

Phenotypic and Functional Plasticity of Murine Intestinal NKp46+ Group 3 Innate Lymphoid Cells

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1	Phenotypic and functional plasticity of murine intestinal NKp46 ⁺ ILC3	
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29 Type 3 Innate Lymphoid Cells (ILC3) actively participate in mucosal defense and 30 homeostasis through prompt secretion of IL-17A, IL-22 and IFN-y. Reports identify 31 two ILC3 lineages : a CCR6⁺T-bet⁻ subset that appears early in embryonic 32 development and promotes lymphoid organogenesis, and a CCR6⁻T-bet⁺ subset that 33 emerges after microbial colonization and harbors NKp46⁺ ILC3. Here, we 34 demonstrate that NKp46 expression in ILC3 subset is highly unstable. Cell fatemapping using $Ncr1^{CreGFP} \ge Rosa26^{RFP}$ mice revealed the existence of an intestinal 35 RFP⁺ ILC3 subset (*Ncr1*^{FM}) lacking NKp46 expression at the transcript and protein 36 level. Ncr1^{FM} ILC3 produced more IL-22 and were distinguishable from NKp46⁺ 37 ILC3 by differential CD117, CD49a, DNAM-1, and surprisingly, CCR6 expression. 38 Ncr1^{FM} ILC3 emerged after birth and persisted in adult mice following broad-39 40 spectrum antibiotic treatment. These results identify an unexpected phenotypic 41 instability within NKp46⁺ ILC3 that suggests a major role for environmental signals 42 in tuning ILC3 functional plasticity.

43 Introduction

44 Innate lymphoid cells (ILCs) comprise an evolutionarily conserved and related 45 set of innate immune effectors. Three ILC groups can be distinguished on the basis of 46 their transcription factors profiles and cytokine production (1-3). T-bet-expressing 47 ILC1 are involved in responses against viruses, intracellular pathogens and tumors 48 and include Eomes-dependent cytotoxic ILC1 (NK cells) and Eomes-independent 49 ILC1, both of which produce interferon- γ (IFN- γ). ILC2 participate in protection 50 against helminthes and viruses via Gata-3-dependent production of interleukins (IL)-51 5, IL-13 and amphiregulin, but may also aggravate allergic responses. ILC3 are 52 enriched at mucosal sites, drive lymphoid tissue organogenesis during fetal life, and 53 maintain intestinal homeostasis in adults through IL-22 production downstream of the 54 transcription factor RORyt. Two ILC3 sublineages have been proposed (4, 5): a 55 CCR6⁺T-bet⁻ subset that includes Lymphoid Tissue inducer (LTi) cells, and CCR6⁻Tbet⁺ subset that develops post-natally in response to microbial stimulation and gives 56 57 rise to cells expressing the natural cytotoxic receptor (NCR) NKp46. These two ILC3 58 subsets share strong functional similarities (6), but occupy distinct niches (7-10). As 59 such their respective specific versus redundant roles in mucosal defense remain 60 unclear.

61 While NKp46 crosslinking triggers cytotoxicity in human and mouse NK cells, 62 the functional role for NCR on ILC3 is poorly understood. Cytokine production in 63 human ILC3 can be stimulated by anti-NKp44 antibodies (11) although the ligands 64 that can mimic this activity *in vivo* remain unknown. In the mouse, *Ncr1* ablation (in 65 *Ncr1*^{GFP/GFP} mice) has little impact on ILC3 homeostasis and cytokine responses to 66 pathogenic *Citrobacter rodentium* are unaffected (12). Modulation of NKp46 67 expression on NK cells occurs during MCMV infection (13) and may 'tune' ILC3 responses in a fashion similar to that proposed for 'licensing' of cytokine andcytotoxic responses of NK cells via NKp46 (14).

70 To address dynamics of NKp46 expression in mucosal ILC3, we have performed in vivo fate-mapping studies using Ncr1^{CreGFP} mice (15) that harbor a 71 72 transgene containing the Ncr1 proximal promoter driving expression of a Green Fluorescent Protein/Cre-recombinase fusion protein (CreGFP). Analysis of Ncr1^{CreGFP} 73 x Rosa26^{RFP} mice (in which NKp46-expressing cells are indelibly marked with RFP 74 expression) revealed the existence of an unusual NKp46⁻GFP⁻RFP⁺ cells uniquely 75 76 within the intestinal mucosa. Here we report the phenotypic and functional characterization of these cells (referred to here as 'Ncr1 Fate-Mapped' ILC3 or 77 *Ncr1*^{FM} ILC3) and discuss the potential implications for NKp46 plasticity in mucosal 78 79 immunity.

80 Material and Methods

81 *Mice*

82 Tg(Ncr1-cre/EGFP)#Cajv mice ($Ncr1^{CreGFP}$) were generated and crossed to $Rosa26^{RFP}$ 83 mice as described (15). $Ncr1^{GFP/+}$ mice (16) were the kind gift from O. Mandelboim 84 (Jerusalem, Israel). Unless specified otherwise, adult mice were used between 7 and 85 13 weeks of age. For E18.5 embryos, mice were co-housed overnight, female mice 86 with a vaginal plug were separated the next day, with embryos considered as E0.5.

87

88 *Antibiotic treatment*

An antibiotic cocktail was used to deplete intestinal commensal microbiota as previously described (4). Ampicillin (1g/L), colistin (1g/L), streptomycin (5g/L) and sucrose (20%; Sigma Aldrich) were diluted into drinking water and then filter sterilized (0.22µm). Plugged female mice were treated with antibiotics; the drinking water was changed each week. Four weeks after birth, pups were weaned and treatment continued until sacrifice 3 weeks later.

95

96 Isolation of Lamina Propria Lymphocytes

97 Small intestine were extracted and perfused with cold RPMI-1640 medium to remove 98 feces. Peyer's patches were removed and the intestine cut open longitudinally, then 99 washed again. Intestine were cut into 1cm pieces and agitated in pre-warmed RPMI-100 1640 medium (Gibco) containing 10mM EDTA (Sigma-aldrich) at 37°C for 15 min, 101 followed by 15 min wash with RPMI-1640 alone at 37°C. Pieces of intestine were 102 harvested, cut into small pieces and digested in RPMI (Gibco) containing 5% FCS 103 and Collagenase VII (0.75mg/mL; Sigma-Aldrich) (twice for 20 min each). After 104 each extraction, supernatants were collected, filtered (100 µm), pooled and 105 centrifuged (300g; 10 min). Lymphocytes were enriched by Percoll 80/40% gradient 106 centrifugation. For stimulation, 1-2 x 10^6 cells were incubated at 37°C with IL-1 β 107 (R&D;100 ng/ml), IL-2 (Peprotech; 100ng/ml), IL-6 (R&D; 20ng/ml), IL-23 (R&D 108 system; 50ng/mL) in RPMI-1640 medium containing 10% FCS, penicillin (Gibco), 109 streptomycin (Gibco) for 1 hour. PMA (50ng/ml) and ionomycin (500ng/ml) were 110 added together with BD GolgiPlugTM (BD Pharmingen; 555029) for the last 3 hours 111 before analysis of intracellular cytokines by FACS.

112

113 Flow cytometry

114 Cell suspensions were incubated for 40 minutes with cold PBS (Gibco) containing 115 10% FCS, Fc receptor block (2.4G2), a viability dye (eFluor® 506, eBioscience #65-116 0866-14) and fluorochrome bound antibodies. To detect intracellular antigens, cells 117 were fixed for 40 minutes with 2% paraformaldehyde followed by a 118 permeabilization/staining step for transcription factors and/or cytokines with the 119 Foxp3 permeabilization kit (eBioscience). The following antibodies were used: CD3 120 (17A2), CD4 (RM4-5), NK1.1 (PK136), CD127 (A7R34), CD117 (2B8), ROR, IL-22 121 (IL22JOP), NKp46 (29A1.4), CCR6 (29-2L17), T-bet (ebio4B10), MHC-II 122 (M5/114.15.2), CD49a (Ha31/8), Ki67 (SoIA15), DNAM (10E5). All the samples 123 were acquired on a LSR FORTESSA (BD), and data were analyzed with FlowJo 124 (TreeStar, version 9.8.5 & 10.0.8).

125

126 Cell Culture of ILC3

127 ILC3 from the SI-LP of *Ncr1*^{GFP/+} mice were isolated as CD45^{int} CD90^{hi} NK1.1⁻, and
128 dissected according to GFP, CD49a and CCR6 expression. Cells were sorted using a
129 FACSAria II (BD) and were cultured on OP9 or OP9-DL1 stromal cells (kind gift of

130	A. Cumano, Institut Pasteur) in IMDM (Lonza) containing 10% FCS, $1 \times$
131	penicillin/streptomycin (Gibco), and 60µM 2-mercaptoethanol (Sigma-Aldrich), rIL-7
132	(20ng/mL) and rSCF (20ng/mL). 2 and 4 days later, cells were harvested and stained
133	(as stated above) for CD45, CD90, NK1.1, CD49a, CCR6, NKp46. Expressions
134	(along with GFP) of these marker were then analyzed by FACS.
135	
136	Statistical analysis
137	Data were compiled and analyzed with Graphpad Prism software (version 6). Paired
138	and unpaired Student's t-test were used to determine statistical differences between
139	groups. All graphs display mean + S.E.M., p values < 0.05 were considered
140	significant.
1/11	

143 **Results**

144 In vivo fate-mapping of Ncr1⁺ innate lymphoid cell subsets

Ncr1^{CreGFP} mice express a Cre-recombinase-GFP fusion protein under the 145 control of a minimal Ncr1 promoter and have been shown to faithfully report GFP 146 expression and Cre recombinase activity in known NKp46⁺ cells, including NK cells, 147 ILC1 and NKp46⁺ ILC3 (15). We crossed $Ncr1^{CreGFP}$ mice with $Rosa26^{RFP}$ Cre 148 reporter mice in order to genetically mark NKp46⁺ cells (RFP) that would allow us to 149 150 investigate the stability of NKp46 expression (Supplemental Figure 1A). In agreement with our previous results (15), RFP⁺ cells isolated from bone marrow, spleen, lymph 151 nodes, salivary glands, liver, and lung of Ncr1^{CreGFP} x Rosa26^{RFP} mice uniformly 152 153 expressed cell surface NKp46 and NK1.1⁺ (Figure 1A, Supplemental Figure 1B) thus 154 identifying these RFP^+ cells as group 1 ILC.

155 In contrast, RFP⁺ cells isolated from gut-associated tissues, including small 156 intestine lamina propria (SI-LP) and Peyer's Patches (PP) were clearly different with 157 a clear fraction of cells lacking cell surface NKp46 and GFP expression (Figure 1A, and Supplemental Figure 1C). These NKp46⁻GFP⁻ cells represented about 30% of the 158 total RFP^+ cells within the SI-LPL or PP, were uniformly $NK1.1^-$ and highly 159 160 expressed RORyt (Figure 1A and Supplemental Figure 1C). As RORyt and Eomes 161 expression in CD3⁻CD19⁻ intestinal cells are mutually exclusive (Supplemental Figure 1D), the NKp46⁻GFP⁻RFP⁺ cells are not related to Eomes⁺ ILC1. As such, 162 RFP⁺NKp46⁻GFP⁻ cells appeared as "ex-NKp46-ILC3' that had lost NKp46 163 expression. We purified NKp46⁻GFP⁻RFP⁺ cells and confirmed that they significantly 164 165 lacked mRNA for *Ncr1* that encodes NKp46 (Figure 1C). We refer to the NKp46⁻ GFP⁻RFP⁺ cells in Ncr1^{CreGFP} x Rosa26^{RFP} mice as 'Ncr1 Fate-Mapped' ILC3 or 166

Ncr1^{FM} ILC3. *Ncr1^{FM}* ILC3 were not detected in the large intestine under steady-state
conditions (Supplemental Figure 1C).

169 Since NKp46 expression levels are lower (yet overlapping) in ILC3 compared 170 to NK/ILC1, a clear separation of these subsets by antibody staining alone is not 171 possible. We therefore examined NKp46 cell surface versus GFP expression in $ROR\gamma t^+$ ILC3 within total RFP⁺ cells (Figure 1B). This approach allowed us to 172 unambiguously identify Ncr1^{FM} ILC3 as NKp46⁻GFP⁻RFP⁺ cells within the RORyt⁺ 173 174 gate. ILC3 subsets co-express CD117 (c-kit) and CD127 (IL-7Ra), albeit at varying levels (Figure 1D). For example, previous studies have shown that CCR6⁺ ILC3 were 175 CD117^{hi} CD127^{hi} as opposed to NKp46⁺CCR6⁻ILC3 that express lower CD117 and 176 CD127 levels (4). We found that *Ncr1*^{FM} ILC3 expressed significantly higher levels 177 of CD127 and CD117 as compared to NKp46⁺ ILC3 (Figure 1E). Further analyses 178 revealed that a fraction of Ncr1^{FM} ILC3 expressed CCR6 (Figure 1F). On average, we 179 found 4 x 10⁴ Ncr1^{FM} ILC3 in the SI-LP from Ncr1^{CreGFP} x Rosa26^{RFP} mice, of which 180 33.5 ± 5.7 % expressed CCR6 (Figure 1G). Thus, CCR6⁺ Ncr1^{FM} ILC3 represented 181 about 10% of total CCR6⁺ ILC3 pool (Figure 1G). Some of these CCR6⁺ Ncr1^{FM} 182 183 ILC3 expressed major histocompatibility complex class II molecules at the cell surface (Supplemental Figure 2A) but these represented a minute fraction of total MHC class 184 II⁺ ILC3 pool. The remaining Ncr1^{FM} ILC3 lacked CCR6 and CD4 expression 185 186 (Supplemental Figure 2B) and would be contained within the 'double negative' (NKp46⁻CCR6⁻) or 'DN' ILC3 gate. Within the total DN ILC3 population, Ncr1^{FM} 187 188 ILC3 accounted for about 17% of cells. Thus, a substantial fraction of previously identified DN ILC3 are in fact, Ncr1^{FM} ILC3 that have lost NKp46 expression. 189

190 As some T cells express NKp46 (17, 18) or ROR γ t (19, 20), we assessed 191 whether *Ncr1*^{FM} ILC3 could represent a peculiar T cell subset that had lost surface expression of CD3 and NKp46. However, neither NKp46⁺RFP⁺ nor NKp46⁻RFP⁺
ILC3 expressed cell surface CD5 or intracellular CD3 protein (Figure 1H).
Importantly, intracellular NKp46 protein was not detected in *Ncr1^{FM}* ILC3, ruling out
that the loss of cell surface NKp46 was due to the protein internalization (Figure 1H).

Taken together, analyses of *Ncr1^{CreGFP}* x *Rosa26^{RFP}* mice provide clear evidence for an intestinal ILC3 subset that has modulated NKp46 expression at both the protein and transcriptional level.

199

200 Functional capacities of Ncr1^{FM} ILC3

Ncr1^{FM} ILC3 did not stain for Ki67 suggesting that they were primarily resting 201 202 cells (Figure 2A, B). Given that one major ILC3 function is the production of IL-22, we assessed whether Ncr1^{FM} ILC3 were able to produce this cytokine upon 203 204 stimulation *in vitro*. Of note, IL-22 protein was readily detectable in both Ncr1⁺ ILC3 and Ncr1^{FM} ILC3 even without IL-23 stimulation (Figure 2C), however, steady-state 205 IL-22 production by *Ncr1*^{FM} ILC3 was significantly greater than that of *Ncr1*⁺ ILC3 206 207 on a population level as well as on a per cell basis (Figure 2D, E). Nevertheless, upon stimulation with IL-23, both Ncr1⁺ ILC3 and Ncr1^{FM} ILC3 produced similar levels of 208 IL-22 (Figures 2C-E). These results suggest that Ncr1^{FM} ILC3 may have received 209 210 activating signals in situ; such pathways might also be involved in NKp46 down-211 modulation, although this remains speculative.

We explored the cytokine production capacities of $Ncr1^{\text{FM}}$ ILC3 using various stimuli. Only group 1 ILC (NKp46⁺GFP⁺ROR γ t⁻) cells produced IFN γ upon stimulation with IL-2, IL-12, and IL-18 (Figure 2F), while both $Ncr1^+$ ILC3 and $Ncr1^{\text{FM}}$ ILC3 produced IL-22 under these conditions (Figure 2F). Interestingly, around 1 out of 4 of the IL-22 producing $Ncr1^+$ ILC3 and $Ncr1^{\text{FM}}$ ILC3 also produced

217 GM-CSF (Supplemental Figure 3A). The frequencies of both IL-22 producing cells 218 and IL-22/GM-CSF producing cells increased when stimulated with a cocktail consisting of IL-1β, IL-2, IL-6, IL-23 and PMA/ionomycin (Figure 2F and 219 Supplemental Figure 3A). Under these conditions, around half of $IL-22^+$ Ncr1⁺ ILC3 220 produced IFNy whereas Ncr1^{FM} ILC3 did not (Supplemental Figure 3A). RFP⁺ cells 221 222 failed to produce IL-17A after stimulation (Supplemental Figure 3B). Consistent with 223 their ability to produce IFN γ , Ncr1⁺ ILC3 express T-bet (Figure 2G) and require this transcription factor for development (5, 22). In contrast, Ncr1^{FM} ILC3 expressed 224 significantly less T-bet protein when compared with NK1.1⁺NK/ILC1 or with Ncr1⁺ 225 226 ILC3 (Figure 2G and H).

227

228 Ontogeny of Ncr1^{FM} ILC3 and the role of microbiota

We next explored at what stage Ncr1^{FM} ILC3 emerged during ontogeny. 229 CCR6⁺ ILC3 have been detected as early as E13.5 (23). However, only few RFP⁺ 230 231 cells were found at E18.5 (Figure 3A) and these cells uniformly expressed NK1.1 232 (Supplemental Figure 4). One week after birth, RFP⁺NK1.1⁻ cells were apparent, the majority of which were Ncr1^{FM} ILC3 (Figure 3B), which indicates that NKp46 233 expression was initiated, but not maintained in these RFP⁺ cells. Interestingly, Ncr1^{FM} 234 235 ILC3 exhibited higher IL-22 production capacity at this stage, both under steady state 236 conditions, and after IL-23 stimulation (Figure 3C), the latter contrasting with the 237 results obtained in adult mice (Figure 2C, D). We also noted an age-dependent decrease in the frequency of $Ncrl^{FM}$ among total ILC3 (13.2 ± 2.9 % and 9.4 ± 2.7%) 238 239 of total ILC3 in 1 week and 7 week-old mice, respectively). This might point to a 240 specific function of these cells during the early postnatal period.

IL-23 driven T-bet expression in CCR6⁻ILC3 was shown to be induced by 241 microbiota resulting in the generation of NKp46⁺ ILC3 (5). As *Ncr1*^{FM} ILC3 emerged 242 243 after birth and increased with age (4), we hypothetized that microbiota might also regulate Ncr1^{FM} ILC3 homeostasis. To address this point, we treated Ncr1^{CreGFP} x 244 $Rosa26^{RFP}$ mice with broad-spectrum antibiotics that have been shown to largely 245 246 ablate commensal microbial communities (9). As expected (9), $CD3^+ ROR\gamma t^+ T$ cells 247 were decreased after antibiotic treatment (Figure 3D). In contrast, we did not observe any changes in proportions of Ncr1⁺ ILC3 or Ncr1^{FM} ILC3 (Figure 3E, F). These 248 249 results indicate that antibiotic-sensitive microbiota are not a major driving force in shaping Ncr1^{FM} ILC3 homeostasis in adult mice. Nevertheless, future studies using 250 germ-free Ncr1^{CreGFP}Rosa26^{RFP} mice should be performed to confirm whether 251 homeostasis of the *Ncr1*^{FM} ILC3 subset is microbiota independent. 252

253

254 CD49a and CD226 expression by ILC3 subsets

255 CD49a (encoded by *Itga1*) is highly expressed by Eomes-independent hepatic ILC1 (DX5⁻NK1.1⁺ cells) but is largely absent on Eomes⁺ DX5⁺ NK cells (24, 25). 256 257 Subsequently, CD49a has been proposed as surface marker to identify ILC1 in various organs (26). As both ILC1 and NKp46⁺ ILC3 require T-bet for their 258 259 development (5, 22, 25, 26), we assessed whether CD49a might also be differentially expressed by intestinal ILC3 subsets. We found that CD49a was uniformly expressed 260 by NKp46⁺ and a subset of DN (NKp46⁻CCR6⁻) ILC3 (Figure 4A). Importantly, 261 262 CD49a was co-expressed with T-bet in both populations, while CCR6 and CD49a 263 expression were mutually exclusive (Figure 4A) consistent with previous transcriptomic analysis of ILC3 subsets (6). Consequently, all Ncr1⁺ ILC3 expressed 264

CD49a (Figure 4B). In contrast, only a fraction of *Ncr1^{FM}* ILC3 stained positive for
this marker (Figure 4B).

267 We next examined whether DNAX accessory molecule-1 (DNAM-1 or CD226) was expressed by Ncr1^{FM} ILC3. CD226 identifies NK/ILC1 subsets with 268 269 enhanced IFNy production (27). DNAM ligands - CD112 and CD155 - are expressed 270 by DCs and other cells (28), and CD226-CD155 interactions have been shown to be 271 involved in NK-DC 'crosstalk' (29). CD226 expression varied on ILC3 subsets: it 272 was expressed at high levels by all CCR6⁻ILC3 (Figure 4C), while the majority of 273 CCR6⁺ ILC3 expressed intermediate levels, with a small fraction of CCR6⁺ ILC3 being CD226⁻ (Figure 4C). We found that Ncr1⁺ ILC3 expressed high levels of 274 CD226, while Ncr1^{FM} ILC3 contained CD226^{hi} and CD226^{int} cells (Figure 4C). High 275 level CD226 expression by Ncr1^{FM} ILC3 was associated with CD49a expression 276 (Figure 4C), while CD49a⁻Ncr1^{FM} ILC3 expressed intermediate levels of CD226 and 277 harbored the CCR6⁺ subset (Figure 4C). Together these data reveal a cluster of 278 279 markers (NKp46, CD49a, CD226) that are highly expressed by T-bet⁺ ILC3 and are down-regulated in Ncr1^{FM} ILC3. 280

281

282 Signals that modulate NKp46 expression on intestinal ILC3

Membrane bound and soluble factors regulate NK cell receptor expression. Concerning NCRs, Notch has been proposed as a key positive regulator for mucosal ILC3 in mice (22), while TGF- β can modulate human NKp30 expression *in vitro* (30). NKp46 expression is reduced in cancer patients (31) and during Influenza and HIV infection (31–33). To begin to investigate the signals that might be involved in the regulation of NKp46 expression in *Ncr1*^{FM} ILC3, we used *Ncr1*^{GFP/+} (16) mice in which one *Ncr1* allele harbors a fluorescent reporter generating high GFP expression

by all NKp46⁺ cells. We then isolated CD49a⁺GFP⁺, CD49a⁺GFP⁻, and CCR6⁺ ILC3 290 291 subsets and monitored GFP expression after cell culture on stromal cells that 292 differentially expressed Notch ligands (22). We could detect a significant decrease in 293 the GFP expression level (mean fluorescence intensity) between day 2 and day 4 by CD49a⁺ GFP⁺ cells cultured on OP9 stromal cells, whereas GFP expression remained 294 295 constant on OP9 DL1 cells (Figure 4D). Addition of TGF-B accelerated GFP 296 downregulation in CD49a⁺ GFP⁺ cells cultured on OP9 but not OP9 DL1 (Figure 4D). 297 In contrast, GFP expression was not induced in cultured CCR6⁺ or CD49a⁺GFP⁻ILC3 298 and no changes in CD49a or CCR6 expression were observed under any conditions 299 (Supplemental Figure 5). These results suggest that TGF-β and Notch-derived signals regulate NKp46 expression in cultured $Ncr1^+$ ILC3 and may be involved in the 300 maintenance of NKp46 expression on Ncr1⁺ ILC3 or the generation of Ncr1^{FM} ILC3. 301

302 Discussion

Using *in vivo* fate-mapping, we have identified an unusual subset of intestinal ILC3 that have lost previous NKp46 expression; these 'ex-NKp46 ILC3' are denoted $Ncr1^{FM}$ ILC3. As a result, a fraction of 'DN' (CD4⁻NKp46⁻) ILC3 are derived from $Ncr1^+$ ILC3 and demonstrate that the NKp46⁺ ILC3 is not a stable or determinant phenotype. Our results also indicate that the 'DN' ILC3 subset is heterogeneous and show that at least two pathways are involved in the generation of this population.

Interestingly, a small subset of Ncr1^{FM} ILC3 express CCR6, thus resembling 309 310 LTi-like cells. It is a currently not clear whether CCR6⁺ ILC3 represent a stable lineage (21). Constantinides et al. identified early ILC precursors using Zbtb16^{CreGFP} 311 312 mice and through fate-mapping, showed that while most NKp46⁺ILC3 were derived from Zbtb16-expressing precursors, a minor fraction of CCR6⁺ ILC3 were also fate-313 mapped (21). Conceivably, the small subset of CCR6⁺ Ncr1^{FM} ILC3 we identified 314 315 might correspond to (or be contained within) the CCR6⁺ Zbtb16-derived ILC3 316 population (21). Since NKp46⁺ ILC3 are CCR6⁻, this receptor appears to be induced on a fraction of Ncr1^{FM} ILC3, possibly in response to environmental cues. As such, 317 the generation of CCR6⁺ Ncr1^{FM} ILC3 may allow for a specific postnatal positioning 318 319 of these IL-22 producing ILC3 cells within the intestine.

320 We found that CD49a and CD226 are differentiatly expressed on intestinal ILC3. Ncr1⁺ ILC3 express both markers, while Ncr1^{FM} ILC3 have reduced levels. As 321 CD49a and CCR6 were exclusively expressed by Ncr1^{FM} ILC3, we propose the 322 RFP⁺NKp46⁺CD49a⁺CD226^{hi}CCR6⁻ -> 323 following RFP⁺NKp46⁻ sequence: CD49a⁺CD226^{hi}CCR6⁻ -> RFP⁺NKp46⁻CD49a⁻CD226^{int}CCR6⁺. The change in 324 CD49a and CCR6 expression might allow for a differential localization of $Ncrl^+$ and 325 Ncr1^{FM} ILC3 within the tissue enabling them to exert their function in the correct 326

327 cellular context. Indeed, $Ncr1^{\text{FM}}$ ILC3s displayed spontaneous production of IL-22 328 suggesting that they may have received stimulatory signals *in situ*. Further studies on 329 the localization of $Ncr1^+$ and $Ncr1^{\text{FM}}$ ILC3 should provide important insights into the 330 specific roles for these ILC subsets in immune homeostasis.

331 Previous work has revealed that under the influence of environmental cues 332 ILC3 acquire the expression of T-bet and concomitant ability to produce IFNy (34). 333 T-bet upregulation is associated with loss of RORyt expression and IL-22 production 334 capabilities, allowing ILC3 to acquire an ILC1-like phenotype ("ex-ILC3" ILC1). In contrast, Ncr1^{FM} ILC3 do not show signs of ILC1-like plasticity. They retain 335 characteristics of ILC3 (RORyt⁺, IL-22 production) in naïve mice and when 336 stimulated in vitro. Furthermore, Ncr1^{FM} ILC3 expressed lower levels of T-bet than 337 $Ncrl^+$ ILC3 and were unable to produce IFNy. However, similar to $Ncrl^+$ ILC3, they 338 can produce GM-CSF. Whether Ncr1^{FM} ILC3 show further plastic features with 339 340 resultant loss of RORyt expression (similar to this ILC3 to ILC1 differentiation) is not known. Of note, our analyses demonstrated that all RFP⁺ group 1 ILC in $Ncrl^{CreGFP}$ x 341 Rosa26^{RFP} mice co-express NK1.1 and NKp46. Thus, if such a plasticity (Ncr1^{FM} 342 343 ILC3 to ILC1) would occur, it would involve concomitant upregulation of NKp46 and 344 NK1.1 by those cells, rendering them indistinguishable from "normal" group 1 ILC.

Taken together, NKp46 modulation in ILC3 likely reflects an intrinsic program influenced by environmental cues. AhR activation by dietary ligands may explain higher CD117 and IL-22 expression in $Ncr1^{FM}$ (35), while cellular partners that emerge after birth such as monocytes derived CX3CR1⁺ macrophages may stabilize NKp46 expression by providing Notch ligands (36). To balance their function, TGF- β might dampens an ILC3 pro-inflammatory program dependent on NKp46 expression. Remarkably, NKp46⁺ ILC3 are the only immune cell to express

- 352 simultaneously Gata-3, RORyt and T-bet. The interplay between these transcription
- 353 factor in ILC3 differentiation is complex and NKp46 expression appears tightly

354 controlled by Gata-3 and T-bet (37). As such, relative amounts of these transcription

- 355 factors, balanced by extrinsic signals, might finely tune the emergence and the
- 356 function of $Ncr1^+$ ILC3 and their transition to $Ncr1^{FM}$ ILC3.

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364

365 **Conflict of Interest Statement**

- 366 JD is a founder and stakeholder in AXENIS (Paris, France). The other authors declare
- 367 no conflict of interest.

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559 Figure 1. An unusual 'ex-NKp46' ILC3 subset in the small intestine. (A) Analysis of NK1.1, RORyt and NKp46 expression in RFP⁺ cells from the indicated organs in 560 *Ncr1*^{CreGFP} x *Rosa26*^{RFP} mice. First column displayed was gated on alive CD45⁺ RFP⁺ 561 562 cells. SI-LP : Small Intestine Lamina Propria. (B) Gating strategy used to identify 563 RFP⁺ NKp46⁻ GFP⁻ ILC3 in small intestine lamina propria lymphocytes from Ncr1^{CreGFP} x Rosa26^{RFP} mice. CD4⁺ LTi cells (CD3⁻ CD127⁺ RORyt⁺ CD4⁺) were 564 565 used as a control to set the negative gate (NKp46 vs GFP). (C) Ncr1 mRNA 566 expression levels by sorted intestinal populations: NK1.1+ -CD3-567 CD45.2+NK1.1+NKp46+GFP+RFP+; Ncr1+ CD3-CD45.2intCD90.2hiCD117+NK1.1-NKp46+GFP+RFP+; Ncr1FM 568 CD3-569 CD45.2intCD90.2hiCD117+NK1.1-NKp46-GFP-RFP+; CD4+ LTi CD3-_ 570 CD45.2intCD90.2hiCD117+NK1.1-NKp46-RFP-CD4+. (D) Representative FACS 571 plot of CD127 versus CD117 in RFP⁺ NK1.1⁻ cells. (E) Expression levels (MFI) of CD127 (left) and CD117 (right) by the indicated populations measured by FACS. (F) 572 573 Representative FACS plot of NKp46, GFP, RFP and CCR6 expression in ILC3 (gated on alive $CD45^+$ $CD3^ CD127^+$ $ROR\gamma t^+$). (G) Distribution of RFP⁺ among intestinal 574 ILC3 (gated on CD3⁻ CD127⁺ RORyt⁺) in percentages (left) and absolute numbers 575 576 (right). (mean + S.E.M.; n = 15). (H) Histograms overlay of the indicated population. Ncr1⁺ (black line) and Ncr1^{FM} (light blue) were gated on as described in (C). T cells 577 578 (grey line) as a negative and positive control for intracellular staining of NKp46 and 579 CD3, respectively.

Figure 2. Ncr1^{FM} ILC3 demonstrate elevated IL-22 production and reduced T-581 582 bet expression. (A). Representative FACS plot of Ki67 versus NKp46 in RFP⁺ NK1.1⁻ cells and CD3⁺ T cells (control). (**B**) Statistical evaluation of Ki67 data 583 584 obtained from analyses as in (A). (C) Representative FACS plot of IL-22 expression by the indicated subsets (among RFP⁺ NK1.1⁻) after 4h stimulation with or without 585 IL-23 (50 ng.ml⁻¹). NS = Non Stimulated. (**D**) Percentages of IL-22 positive cells in 586 RFP^+ NKp46⁺ (empty bar) and RFP^+ NKp46⁻ (black bar) (mean + S.E.M.; n=9), *** 587 p < 0.001. (E) Expression levels (MFI) of intracellular IL-22 protein in unstimulated 588 589 or IL-23-stimulated cells from the indicated populations. (F) Representative FACS 590 plot of IFNy and IL-22 expression after stimulation with various cytokine mixes as 591 indicated. Of note, cells were first incubated for 1h with IL-1β, IL-2, IL-6, and IL-23 592 before PMA and ionomycin were added for the remaining 3hrs of incubation. (G) Histograms overlay of T-bet expression in RFP⁺ NK1.1⁺ (red), RFP⁺ NKp46⁺ (grey), 593 RFP⁺ NKp46⁻ (light blue) and CD4⁺ LTi (dark grey). Mean fluorescence intensity 594 (MFI) were normalized to RFP^+ NK1.1⁺ and are displayed in (H). (mean + S.E.M.; 595 n=4), ** p < 0.01. 596

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Figure 3. Impact of ontogeny and microbiota on Ncr1^{FM} ILC3 generation. (A) 598 599 Representative FACS plot of CD4, RFP, NKp46 and GFP expression in ILC3 (gated on alive CD45⁺ CD3⁻ CD127⁺ ROR γ t⁺) from the SI-LP of Ncr1^{CreGFP} x Rosa26^{RFP} 600 mice at the indicated time points. (B) Percentages of $Ncrl^+$ and $Ncrl^{FM}$ ILC3 among 601 NK1.1⁻ cells, (mean + S.E.M.; $n \ge 9$, **** p < 0.0001). (C) Percentages of IL-22 602 positive cells in Ncr1⁺ (empty bar) and Ncr1^{FM} (black bar) in SI-LPL from 1 week-old 603 604 mice (mean + S.E.M.; n=9), ** p < 0.01 stimulated or not with IL-23 as indicated. 605 (**D**). Percentages of ROR γ t⁺ among CD3⁺ T cells with or without antibiotic treatment in control (empty bars) or antibiotic treated (black bars) mice (mean + s.d., n = 3), ** p < 0.01. Antibiotics were administered in drinking water to pregnant mice and mice after birth until sacrifice (6-7 weeks old). (E) Representative FACS plot of RFP⁺ NK1.1⁻ ILC3 with or without antibiotic treatment. (F) Percentages of *Ncr1*⁺ and *Ncr1*^{FM} ILC3 among NK1.1⁻ RFP⁺ (E) in control (empty bars) or antibiotic treated (black bars) mice (mean + s.d., n = 3).

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Figure 4. Phenotype and plasticity of Ncr1^{FM} ILC3. (A) Representative FACS plot 613 614 of CD49a and T-bet expression in the indicated ILC3 subsets from the SI-LP of 615 C57BL/6 mice. (B) Representative FACS plot of CD49a versus CCR6 expression in $Ncrl^+$ (left) and $Ncrl^{FM}$ (right) from the SI-LPL of $Ncrl^{CreGFP} \propto Rosa26^{RFP}$ mice. (C) 616 617 Representative FACS plot of DNAM-1 versus CD49a and CCR6 expression by cells from the indicated subsets from Ncr1^{CreGFP} x Rosa26^{RFP} mice. (D) CD45^{int} CD90^{hi} 618 NK1.1⁻ CD49a⁺ GFP⁺ were sorted from the SI-LP of Rag2^{-/-} Ncr1^{GFP/+} mice and 619 620 cultured on OP9 or OP9-DL1 expressing stromal cells in the presence (grey bar) or absence (black bar) of TGF- β (100 ng.ml⁻¹) for up to four days. Mean Fluorescence 621 Intensity (MFI) of GFP in sorted CD49a⁺ GFP⁺ cells was assessed by flow cytometry 622 at the indicated time points. (mean + s.d.; n=3), * p < 0.05, ** p < 0.01. 623

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626 Supplemental Figure Legends

627 Supplemental Figure 1. Relates to Figure 1. (A) Experimental approach for fatemapping NKp46⁺ cells. (**B**) CD45⁺ RFP+ cells of the indicated organs were stained 628 for NKp46 and NK1.1. Dotplots on the left show NKp46 versus RFP expression of 629 630 these cells isolated from the indicated organs. Histograms on the right show the 631 expression of NK1.1 by the same cells. BM- bone marrow, SG- salivary glands, aLN-632 axial lymph nodes, mLN- mesenteric lymph nodes. Histograms overlays show NK1.1 633 expression by CD19+ cells (grey, negative control) and RFP+ cells (black). (C) Analysis of NK1.1, RORyt and NKp46 expression in RFP⁺ cells from the indicated 634 organs in $Ncr1^{CreGFP}$ x $Rosa26^{RFP}$ mice. First column displayed was gated on alive 635 636 $CD45^{+}RFP^{+}$ cells. PP : Peyer's Patches; LI-LP : Large Intestine Lamina Propria. (**D**) 637 FACS plots showing the expression of NKp46 versus intracellular Eomes (left) and 638 intracellular RORyt versus intracellular Eomes (right) by CD3-CD19- SI-LP from 639 C57BL/6 mouse.

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641 Supplemental Figure 2. Relates to Figure 1. (A) Expression of MHC-II versus CCR6
642 by the indicated cell populations. (B) Expression of CD4 versus CCR6 by the
643 indicated cell populations.

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Supplemental Figure 3. Relates to Figure 2. (A) Related to Figure 2F. Intracellular IL-22 versus GM-CSF expression by the indicated cell populations and stimulated as indicated. Cells were stimulated as detailed in figure legend 2F. (B) SI-LPL from $Ncr1^{CreGFP} \ge Rosa26^{RFP}$ mice were stimulated for 4hrs with PMA and ionomycin and then stained for intracellular IL-17. Shown are IL-17 versus RFP (left; of note, there

- are no double positive cells) and IL-17 versus CD3 (right; 25% of CD3⁺ cells express
 IL-17).
- 652
- 653 Supplemental Figure 4. Relates to Figure 3A. (A)FACS plots showing the expression
- of NKp46 and NK1.1 by total RFP+ cells (left) and ILC3 (right) in E18.5 fetal livers.
- 655 (B) Representative FACS plots of expression of CCR6 and CD49a by cells cultured
- 656 for 4 days on OP9 feeder cells with or without the Notch-logand DL-1 and with or
- 657 without TGF β as indicated. Grey overlay represents RFP⁻CCR6⁺ ILC3, red *Ncr1*⁺
- 658 ILC3, blue overlay represents *Ncr1*^{FM} ILC3.











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RFP⁺





SUPPLEMENTAL FIGURE 1











В

