cell granulomas in the dermis (Figure S1D). Cultures were negative for bacteria and fungi and histochemical stainings did not reveal fungal mycelia or acid-fast bacteria. PCR was performed for mycobacteria and viruses including cytomegalovirus, varicella zoster, mumps, measles and, based on recent reports, rubella virus (RV) (10,12). Indeed, only the PCR for RV was positive and subsequent immunohistological stainings showed RV-positive cells in the center of the granulomas (Figure S1D). The extended patient history revealed measles, mumps and rubella (MMR) vaccinations at 15 and 25 months of age. Rubella was not detected in respiratory secretions or blood and the patient had a protective IgG titer against rubella.

Skin and visceral granulomas are common in patients with genetic defects in cytotoxicity

While rubella-associated granulomas have been described mostly in IEI patients with defective T-cell immunity, they have so far not been reported in patients with GS2. To address whether RAB27A deficiency and potentially other genetic defects in cytotoxicity predispose to rubella granulomas, we combined a literature search with an

analysis of the databases of the German HLH reference centers and enquiries through an international network of colleagues. Twenty additional patients with a history of skin or visceral granuloma were identified based on a clinical description given by referring physicians, the medical reports or publications (Table 1). We evaluated three additional criteria to verify the diagnosis in these 20 patients: (i) photo documentation compatible with skin granulomas, (ii) histological confirmation of granulomas and (iii) identification of rubella by immunohistochemistry and/or PCR. We had access to formalin-fixed paraffin embedded skin biopsies from 10 patients (P2,3,6,8,9,12,15,17,18,19) and detected the RV antigen by immunohistochemistry in all samples. Additionally, rubella was identified in 3 of these patients (P1,6,15) by RT-PCR analysis of fresh skin biopsies. Sequence analysis of the PCR products in 2 patients (P6,15) revealed a sequence identical (P6, 131 nt sequenced) or nearly identical (P15, 739 nt sequenced, 98.2% alignment) with that of the rubella RA27/3 vaccine strain. Overall, 7/21 patients fulfilled 2 criteria, 3/21 fulfilled 3 criteria and 10/21 fulfilled all 4 criteria (Table 1). In addition to skin granulomas, some patients had rubella-positive extracutaneous granulomas in the liver (P12) or lung (P8,19), detected in the context of liver or interstitial lung disease. P14 only presented with lung granulomas (Table 1). Among the 21 patients in this “granuloma cohort”, 13 had RAB27A deficiency, 5 had MUNC13-4 deficiency, 2 had PRF1 deficiency and 1 had MUNC18-2 deficiency (Table 1). Patients with FHL2 (perforin deficiency), FHL3 (MUNC13-4 deficiency) or FHL5 (MUNC18-2 deficiency) had at least one point or splice site mutation (Suppl. Table 1) possibly indicating hypomorphic disease variants.

Clinical and immunohistochemical variability of granulomas

Most patients presented with isolated, non-confluent maculopapular or pustular lesions on arms, legs and face, which in some cases developed into hyperkeratotic papules (P6, P15). However, papules were also found on the trunk or buttocks (P3, P7, P8,

P10, P11, P13, P21) (Figure 1), 2 patients only had a single granulomatous lesion on the leg (P17, P19) which gradually increased in size. Overall, clinical manifestations were similar in patients with different cytotoxicity defects (Figure 1, particularly P7, P18 and P3, P15). Positive RV immunostaining was found in granulomas in the dermis and granuloma-like histiocytic aggregates in the dermis, lung and liver (Figure 2). The quantity of RV positive cells varied considerably between patients. Whereas in some sections the RV antigen could be detected in many epithelioid cells in the center of granulomas, others only showed a few positive cells. RV-positive cells also stained positive for the macrophage marker CD68 (Figure 2), demonstrating that macrophages were the cell type harboring the virus. Granulomas in the dermis were usually epithelioid and occasionally contained multinucleated giant cells (Figure 2A). Some granulomas also showed central necrosis. Lymphocytic infiltrates (including CD8+ T cells) were located in the periphery of the granulomas (Figure 2A). Hyper(para)keratosis of the epidermis was seen in 5/11 skin samples.

Clinical course of granulomas and response to treatment

The temporal relationship between vaccination, onset and persistence of granulomas is summarized in Table 1 and Figure 3. Patients had received their first MMR dose between 12-52 months of age (median 13.5 M, n = 14) and developed granulomas 4 months (median, range 0.5 M – 3 Y, n = 14) later. The median age at granuloma onset was 20 months (n = 18) and preceded HLH development in all 16 patients who eventually developed HLH. Only 4 patients showed HLH symptoms within 3 months after granuloma onset. In P8, *Pseudomonas aeruginosa* superinfection of the skin lesions was a possible trigger of incomplete HLH and in P4 HLH onset was linked to EBV infection. No possible infectious triggers were given for P20 and P21, raising the possibility that the rubella vaccination could have triggered the HLH episode. Apart from EBV-associated HLH in P4, P7, P11, VZV-associated “CNS-only” HLH in P2 and

CMV-associated hepatitis in P18, none of the patients were reported to have suffered from severe or unusual courses of viral infections.

Skin granulomas persisted over months or even years (range 1.5 M – 6.5 Y, n = 18), but eventually spontaneously resolved with variable residual scars in 7/21 patients. P17 had a single lesion increasing in size over the course of 6.5 years which was excised in total and did not reoccur. Systemic glucocorticoids were associated with improvement of skin lesions in 5/6 patients. In 4 patients (P3, P4, P20, P21), HLH-directed therapy (mostly HLH94) was not associated with deterioration of the granulomas but induced partial or complete remission in P3 and P4. Five patients entered HSCT with skin lesions, which completely resolved in 4 within a few weeks. P3 had resolution of the erythematous component, but atrophic papules persisted and were later surgically excised.

Risk of rubella granuloma varies with genetic diagnosis

To understand how frequently patients with cytotoxicity defects are affected by skin granulomas, we identified 61 patients with genetically confirmed cytotoxicity defects and a minimum age of 18 months before clinical onset of HLH via the databases of the German national HLH study (Figure 4A). In this “at risk cohort” (including 9 patients that were also part of the “granuloma cohort”), rubella vaccination or antibody titers against rubella had been explicitly documented in 43 patients. In the remaining 18 patients, rubella vaccination was presumed based on 97% MMR vaccination rates in Germany (15). Patient reports revealed skin lesions consistent with rubella granulomas in 9/61 patients (14.7%) (Figure 4B) and RV could be demonstrated in all 5 accessible biopsies. Skin lesions were described in 1/11 FHL3, 1/14 FHL5 and 7/14 GS2 patients (Figure 4B), but not in patients with CHS, FHL2 or FHL4.

No evidence for impaired T-cell migration in patients with genetic defects of lymphocyte cytotoxicity

Literature indicates a role for RAB27A in cell motility (19–22) and GS2 patients were shown to have absent delayed-type cutaneous hypersensitivity (DTH) responses (23,24). Since nearly all patients had deficiency in RAB27A or the RAB27A interaction partner MUNC13-4, we considered that the mutations impair T-cell migration and therefore rubella clearance in the skin.

Since the chemokine receptor CXCR3 and its ligands are important for directed migration of T cells and NK cells into inflamed tissues and contribute to virus control in the skin (25–27), we first tested expression and regulation of CXCR3 (Figure 5A). Surface expression of CXCR3 on CD8+ T cells was similar in patient and control cells and stimulation with CXCL11 caused a comparable downregulation and re-expression kinetics of CXCR3. Moreover, patient cells showed concentration-dependent migration towards the chemokine CXCL10 that was comparable with control cells (Figure 5B). To investigate the motility of RAB27A- and MUNC13-4-deficient CD8+ T cells in a more confined environment, we examined the migration of fluorescently labelled T-cell blasts along 4 µm wide micro-channels (Figure 5C). Patient T cells moved at the same speed as control cells. Also, when the skin's dermal layer was mimicked by using a three-dimensional (3D) collagen gel matrix, time-lapse video microscopy revealed no differences in speed between CD8+ T cells from patients and healthy controls (Figure 5D). In summary, we could not provide evidence that RAB27A- and MUNC13-4-deficiency affects the spontaneous or chemokine-induced motility of CD8+ T cells.

Discussion

Here we show that rubella vaccine-induced granulomas are a novel phenotype with incomplete penetrance in patients with defects of cytotoxicity. This is illustrated by long-term persistence of attenuated rubella vaccine strain in skin and organ granulomas in some, but not all rubella-vaccinated patients with various genetic defects of cytotoxicity.

Rubella-associated granulomas are a known complication in IEIs (10–12,28–32). In a series of 66 patients, 17 suffered from AT, 16 from atypical SCID, 25 from various forms of combined immunodeficiencies, 6 patients from CVID and 2 patients from X-linked agammaglobulinemia (13). Apart from the latter two patients, the common theme in all of these diseases are defects in T-cell immunity, in most cases associated with T-cell lymphopenia. We describe here for the first time such lesions in a cohort of patients with normal T-cell numbers, but various defects in lymphocyte cytotoxicity. These observations strongly suggest that cytotoxicity is a key effector mechanism in T cell mediated control of rubella virus. This raises the question, whether impaired cytotoxicity may also contribute to granuloma development in other IEI such as AT. However, there is no evidence that lymphocyte cytotoxicity is impaired in AT patients (33). Moreover, in most AT patients presenting with rubella granulomas, T-cell lymphopenia was documented, suggesting that similar to atypical SCID/CID patients, the numeric T-cell deficiency is a key factor in these diseases.

The clinical characteristics of the granulomas in our cohort showed many similarities with those of the published IEI patients. We also observed a time lag between MMR vaccination and granuloma onset of several months to years. We found similar immunohistological characteristics with positive rubella staining of variable extent and not correlating with granuloma severity, located in macrophages within granulomas.

Overall, lesions in our patients were less severe than some of those described in published IEI patients. Several patients had only a few non-ulcerated, disseminated nodules or superficial plaques, that spontaneously resolved with variable scar formation without treatment after several months in one third of the cases. However, in the majority of patients, granulomas persisted longer and we also found rubella-antigen in visceral organ granulomas (liver and lung) in 5/21 patients years after vaccination. Notably, the immunosuppressive treatment for HLH in some patients led to partial remission and, as observed previously (32), HSCT performed to treat the underlying genetic defect also cured the granulomas in 4/5 patients. This suggests that the ongoing inflammatory response is an important factor of the tissue pathology and although this is likely to be driven by the virus, the risk of secondary viral dissemination is low.

One limitation of our study was that we were able to analyze skin and visceral biopsies for the presence of the rubella virus only in 12/21 patients by immunohistochemistry. However, we could demonstrate viral protein in all of them. In 3 patients we had additional access to fresh biopsies, enabling the detection of RV by RT-PCR in all analyzed patients. For the remaining 9/21 patients, we did not have access to biopsies. However, in an effort to minimize misdiagnosis, we carefully considered their clinical morphology, the histopathological findings and the time of appearance relative to rubella vaccination.

Why have skin granulomas so far only rarely been reported in patients with cytotoxicity defects? To address the question, whether granuloma formation was a more common phenomenon, we analyzed an “at risk cohort” of 61 patients with genetically confirmed cytotoxicity defect and documented or likely exposure to the MMR vaccine. Based on this analysis, patients with cytotoxicity defects had at least a 1:7 risk to develop skin

granulomas after MMR vaccination. Patients with GS2 had the highest risk with 1:2 patients eventually developing granulomas during childhood. This indicates that, as in AT, granuloma development in patients with cytotoxicity defects is a phenotype with partial penetrance. Notably, NK cell degranulation was similar in GS2 patients with or without granulomas, suggesting that so far unknown factors in addition to the impaired cytotoxicity determine whether MMR vaccination results in granuloma formation. These could also include factors associated with the vaccine preparation or site of injection.

To further explore why GS2 patients were overrepresented in our cohort, we considered the hypothesis that impaired rubella control in the skin could be attributed in part to a T-cell migration defect. This was based on the observation that patients with RAB27A deficiency lack delayed-type skin hypersensitivity reactions (23,24). Moreover, human T cells with RAB27A deficiency were reported to display decreased chemotaxis towards CXCL12 (19,21) and RAB27A-deficient murine neutrophils show diminished migration *in vitro* and *in vivo* (34). In contrast to these findings, we could not demonstrate decreased chemotaxis towards CXCL12 of RAB27A-deficient or MUNC13-4-deficient CD8+ T cells. One explanation for this discrepancy could be the differences in assay setup. Indeed, Franciszkiewic et al. observed diminished migration towards CXCL12 only after recent TCR activation, which they attributed to disturbed synaptic secretion of CCL5 in RAB27A-deficient cells, which is needed for regulated increase of CXCR4 surface expression (21). Since the collagen fiber network of the dermis imposes significant physical constraints on immune cells, we also tested the motility of patient T cells in microchannels and a more complex 3D collagen gel environment. However, also in these assays we found no evidence of impaired T-cell migration in the absence of RAB27A or MUNC13-4.

Importantly, rubella granulomas were not only detected in patients with defects at variable steps of the degranulation machinery but also in patients with perforin deficiency. The common mechanistic basis of these diseases is impairment of lymphocyte cytotoxicity. Given that most other IEI-related granulomas were observed in patients with T-cell deficiencies, it is therefore plausible to assume that T cell mediated cytotoxicity is a key effector mechanism to control rubella infection. Hence, the overrepresentation of patients with GS2, FHL3, FHL5 and some patients with hypomorphic FHL2 may be explained by the fact that the residual cytotoxicity in these patients is sufficient to reach the age of MMR vaccination without HLH manifestation, but insufficient to reliably control rubella virus. In contrast, as shown previously, patients with FHL4 and CHS have a milder impairment of cytotoxicity (35,36) and may therefore be able to control the vaccine.

It is notable that all patients with rubella granulomas controlled the concomitantly applied attenuated mumps, measles and in several cases varicella live vaccines without obvious clinical sequelae, as shown also in other IEI associated with rubella granulomas (10,12,14). This is unlikely due to different tissue tropism, since not only rubella virus, but also measles and varicella vaccine virus can be isolated from the skin of immunodeficient patients with vaccine-induced rashes (37,38). Moreover, our patients did not suffer from generally increased susceptibility to severe or recurrent viral infections, although exposure to wild-type viruses is likely to require more active effector functions than challenge with an attenuated live vaccine. A notable exception was EBV, which was implicated as a trigger of HLH in 3/16 patients who eventually developed this disease.

Is lymphocyte cytotoxicity maybe not as broadly required for control of viral infections as generally assumed? Doubts about this concept also emerge from murine studies.

Perforin-deficient (PKO) mice fail to control infection with lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus (MCMV), Theiler's Murine Encephalomyelitis or ectromelia virus (39–43). However, apart from these prominent examples, cytotoxicity is redundant for the elimination of a large number of other murine host-specific viruses (pneumonia virus of mice, murine gammaherpesvirus 68, JHMV strain of mouse hepatitis virus) (44–46) and viruses not restricted to mice (Vesicular stomatitis virus, Semliki Forest virus, vaccinia virus, cowpox virus, influenza virus, respiratory syncytial virus, Rotavirus) (39,43,47–49). Thus, our human data support the view that cytotoxicity is not needed as an effector mechanism against viruses in general, but rather has a selective role against a few specific viruses (50), including rubella virus.

In summary, we document that patients with cytotoxicity defect are at risk of developing rubella virus containing skin and visceral granulomas after MMR vaccination, but do not seem at risk for developing complications associated with uncontrolled measles, mumps or varicella infection. Skin granulomas can be the presenting manifestation of these genetic defects and they should be considered in the differential diagnosis in young children with persistent granulomas of unknown cause. Importantly, children with known cytotoxicity defects should not be given live MMR vaccines.

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Tables

Table 1: Clinical Characteristics of Patients with Cytotoxicity Defects and Clinical Granulomas

Patient	Diagnosis	Age at Vaccination [Months]	Age at granuloma onset	Age at HLH onset	Description of skin alteration*	Extracutaneous granulomas	Consistent Photodocu-mentation	Granulomas confirmed by histology	Rubella identified	Criteria fulfilled	Identified via
P1	GS2	15 + 25	22 M	no HLH	papulopustular, granulomatous exanthema on limbs and face	N/R	yes	yes	IHC + PCR (skin)	4/4	Index Patient, R
P2	GS2	15 + 72	17 M	no HLH	skin rash, macules evolving into papules on face and limbs	non-specific granulomatous microfoci in CNS	yes	yes	IHC (skin)	4/4	P4 in (51)
P3	GS2	N/A	36 M	4.5 Y	papular, inflammatory lesions on lower limbs, buttocks and cheeks, some ulcerating	N/R	yes	yes	IHC (skin)	4/4	(52)
P4	GS2	12§	12 - 21 M	> 2 M after granuloma onset	multiple red, crusted papules on cheeks and thighs	N/R	yes	yes	N/A	3/4	(53)
P5	GS2	18	24 M	14.5 Y	skin granulomas on arms and legs	N/R	N/A	yes	N/A	2/4	P2 in (51)
P6	GS2	16 + 18	22 M	no HLH	pustules on limbs	N/R	yes	yes	IHC + PCR (skin)	4/4	R
P7	GS2	12	16 M	17.3 Y	pustules on limbs, face, buttocks	N/R	yes	N/A	N/A	2/4	R
P8	GS2	17 + 78	92 M	incomplete HLH (7.8 Y)	skin rash / extensive skin lesions	lung	yes	yes	IHC (skin + lung)	4/4	Lithuania
P9	GS2	12	13 M	8.7 Y	erythematous papules, granulomatous inflammation	N/R	N/A	yes	IHC (skin)	3/4	R
P10	GS2	12	13 M	no HLH	papules on trunk and limbs	N/R	yes	N/A	N/A	2/4	R
P11	GS2	12	14 M	3.1 Y	red papules on face, limbs and buttocks	N/R	N/A	yes	N/A	2/4	R
P12	GS2	N/A	since childhood	no HLH	red papules	liver, muscle	yes	yes	IHC (skin + liver)	4/4	R
P13	GS2	16	16.5 M	5 Y	non-pruritic, erythematous-violaceous papules on cheeks, limbs and buttocks	N/A	yes	yes	N/A	3/4	(54)
P14	FHL2	52	72 M	8 Y (CNS only)	no skin granulomas	lung	N/A	yes	IHC (lung)	2/4	Australia
P15	FHL2	16 - 40	28 - 40 M	8.5 Y	erythematous papular and hyperkeratotic skin lesions on arm and face	N/R	yes	yes	IHC + PCR (skin)	4/4	France
P16	FHL3	N/A	18 M	after granuloma onset	necrotizing palisaded granulomatous dermatitis, small papules on arms and legs	N/R	N/A	yes	N/A	2/4	(55)
P17	FHL3	12 + 22	24 M	incomplete HLH (10 Y)	small granuloma on right upper thigh, increasing in size over time to 4x3 cm	N/R	yes	yes	IHC (skin)	4/4	R
P18	FHL3	12	16 M	3.6 Y	individual purplish papules with some overlying scale on the face, arms and legs	N/R	yes	yes	IHC (skin)	4/4	Australia
P19	FHL3	12	48 M	6 Y	irregular single maculopapular lesion on left calf	lung	yes	yes	IHC (skin + lung)	4/4	Australia
P20	FHL3	N/A	18 M	21 M	dense rash on arms, legs and face	N/A	yes	N/A	N/A	2/4	P1 in (56)
P21	FHL5	N/A	43 M	45 M	erythematous papules on lower limbs and occasionally on belly	N/R	N/A	N/A	N/A	1/4	R

*R = German HLH reference centers, patient included in "AT RISK" cohort, *information taken from medical files and letters, § exact age not given in reference, however MMR vaccination is compulsory in Chile and recommended at 12 M*

711 *Abbreviations: N/R = not reported, N/A = not available, CNS = central nervous system, GS2 = Griscelli syndrome type 2, FHL = familial hemophagocytic lymphohistiocytosis, IHC =*
712 *immunohistochemistry*
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Figure legends:

Figure 1: Clinical pictures of cutaneous skin lesions in patients with cytotoxicity defects.

Figure 2: Histopathological findings in patients with rubella-associated granulomas. **(A)** Skin biopsy of P2 stained with HE, showing multiple dermal epithelioid granulomas and epidermal hyperkeratosis. Double staining with anti-CD68 (red) and anti-RV-C (brown) revealed double positive cells, including giant cells, in the center of the granulomas. Anti-CD8 staining showed infiltrating CD8+ T cells in the periphery of the granulomas. Scale bar = 200 µm (left), 20 µm (right). **(B)** Double staining of the lung biopsy of P14. Scale bar = 50 µm (upper), 20 µm (lower). **(C)** Distribution of RV capsid positive cells in skin samples. Positive cells were located within granulomas or loosely formed granuloma-like histiocytic aggregates. Scale bar = 20 µm.

Figure 3: Clinical course of granulomas in patients with cytotoxicity defects. Time periods during which granulomas were reported are depicted as grey boxes, whereas white boxes (labelled with "?") indicate that detailed information on granuloma persistence was not available. Age at vaccination is indicated by triangles, age at granuloma onset (if known) is indicated by an arrow, time point of HSCT by a cross.

Figure 4: Risk of granuloma development for patients with cytotoxicity defects. **(A)** Recruitment scheme for patients in the "AT RISK" cohort. **(B)** Percentage of cases with skin alterations consistent with rubella granulomas, categorized by underlying genetic disorder. (C) Activity of NK cells from GS2 patients with (+ gran.) and without (- gran.) granulomas measured by degranulation assay of resting NK cells. ΔCD107a indicates the difference in the fraction of NK cells expressing CD107a under

unstimulated and stimulated conditions. Grey shaded area represents normal values (10^{th} percentile of 94 healthy controls). **(D)** Distribution of granuloma cases among patients with different cytotoxicity defects. Left: Patients included in “AT RISK” cohort, right: all patients with clinical granulomas (skin and visceral).

Figure 5: T cell chemotaxis and migration are not affected by mutations in RAB27A or UNC13D. (A) CXCR3 surface expression on CD8+ T cell lines from healthy donors (HD) and patients with mutations in RAB27A, UNC13D or ATM after stimulation with 100 nM CXCL11. Results from 2 individual experiments are summarized on the left (mean +/- SD), representative histograms are shown on the right. **(B)** Transwell migration of CD8+ T-cell lines towards CXCL10 or CXCL12. Each experiment was performed once. **(C)** Scheme of the micro-channel device used to assess cell speed. Cells are loaded in the round loading chamber and from there spontaneously migrate into the micro-channels (upper section). Lower section depicts CD8+ T-cell lines (labelled with Hoechst33342) moving within 4 µm wide micro-channels. Scale bar = 100 µm. Scheme adapted from Vargas et al. 2016 (57). Mean speeds of 3 individual experiments for 3 HD and patients with mutations in PRF1 (n=1), STXBP2 (n=1), RAB27A (n=2), UNC13D (n=3) are shown as individual values, bars represent mean speed of pooled results +/- SD. **(D)** Schematic drawing of the custom-made PDMS chamber loaded with a mixture of cells and collagen (grey lines), adapted from Sáez et al., 2018 (18). Bright field image of CD8+ T-cell lines moving within the 3D collagen matrix (3 mg/ml rat tail collagen type I). Cell tracks after 60 min of cell migration are overlaid as colored lines. Scale bar = 200 µm. 120 cells were tracked per experiment, bars represent mean speed of pooled results +/- SD. (C+D) Open circles represent results obtained for T-cell lines derived from patients with skin granulomas. Statistical analysis: Ordinary one-way ANOVA with Dunnett’s multiple comparisons test.

Fig. 1

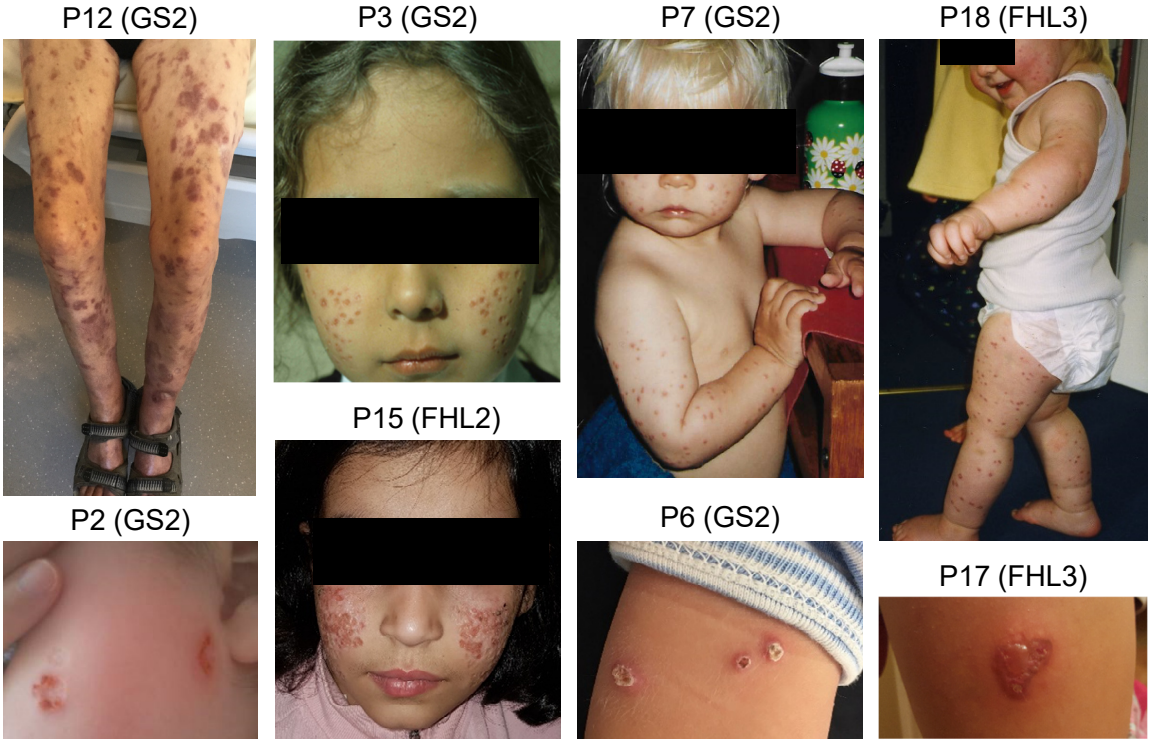


Fig. 2

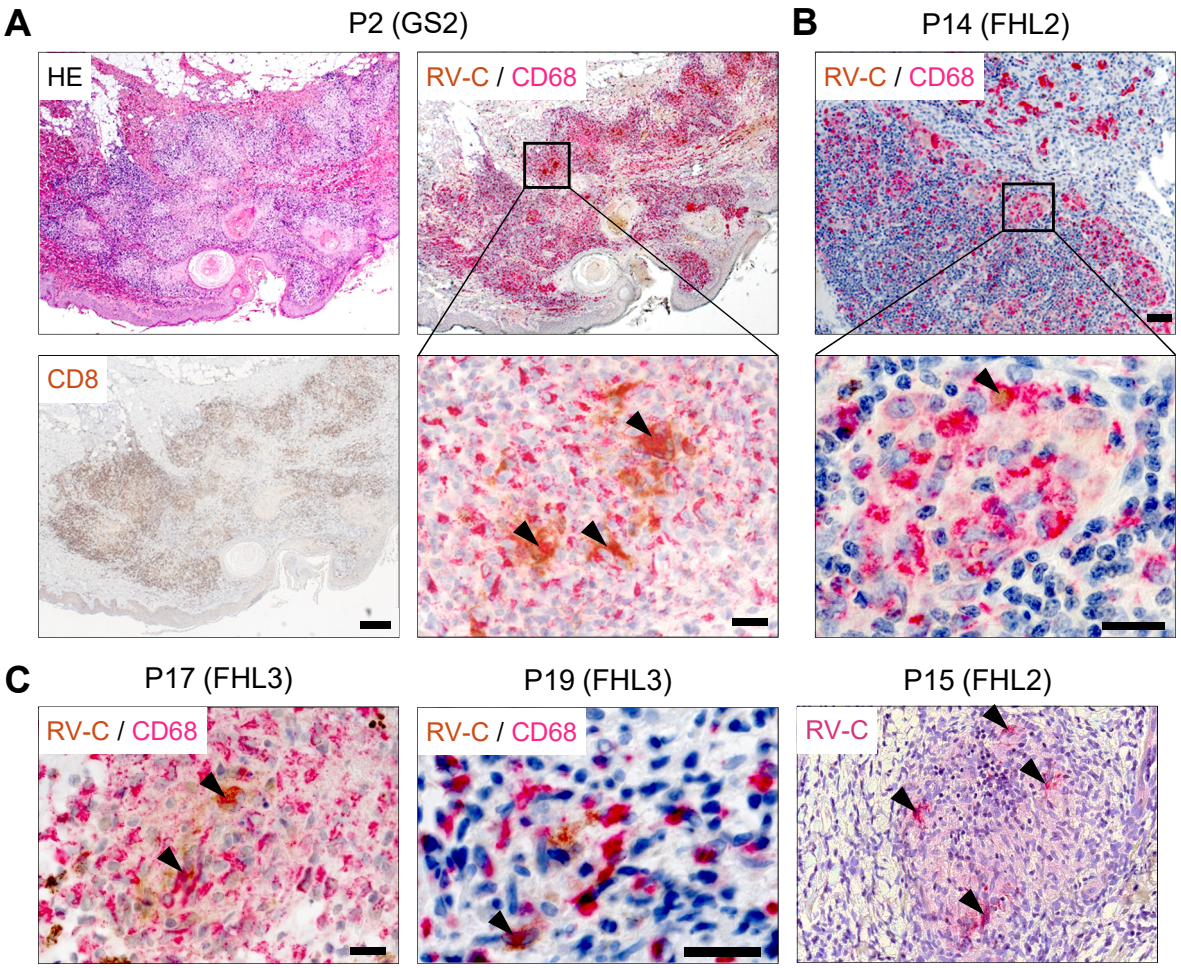
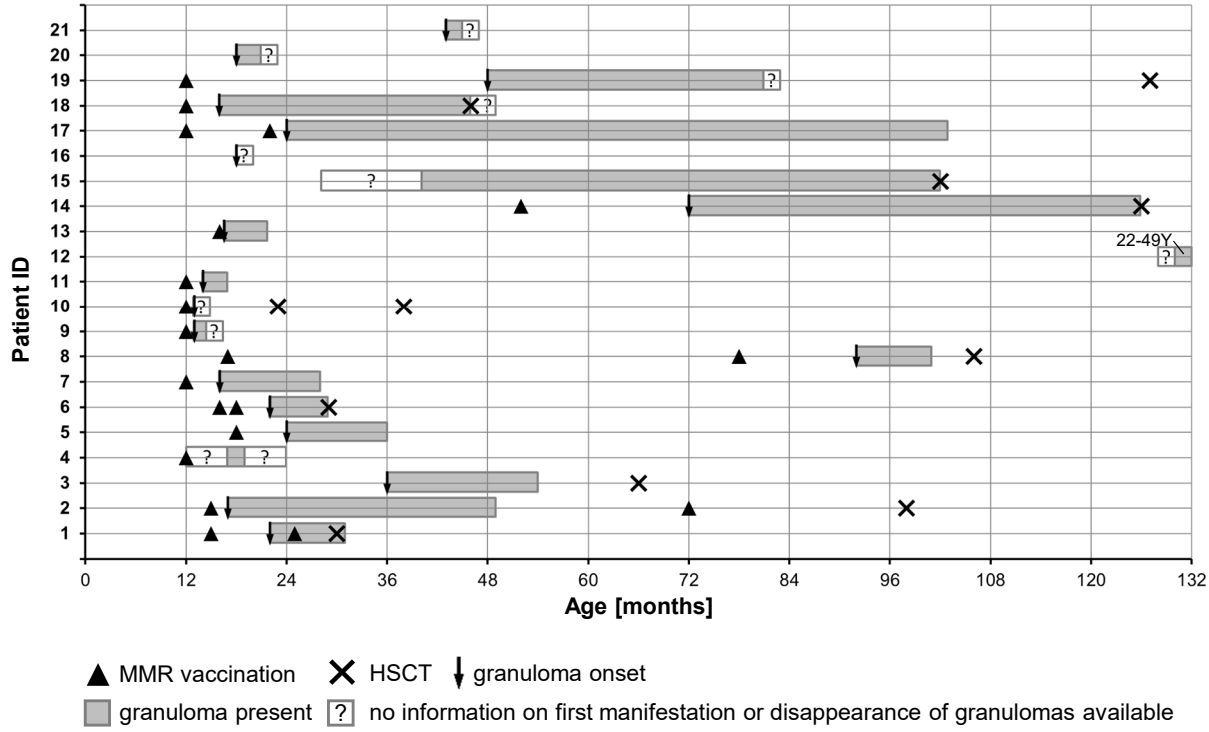

A
P2 (GS2)
HE
RV-C / CD68
CD8
B
P14 (FHL2)
RV-C / CD68
C
P17 (FHL3)
RV-C / CD68
P19 (FHL3)
RV-C / CD68
P15 (FHL2)
RV-C

Fig. 3


Patient ID
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120
132
Age [months]
22-49Y
MMR vaccination
HSCT
granuloma onset
granuloma present
no information on first manifestation or disappearance of granulomas available

Fig. 4

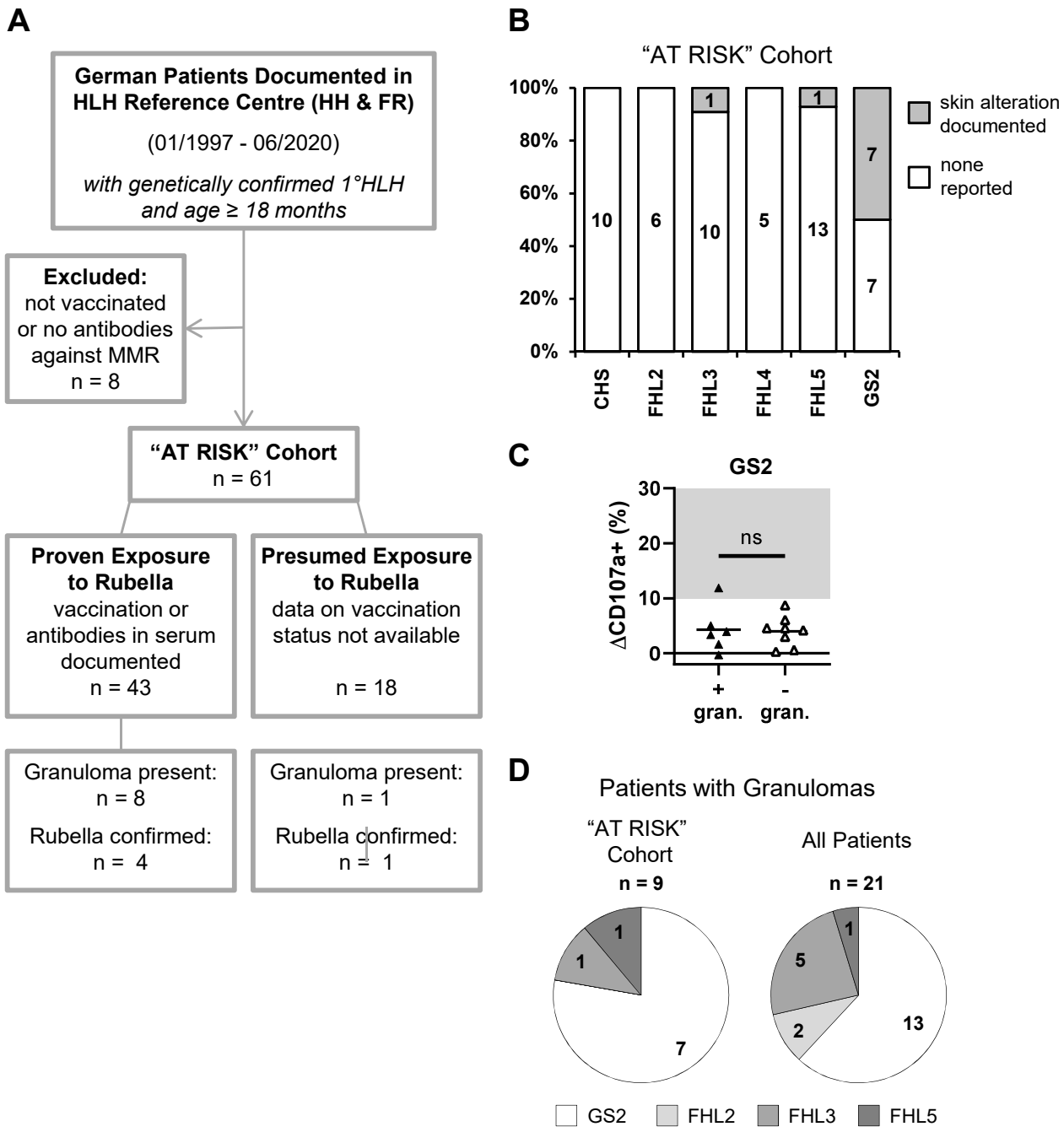
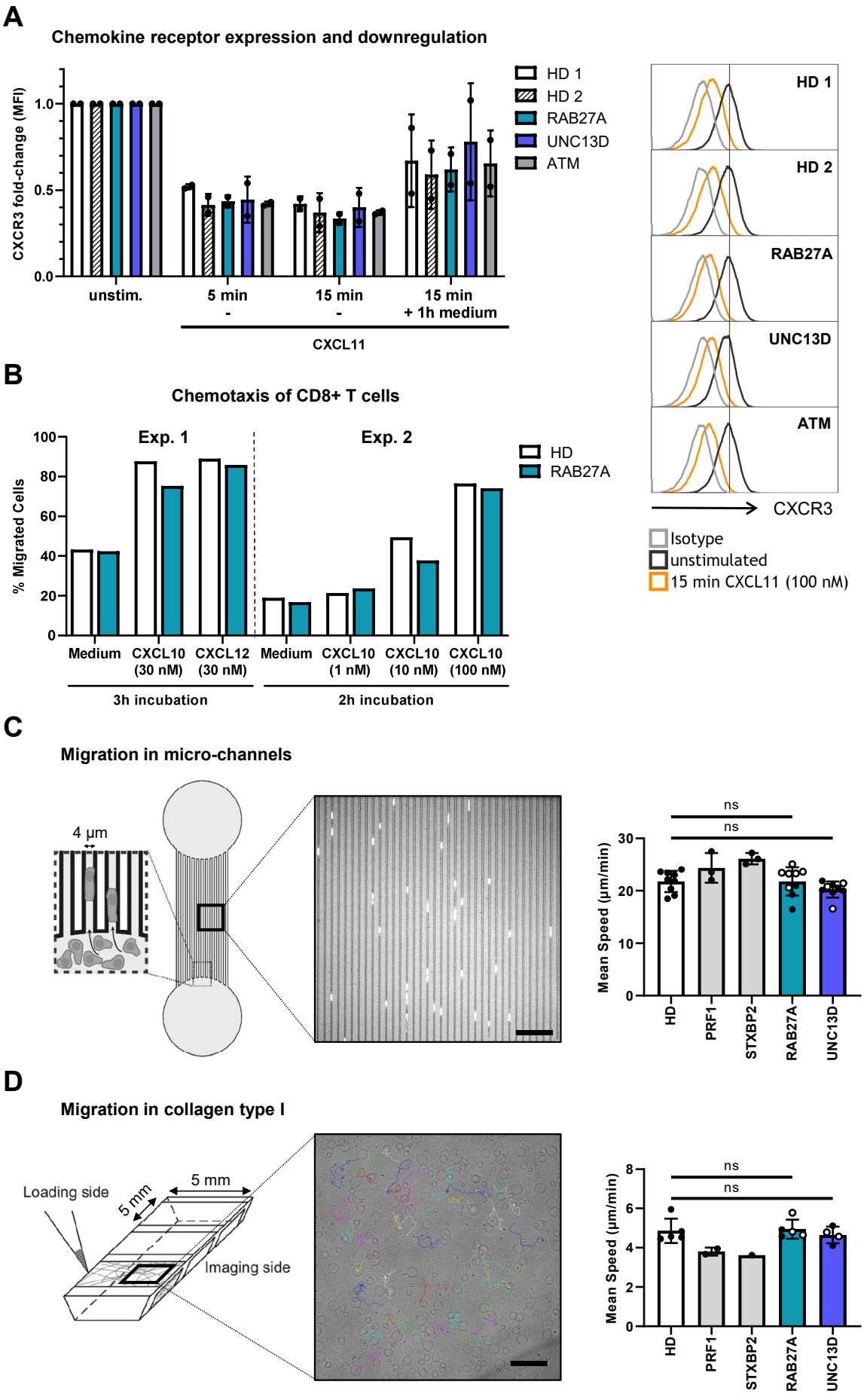

A
German Patients Documented in HLH Reference Centre (HH & FR)
(01/1997 - 06/2020)
with genetically confirmed 1°HLH and age ≥ 18 months
Excluded: not vaccinated or no antibodies against MMR n = 8
"AT RISK" Cohort n = 61
Proven Exposure to Rubella vaccination or antibodies in serum documented n = 43
Presumed Exposure to Rubella data on vaccination status not available n = 18
Granuloma present: n = 8
Rubella confirmed: n = 4
Granuloma present: n = 1
Rubella confirmed: n = 1
B
"AT RISK" Cohort
100%
80%
60%
40%
20%
0%
10
6
1
10
5
1
13
7
7
skin alteration documented
none reported
CHS
FHL2
FHL3
FHL4
FHL5
GS2
C
GS2
ΔCD107a+ (%)
30
20
10
0
ns
+ gran.
- gran.
D
Patients with Granulomas
"AT RISK" Cohort
n = 9
All Patients
n = 21
1
1
7
1
5
2
13
GS2
FHL2
FHL3
FHL5

Fig. 5


A
Chemokine receptor expression and downregulation
CXCR3 fold-change (MFI)
1.0
0.5
0.0
unstim.
5 min
-
15 min
-
15 min
+ 1h medium
CXCL11
HD 1
HD 2
RAB27A
UNC13D
ATM
HD 1
HD 2
RAB27A
UNC13D
ATM
CXCR3
Isotype
unstimulated
15 min CXCL11 (100 nM)
B
Chemotaxis of CD8+ T cells
% Migrated Cells
100
80
60
40
20
0
Exp. 1
Exp. 2
HD
RAB27A
Medium
CXCL10 (30 nM)
CXCL12 (30 nM)
Medium
CXCL10 (1 nM)
CXCL10 (10 nM)
CXCL10 (100 nM)
3h incubation
2h incubation
C
Migration in micro-channels
4 µm
ns
ns
Mean Speed (µm/min)
30
20
10
0
HD
PRF1
STXBP2
RAB27A
UNC13D
D
Migration in collagen type I
Loading side
5 mm
5 mm
Imaging side
ns
ns
Mean Speed (µm/min)
8
6
4
2
0
HD
PRF1
STXBP2
RAB27A
UNC13D