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1 **Rubella vaccine-induced granulomas are a novel phenotype with**  
2 **incomplete penetrance of genetic defects in cytotoxicity**

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119 **Abstract** (246 words)

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

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

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

130 **Results:** We identified 21 patients with various genetically confirmed cytotoxicity  
131 defects, who presented with skin and visceral granulomas. Rubella virus was  
132 demonstrated in all 12 accessible biopsies. Granuloma onset was typically before age  
133 2 years and lesions persisted from months to years. Granulomas were particularly  
134 frequent in MUNC13-4 and RAB27A deficiency, where 50% of patients at risk were  
135 affected. Although these proteins have also been implicated in lymphocyte migration,  
136 3D migration assays revealed no evidence of impaired migration of patient T cells.  
137 Notably, patients showed no evidence of reduced control of concomitantly given  
138 measles, mumps or varicella live-attenuated vaccine or severe infections with other  
139 viruses.

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

144

145 **Clinical Implications (28 words):**

146 Lymphocyte cytotoxicity is important for control of rubella vaccine virus persistence.

147 Patients with genetic defects of cytotoxicity including albinism syndromes should not  
148 be vaccinated with rubella live vaccine.

149

150 **Capsule Summary (35 words).**151 Lymphocyte cytotoxicity is important for control of rubella vaccine without evidence for  
152 its need to control concomitantly given live vaccines. Rubella vaccine-induced  
153 granulomas are a novel phenotype with incomplete penetrance of disorders of  
154 cytotoxicity.

155

156 **Key words (up to 10):**157 Cytotoxicity, rubella virus, live vaccine, granuloma, primary immunodeficiency,  
158 hemophagocytic lymphohistiocytosis, Griscelli syndrome type 2

159

160 **Abbreviations.**

161 AT – Ataxia telangiectasia

162 CHS – Chédiak-Higashi syndrome

163 CMV - Cytomegalovirus

164 CNS – central nervous system

165 CTL – cytotoxic T lymphocyte

166 DTH – delayed type hypersensitivity

167 EBV – Epstein-Barr virus

168 fHLH – familial hemophagocytic lymphohistiocytosis



- 169 HLH - hemophagocytic lymphohistiocytosis
- 170 HSCT- hematopoietic stem cell transplantation
- 171 IEI - inborn errors of immunity
- 172 GS2 – Griscelli syndrome type 2
- 173 LCMV - lymphocytic choriomeningitis virus
- 174 MMR(V) – measles, mumps, rubella, (varicella) live-attenuated vaccine
- 175 PBMCs - Peripheral blood mononuclear cells
- 176 PCR – polymerase chain reaction
- 177 PID – primary immunodeficiency
- 178 RV – rubella virus
- 179 RV-C - rubella virus capsid
- 180 VZV- varicella-zoster virus

## 181 **Introduction**

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

200 Granulomas are organized structures formed by macrophages and other immune cells  
201 that participate in antimicrobial defense by preventing spread of a persistent pathogen  
202 (8). This also includes viral infections such as measles and EBV (9). Recently, elegant  
203 work has demonstrated persistence of rubella virus (RV) vaccine strain RA27/3 in skin  
204 and visceral granulomas of patients with inborn errors of immunity (IEI) over decades  
205 (10–12). Most commonly, these RV associated granulomas were detected in patients  
206 with Ataxia-telangiectasia (AT) and other DNA repair disorders, followed by patients

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

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

## 224 **Materials and Methods**

### 225 **Patient Recruitment**

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

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

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

## 249 **Immunohistochemistry**

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

## 258 **Molecular Analysis**

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

## 261 **Cell Culture**

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

## 268 **Flow Cytometry Based Assays**

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

**273 T-cell Migration Assays**

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

280

## 281 **Results**

### 282 **A patient with Griscelli syndrome type 2 and rubella granulomas**

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

### 300 **Skin and visceral granulomas are common in patients with genetic defects in** 301 **cytotoxicity**

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

306 analysis of the databases of the German HLH reference centers and enquiries through  
307 an international network of colleagues. Twenty additional patients with a history of skin  
308 or visceral granuloma were identified based on a clinical description given by referring  
309 physicians, the medical reports or publications (Table 1). We evaluated three additional  
310 criteria to verify the diagnosis in these 20 patients: (i) photo documentation compatible  
311 with skin granulomas, (ii) histological confirmation of granulomas and (iii) identification  
312 of rubella by immunohistochemistry and/or PCR. We had access to formalin-fixed  
313 paraffin embedded skin biopsies from 10 patients (P2,3,6,8,9,12,15,17,18,19) and  
314 detected the RV antigen by immunohistochemistry in all samples. Additionally, rubella  
315 was identified in 3 of these patients (P1,6,15) by RT-PCR analysis of fresh skin  
316 biopsies. Sequence analysis of the PCR products in 2 patients (P6,15) revealed a  
317 sequence identical (P6, 131 nt sequenced) or nearly identical (P15, 739 nt sequenced,  
318 98.2% alignment) with that of the rubella RA27/3 vaccine strain. Overall, 7/21 patients  
319 fulfilled 2 criteria, 3/21 fulfilled 3 criteria and 10/21 fulfilled all 4 criteria (Table 1). In  
320 addition to skin granulomas, some patients had rubella-positive extracutaneous  
321 granulomas in the liver (P12) or lung (P8,19), detected in the context of liver or  
322 interstitial lung disease. P14 only presented with lung granulomas (Table 1). Among  
323 the 21 patients in this “granuloma cohort”, 13 had RAB27A deficiency, 5 had MUNC13-  
324 4 deficiency, 2 had PRF1 deficiency and 1 had MUNC18-2 deficiency (Table 1).  
325 Patients with FHL2 (perforin deficiency), FHL3 (MUNC13-4 deficiency) or FHL5  
326 (MUNC18-2 deficiency) had at least one point or splice site mutation (Suppl. Table 1)  
327 possibly indicating hypomorphic disease variants.

### 328 **Clinical and immunohistochemical variability of granulomas**

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



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

#### 346 **Clinical course of granulomas and response to treatment**

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

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

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

### 370 **Risk of rubella granuloma varies with genetic diagnosis**

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

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

384 Literature indicates a role for RAB27A in cell motility (19–22) and GS2 patients were  
385 shown to have absent delayed-type cutaneous hypersensitivity (DTH) responses  
386 (23,24). Since nearly all patients had deficiency in RAB27A or the RAB27A interaction  
387 partner MUNC13-4, we considered that the mutations impair T-cell migration and  
388 therefore rubella clearance in the skin.

389 Since the chemokine receptor CXCR3 and its ligands are important for directed  
390 migration of T cells and NK cells into inflamed tissues and contribute to virus control in  
391 the skin (25–27), we first tested expression and regulation of CXCR3 (Figure 5A).  
392 Surface expression of CXCR3 on CD8+ T cells was similar in patient and control cells  
393 and stimulation with CXCL11 caused a comparable downregulation and re-expression  
394 kinetics of CXCR3. Moreover, patient cells showed concentration-dependent migration  
395 towards the chemokine CXCL10 that was comparable with control cells (Figure 5B).  
396 To investigate the motility of RAB27A- and MUNC13-4-deficient CD8+ T cells in a more  
397 confined environment, we examined the migration of fluorescently labelled T-cell blasts  
398 along 4 µm wide micro-channels (Figure 5C). Patient T cells moved at the same speed  
399 as control cells. Also, when the skin's dermal layer was mimicked by using a three-  
400 dimensional (3D) collagen gel matrix, time-lapse video microscopy revealed no  
401 differences in speed between CD8+ T cells from patients and healthy controls (Figure  
402 5D). In summary, we could not provide evidence that RAB27A- and MUNC13-4-  
403 deficiency affects the spontaneous or chemokine-induced motility of CD8+ T cells.

404

## 405 **Discussion**

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

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

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

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

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

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

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

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

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

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

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

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

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

522

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530



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706 **Tables**707 **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 Photodocumentation	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 <sup>§</sup>	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

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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*  
713

714

715 **Figure legends:**

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

718 **Figure 2: Histopathological findings in patients with rubella-associated**  
719 **granulomas. (A)** Skin biopsy of P2 stained with HE, showing multiple dermal  
720 epithelioid granulomas and epidermal hyperkeratosis. Double staining with anti-CD68  
721 (red) and anti-RV-C (brown) revealed double positive cells, including giant cells, in the  
722 center of the granulomas. Anti-CD8 staining showed infiltrating CD8+ T cells in the  
723 periphery of the granulomas. Scale bar = 200  $\mu\text{m}$  (left), 20  $\mu\text{m}$  (right). **(B)** Double  
724 staining of the lung biopsy of P14. Scale bar = 50  $\mu\text{m}$  (upper), 20  $\mu\text{m}$  (lower). **(C)**  
725 Distribution of RV capsid positive cells in skin samples. Positive cells were located  
726 within granulomas or loosely formed granuloma-like histiocytic aggregates. Scale bar  
727 = 20  $\mu\text{m}$ .

728 **Figure 3: Clinical course of granulomas in patients with cytotoxicity defects.**

729 Time periods during which granulomas were reported are depicted as grey boxes,  
730 whereas white boxes (labelled with “?”) indicate that detailed information on granuloma  
731 persistence was not available. Age at vaccination is indicated by triangles, age at  
732 granuloma onset (if known) is indicated by an arrow, time point of HSCT by a cross.

733 **Figure 4: Risk of granuloma development for patients with cytotoxicity defects.**

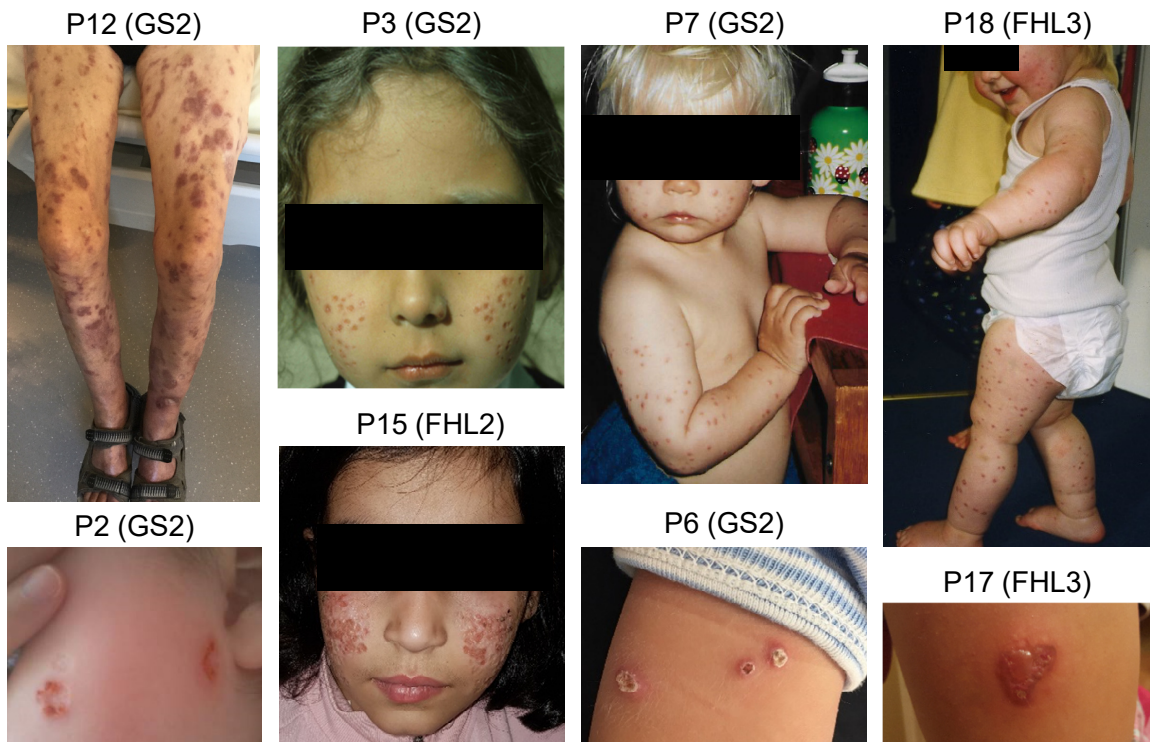
734 **(A)** Recruitment scheme for patients in the “AT RISK” cohort. **(B)** Percentage of cases  
735 with skin alterations consistent with rubella granulomas, categorized by underlying  
736 genetic disorder. **(C)** Activity of NK cells from GS2 patients with (+ gran.) and without  
737 (- gran.) granulomas measured by degranulation assay of resting NK cells.  $\Delta\text{CD107a}$   
738 indicates the difference in the fraction of NK cells expressing CD107a under



739 unstimulated and stimulated conditions. Grey shaded area represents normal values  
740 (10<sup>th</sup> percentile of 94 healthy controls). **(D)** Distribution of granuloma cases among  
741 patients with different cytotoxicity defects. Left: Patients included in “AT RISK” cohort,  
742 right: all patients with clinical granulomas (skin and visceral).

743 **Figure 5: T cell chemotaxis and migration are not affected by mutations in**  
744 **RAB27A or UNC13D. (A)** CXCR3 surface expression on CD8+ T cell lines from  
745 healthy donors (HD) and patients with mutations in RAB27A, UNC13D or ATM after  
746 stimulation with 100 nM CXCL11. Results from 2 individual experiments are  
747 summarized on the left (mean +/- SD), representative histograms are shown on the  
748 right. **(B)** Transwell migration of CD8+ T-cell lines towards CXCL10 or CXCL12. Each  
749 experiment was performed once. **(C)** Scheme of the micro-channel device used to  
750 assess cell speed. Cells are loaded in the round loading chamber and from there  
751 spontaneously migrate into the micro-channels (upper section). Lower section depicts  
752 CD8+ T-cell lines (labelled with Hoechst33342) moving within 4 µm wide micro-  
753 channels. Scale bar = 100 µm. Scheme adapted from Vargas et al. 2016 (57). Mean  
754 speeds of 3 individual experiments for 3 HD and patients with mutations in PRF1 (n=1),  
755 STXBP2 (n=1), RAB27A (n=2), UNC13D (n=3) are shown as individual values, bars  
756 represent mean speed of pooled results +/- SD. **(D)** Schematic drawing of the custom-  
757 made PDMS chamber loaded with a mixture of cells and collagen (grey lines), adapted  
758 from Sáez et al., 2018 (18). Bright field image of CD8+ T-cell lines moving within the  
759 3D collagen matrix (3 mg/ml rat tail collagen type I). Cell tracks after 60 min of cell  
760 migration are overlaid as colored lines. Scale bar = 200 µm. 120 cells were tracked per  
761 experiment, bars represent mean speed of pooled results +/- SD. (C+D) Open circles  
762 represent results obtained for T-cell lines derived from patients with skin granulomas.  
763 Statistical analysis: Ordinary one-way ANOVA with Dunnett’s multiple comparisons  
764 test.

**Fig. 1**



**Fig. 2**

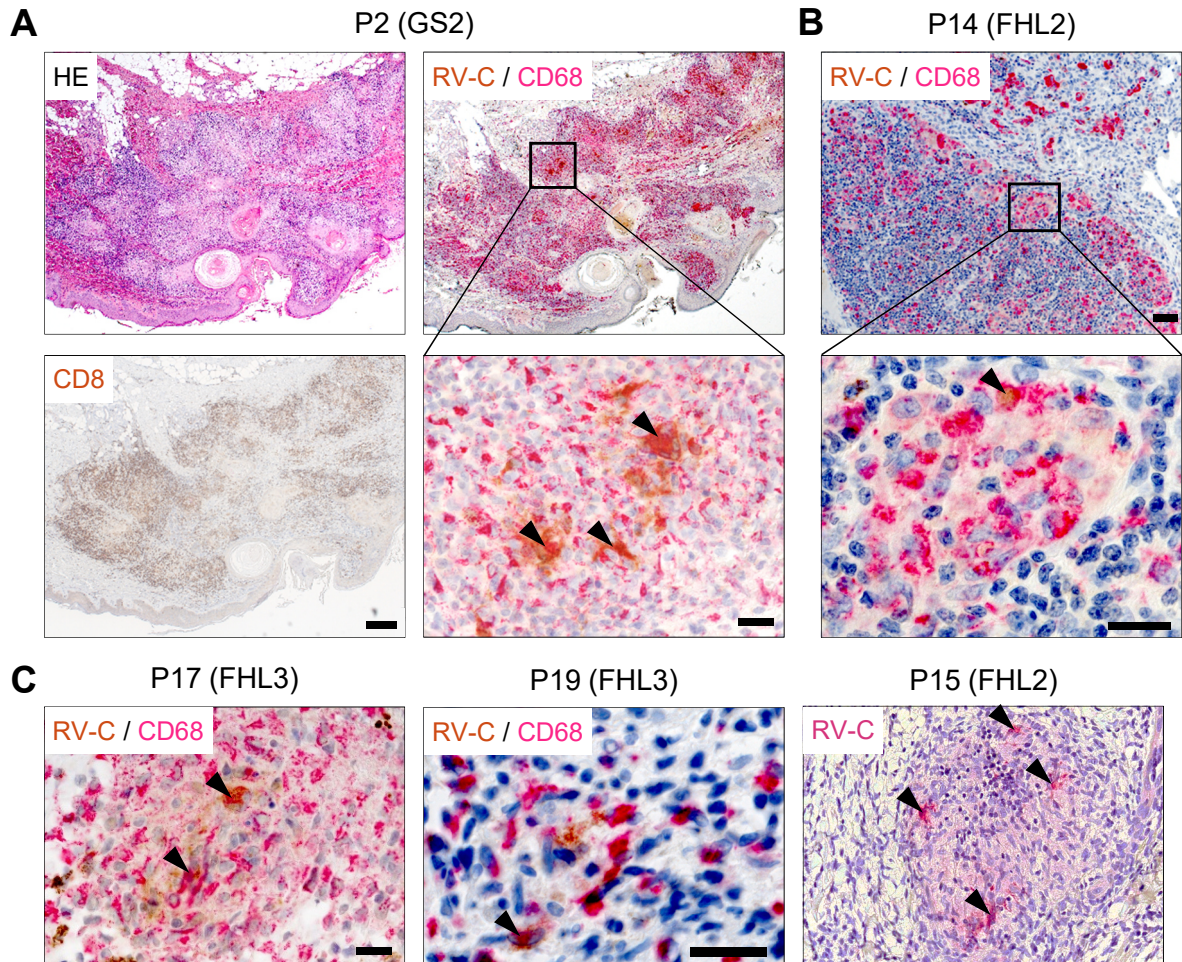
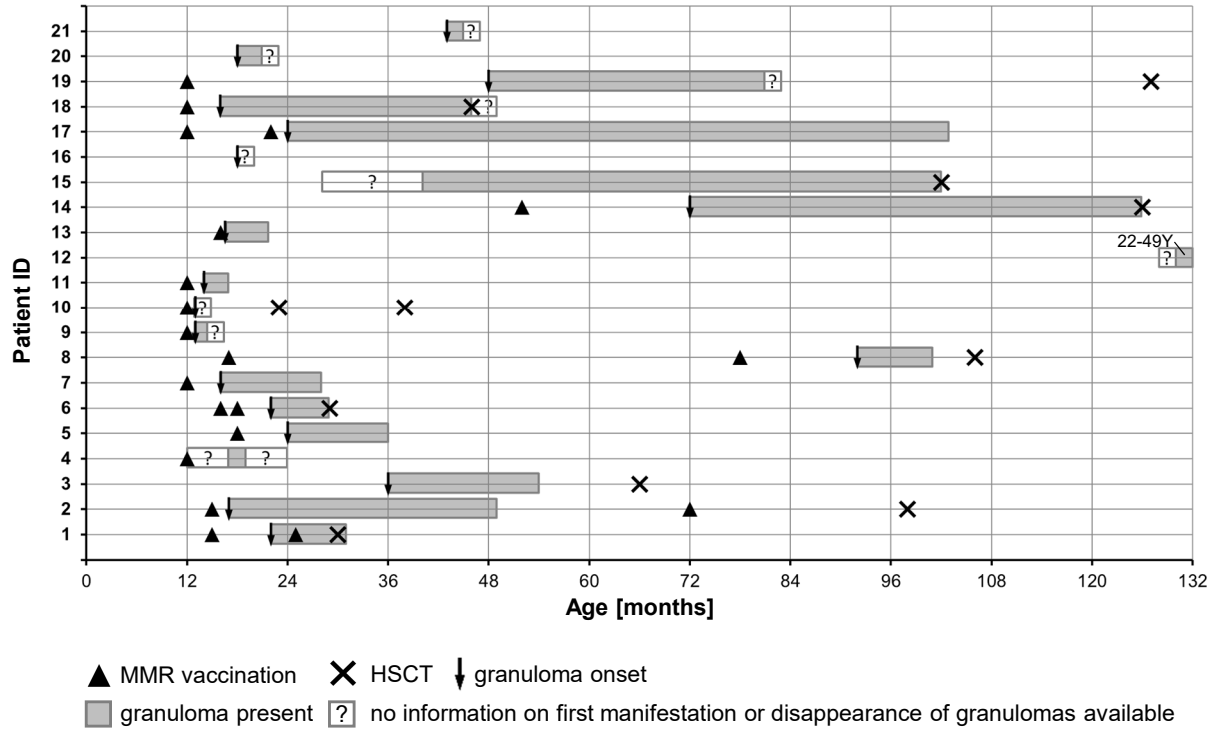
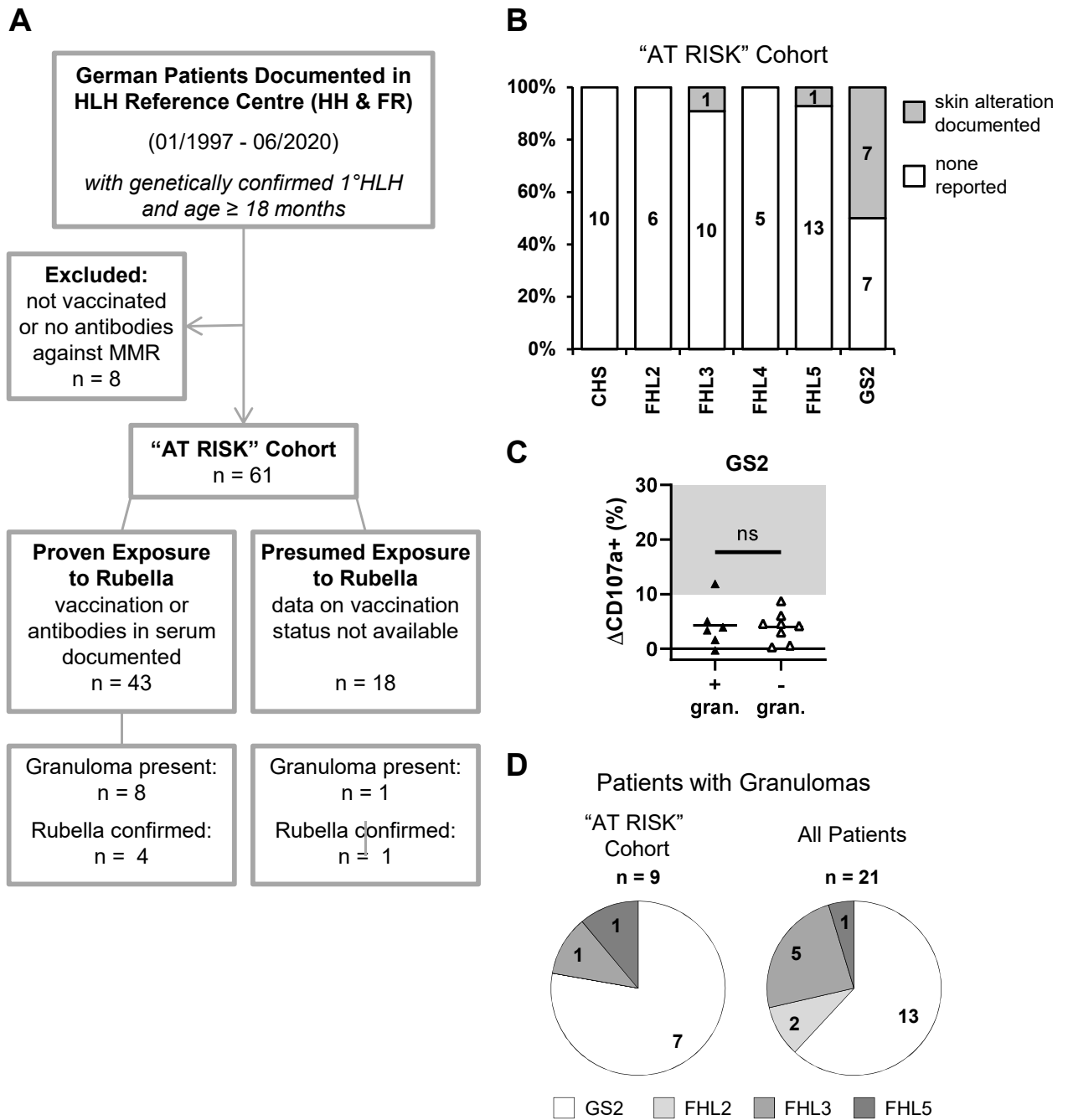


Fig. 3

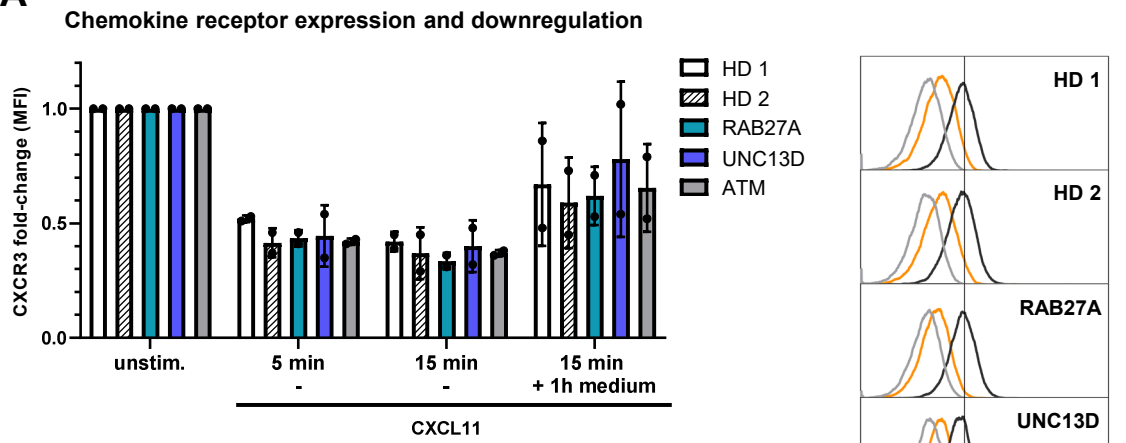


**Fig. 4**

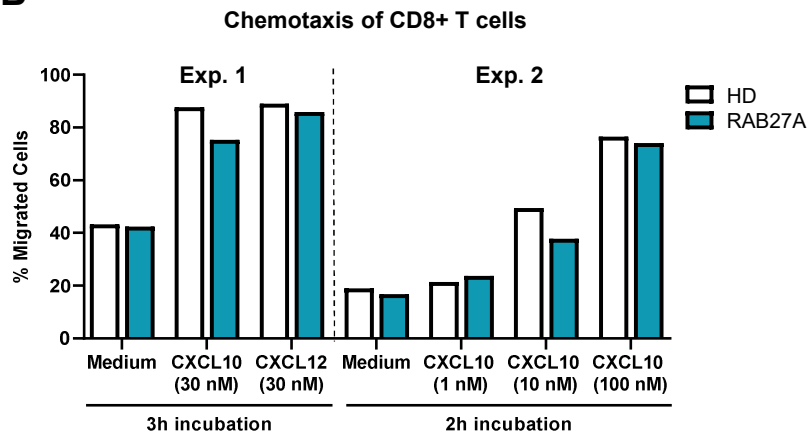


# Fig. 5

**A**

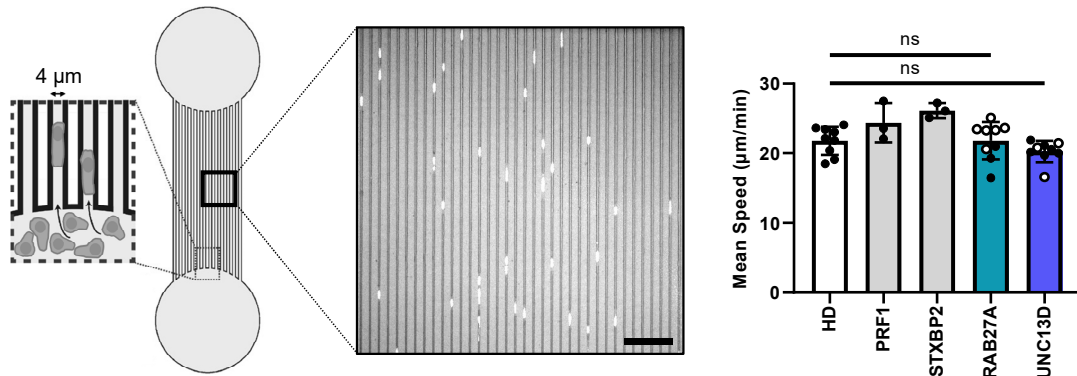


**B**



**C**

**Migration in micro-channels**



**D**

**Migration in collagen type I**

