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Mathilde Pohin, Carolina Veaute, Julien Garnier, Christine Barrault, Laure Cronier, Vincent Huguier, Laure Favot, Jiad Mcheik, François-Xavier Bernard, Jean-Claude Lecron, et al.

### ► To cite this version:

Mathilde Pohin, Carolina Veaute, Julien Garnier, Christine Barrault, Laure Cronier, et al.. Development of a new model of reconstituted mouse epidermis and characterization of its response to proinflammatory cytokines. *Journal of Tissue Engineering and Regenerative Medicine*, 2018, 12, pp.e1098-e1107. 10.1002/term.2442 . hal-01699562

**HAL Id: hal-01699562**

**<https://hal.science/hal-01699562>**

Submitted on 4 Oct 2023

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3 **1 Development of a new model of reconstituted mouse epidermis and**  
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6 **2 characterization of its response to proinflammatory cytokines**  
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35 Running title: New model of reconstituted mouse epidermis.  
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39 14 Keywords: three-dimensional culture, keratinocyte, reconstituted epidermis, skin  
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41 15 inflammation, cytokine.  
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1  
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3 21 **Abstract**  
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5 22 The development of three-dimensional models of reconstituted mouse epidermis (RME) has  
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7 23 been hampered by the difficulty to maintain murine primary keratinocyte cultures and to  
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9 24 achieve a complete epidermal stratification. In this study, we propose a new protocol for the  
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11 25 rapid and convenient generation of RME, which reproduces accurately the architecture of a  
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13 26 normal mouse epidermis. During RME morphogenesis, the expression of differentiation  
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15 27 markers such as keratins, loricrin, filaggrin, E-cadherin and connexins was followed, showing  
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17 28 that RME structure at day 5 was similar to those of a normal mouse epidermis, with the  
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19 29 acquisition of the natural barrier function. We also demonstrated that RME responded to skin-  
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21 30 relevant proinflammatory cytokines by increasing the expression of antimicrobial peptides  
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23 31 and chemokines, and inhibiting epidermal differentiation markers, as in the human system.  
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25 32 This new model of RME is therefore suitable to further investigate mouse epidermis  
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27 33 physiology and opens new perspectives to generate reconstituted epidermis from transgenic  
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29 34 mice.  
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## 1. Introduction

In the field of immunodermatology research, standard monolayer cell cultures of human keratinocytes are commonly used for *in vitro* studies, although they do not completely recapitulate the physiological skin architecture and the features of the different states of cell differentiation. The stratification of keratinocytes is essential to study the physiological response of the epidermis since keratinocytes from the basal layer are widely different from corneocytes in morphology but also in metabolism and response to environmental factors. Regarding those limits, three-dimensional models of reconstituted human epidermis (RHE) have been therefore developed and proved to be more suitable to test cosmetics products, for drug development or cutaneous disease modelling. Mouse is also commonly used to investigate human diseases *in vivo*, offering a large possibility of spontaneous, genetically engineered or inducible murine models of skin pathologies. By contrast to human primary keratinocytes, murine keratinocytes have always been difficult to isolate and to maintain in culture. Likewise, the development of reconstituted murine epidermis (RME) seems to be more problematic to achieve a complete stratification than RHE.

Over the last three decades, the development of RHE consisted in determining the optimal culture conditions to reach a rapid and complete epidermal differentiation. The first attempts described the need for cultivating keratinocytes on irradiated fibroblastic feeder cells, in culture medium supplemented with epidermal growth factor and hydrocortisone, but resulted in an incomplete stratification of keratinocyte colonies (Rheinwald and Green, 1975). The capacity of keratinocytes cultivated in monolayer to differentiate into three-dimensional epidermis was further shown to require their transition to an air/liquid interface (Prunieras, *et al.*, 1983, Rosdy and Clauss, 1990). Then, the modulation of calcium concentrations in culture medium has been identified as key element in regulating the proliferation/differentiation



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3 60 balance (Hennings, *et al.*, 1980). Calcium has also been reported to play a role in adherent  
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5 61 junctions through the assembly of the E-cadherins (Tu and Bikle, 2013, Tu, *et al.*, 2008).  
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7 62 Finally, the substitution of the classic fetal bovine serum in culture media by specific growth  
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9 63 factors such as keratinocyte growth factor (KGF), bovine pituitary extract, Insulin Growth  
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11 64 Factor and L-acid ascorbic allowed to get rid of fibroblastic feeders and was shown to induce  
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13 65 a better epidermal stratification with the restoration of the natural barrier function (Bertolero,  
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15 66 *et al.*, 1984, Borowiec, *et al.*, 2013, Frankart, *et al.*, 2012, Ponec, *et al.*, 1997, Rosdy and  
16  
17 67 Clauss, 1990).

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21 68 Attempting to develop RME, some authors used **human de-epidermized dermis** (Carroll and  
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23 69 Moles, 2000) or employed mouse dermal fibroblasts as feeders for keratinocyte attachment  
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25 70 and growth (Ikuta, *et al.*, 2006, Rosenberger, *et al.*, 2014). This fibroblastic support was  
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27 71 demonstrated to favor the establishment of the epidermis and to improve long term skin  
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29 72 equivalents (Boehnke, *et al.*, 2007). With technical progress in the characterization of stem  
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31 73 cells, some studies described long-term protocol to generate mouse epidermal stem and  
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33 74 precursor cell lines (Segrelles, *et al.*, 2011, Vollmers, *et al.*, 2012). After several passages,  
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35 75 epidermal progenitor cell lines were successfully obtained and used to generate mouse  
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37 76 reconstructed epidermis. In these models, the epidermal differentiation remained limited as  
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39 77 indicated by the discontinued expression of some differentiation markers such as keratin (K)  
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41 78 10, involucrin or loricrin by suprabasal keratinocytes.  
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47 79 The differentiation of keratinocytes is a complex molecular process which leads to the  
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49 80 establishment of a protective barrier. It results in a spatiotemporal expression of epidermal  
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51 81 differentiation markers. In the basal layer, K5/K14 are expressed by undifferentiated  
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53 82 keratinocytes and K6/16 expressions are restricted to hyperproliferative or activated  
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55 83 keratinocytes (Moll, *et al.*, 2008), while K1/K10 expressions are found in the differentiated  
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57 84 keratinocytes of the suprabasal layers (Simpson, *et al.*, 2011). In the granular layer,  
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3 85 keratinocytes expressed progressively involucrin and loricrin which are involved in the  
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5 86 formation of the mature cornified envelope. Granular keratinocytes are also characterized by  
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7 87 the presence of keratohyalin granules in their cytoplasm, containing the pro-filaggrin. In the  
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9 88 upper layers, filaggrin aggregates with the intermediate K1/K10 filaments to form mature  
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11 89 corneocytes and contributes to the epidermal barrier function (Candi, *et al.*, 2005, Pendaries,  
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13 90 *et al.*, 2014). In addition, GAP junctions were shown to be essential for the communication  
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15 91 between keratinocytes throughout the epidermis. Among the connexins (Cx) found in the  
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17 92 epidermis, Cx43 is highly expressed in the basal and spinous layers, whereas Cx26 expression  
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19 93 is restricted to the granular layer (Kandyba, *et al.*, 2008, Scott, *et al.*, 2012).

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24 94 In inflammatory conditions, this epidermal differentiation is altered and the protective barrier  
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26 95 may be disrupted. RHE have been commonly used *in vitro* to study the effect of  
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28 96 proinflammatory mediators which were reported to be overexpressed in different skin  
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30 97 pathologies. We and others reported the activity of cytokines, such as IL17A, IL-1 $\alpha$ , TNF $\alpha$ ,  
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32 98 IL-22 and Oncostatin M (OSM), alone or in synergy, on epidermal differentiation,  
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34 99 recapitulating some features of psoriasis or atopic dermatitis (Boniface, *et al.*, 2005, Boniface,  
35  
36 100 *et al.*, 2007, Guilloteau, *et al.*, 2010, Pohin, *et al.*, 2016, Rabeony, *et al.*, 2014, Rabeony, *et*  
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38 101 *al.*, 2015, Wilson, *et al.*, 2007). OSM and IL-22 induced the expression of K6, classically  
39  
40 102 associated with keratinocyte proliferation, and inhibited the expression of differentiation  
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42 103 markers such as K10, filaggrin, involucrin and loricrin leading to epidermis hyperplasia  
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44 104 (Rabeony, *et al.*, 2014). By contrast, IL-1 $\alpha$ , IL-17A and TNF- $\alpha$  were found to be more  
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46 105 important for innate immune activation, by inducing higher expressions of proinflammatory  
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48 106 mediators by keratinocytes such as chemokines (CXCL-3), or the antimicrobial peptides  $\beta$ -  
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50 107 defensin 3 (BD3) and S100A9 (Guilloteau, *et al.*, 2010).

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56 108 Here, we described a fast and convenient method to generate RME using primary keratinocyte  
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58 109 cultures from newborn mouse skin, without fibroblastic support, which reproduce accurately  
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3 110 the morphological features of a normal murine epidermis. In this study, we characterized the  
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5 111 progressive morphogenesis of the epidermis and the expression profiles of proteins involved  
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7 112 in differentiation, as well as the expression of proinflammatory cytokine receptors. Finally,  
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9 113 we performed functional studies on RME, by analyzing their natural barrier function and their  
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11 114 response to proinflammatory cytokines.  
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## 116 2. Materials and methods

### 117 2.1. Generation of Reconstituted Murine Epidermis

118 Normal Murine Epidermal Keratinocytes (NMEK) were isolated from pooled skins of  
119 C57BL/6J newborn mice and cultivated in CnT07 medium (CELLnTEC) as previously  
120 described (Pohin, *et al.*, 2016). After 5 days, when cells reached 80% of confluency, they  
121 were detached with Trypsin LE-express (Invitrogen) and centrifuged at 1500 rpm for 5 min,  
122 before resuspension at  $1.125 \times 10^6$  cells/ml in CnT07 medium. Polycarbonate culture inserts  
123 ( $0.63 \text{ cm}^2$  of area containing  $0.4 \mu\text{m}$  diameter pore size, Millipore) were seeded with  $400 \mu\text{l}$  of  
124 cell suspension ( $0.45 \times 10^6$  cells) and deposited in the bottom of six-well plates containing 3 ml  
125 of CnT07 medium with 1.5 mM of calcium. After 24 h of incubation at  $37^\circ\text{C}$  in a humidified  
126 atmosphere containing 5%  $\text{CO}_2$ , keratinocytes were exposed to the air/liquid interface by  
127 adding a sterile gaze under the insert in each well and replacing the CnT07 medium by 3 ml  
128 of differentiation medium composed of Epilife medium with HKGS supplements (Cascade  
129 biologics), 1.5 mM of calcium,  $50 \mu\text{g/ml}$  of L-ascorbic acid (Sigma) and  $3 \text{ ng/ml}$  of murine  
130 recombinant KGF (R&D systems). This medium was renewed every 2 days until the use of  
131 RME.

### 132 2.2. Cytokines stimulation

133 Five days after the air/liquid interface exposure, RME were stimulated with the murine  
134 cytokines OSM, IL-22, IL-17A, IL- $1\alpha$  and TNF- $\alpha$  ( $10 \text{ ng/ml}$ , R&D systems) or a mix of the  
135 five cytokines at  $10 \text{ ng/ml}$  or  $1 \text{ ng/ml}$  each, for 24 h before transcriptional analyses or for 72 h  
136 before histology, ELISA and permeability studies.

### 137 2.3. RNA and real-time quantitative RT-PCR

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3 138 Total RNA was extracted from RME using Nucleospin RNA XS kit (Macherey-Nagel). RNA  
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5 139 was reverse-transcribed with Superscript II Reverse Transcriptase (Invitrogen) and transcripts  
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7 140 were amplified and quantified using the LightCycler-FastStart DNA Master Plus SYBR  
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9 141 Green I kit on a LightCycler 480 instrument (Roche). Oligonucleotides were designed with  
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11 142 Primer-Blast software, purchased from Eurogentec and listed in Supporting Information Table  
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13  
14 143 1. Samples were normalized to the expression of the control housekeeping gene, *GAPDH*, or  
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16 144 reported according to the  $\Delta\Delta CT$  method as RNA fold change:  $2^{\Delta\Delta CT} = 2^{\Delta CT_{\text{sample}} - \Delta CT_{\text{reference}}}$ , as  
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18 145 indicated in the figure legends.

#### 21 146 **2.4. Histology and immunohistofluorescence**

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25 147 For histological examination, RME were fixed in 4% formaldehyde before inclusion in  
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27 148 paraffin and H&E coloration. For immunohistofluorescence, frozen sections (8  $\mu\text{m}$ -thick)  
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29 149 embedded in Cryomatrix compound (Shandon) were fixed either in cold acetone/methanol  
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31 150 (20/80, v/v) for 10 min or in 4% paraformaldehyde/PBS solution (only for Cx26, Cx43 and E-  
32  
33 151 Cadherin immunostainings). After blocking with 0.5 to 2% FBS or BSA solutions and  
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35 152 permeabilization in 0,5% Triton X-100 diluted in PBS, samples were incubated with primary  
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37 153 antibodies directed against the following mouse antigens: K6 (Thermo Scientific), K14, K10,  
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39 154 filaggrin, loricrin (all from Covance), Cx26 (Invitrogen), Cx43 (BD Biosciences), E-cadherin  
40  
41 155 (SC-7870, Santa-Cruz). RRX- or FITC- conjugated goat or donkey antibodies were used as  
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43 156 secondary antibodies (Jackson Immunoresearch). Cell nuclei were stained with TOPRO-3  
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45 157 (Molecular Probes). Image acquisition was performed with an Olympus FV1000 confocal  
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47 158 microscope using FluoView software (ImageUP platform, Université de Poitiers).

#### 51 159 **2.5. Lucifer Yellow permeability assay and ELISA**

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55 160 Lucifer Yellow (LY, 200  $\mu\text{l}$  of 1 mM solution; Sigma) was deposited inside the insert on the  
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57 161 surface of RME and incubated for 6 h at 37°C, 5% CO<sub>2</sub>. Frozen sections were fixed in 4%  
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3 162 formaldehyde. Image acquisition was performed with an Olympus FV1000 confocal  
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5 163 microscope using FluoView software (ImageUP platform, Université de Poitiers). To assess  
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7 164 RME permeability, concentrations of LY that passed through RME were measured in culture  
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9 165 medium using a Varioskan Flash at 550 nm emission (Thermo scientific). To investigate the  
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11 166 role of connexins in the barrier function of RME, carbenoxolone (CBX , Sigma) was used to  
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13 167 block gap junctions at a non-cytotoxic concentration of 60  $\mu$ M.  
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17 168 ELISA for mouse S100A9 detection was provided with R&D Systems and carried out  
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19 169 according to the manufacturer's instructions.  
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### 171 3. Results

#### 172 3.1. Epidermal morphogenesis and localization of differentiation markers in RME 173 mimic a normal epidermis

174 We have designed a new protocol for the rapid, convenient and reproducible generation of  
175 RME using keratinocytes isolated from newborn mouse skin, requiring no fibroblastic support  
176 or de-epidermised dermis, which allows studying exclusively the physiology of epidermis  
177 independently of dermis influence. Freshly isolated murine primary keratinocytes were  
178 directly seeded in polycarbonate membrane inserts to form the future RME as described in the  
179 experimental procedures (Figure 1A). The protocol to reach a complete epidermal  
180 stratification in the mouse system has been adapted from protocols previously described for  
181 human three-dimensional equivalents (Frankart, *et al.*, 2012, Guenou, *et al.*, 2009, Poumay  
182 and Coquette, 2007). This adaptation for RME consisted mainly in incubating firstly  
183 keratinocytes in a seeding medium composed of CnT07 medium, adapted for murine cell  
184 culture, with an increased calcium concentration (1.5 mM) to form a regular cell monolayer,  
185 then in incubating the cells, after their passage to the air-liquid interface, in a differentiation  
186 medium composed of complete Epilife medium supplemented with murine KGF, L-ascorbic  
187 acid and high calcium concentration. In order to monitor the epidermal morphogenesis of the  
188 RME, histological analyses and fluorescent immunostaining of several differentiation markers  
189 were performed at day 3, 5 and 8 after exposure to the air/liquid interface, and compared with  
190 normal epidermis (Figure 1B). Histological examination after H&E staining showed that  
191 RME exhibited a single layer of confluent keratinocytes at day 3, covered by a protective  
192 layer of acidophilic keratinocytes as previously described in human RHE (Frankart, *et al.*,  
193 2012). At day 5, RME were ~ 20 µm thick and displayed a complete epidermis  
194 morphogenesis with the presence of the four expected layers. Notably, we could observe the

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3 195 presence of keratohyalin granules in the granular layer and a thin corneous layer. After 8 days,  
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5 196 histological analyses of RME showed a dense corneous layer, RME degenerating  
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7 197 progressively in differentiated corneocytes. K6 expression is normally restricted to  
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9 198 hyperproliferative cells of the basal layer in normal epidermis but it was expressed throughout  
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11 199 the RME at day 3. This expression was maintained at day 5 and clearly lowered at day 8. As  
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13 200 expected, K14 expression was restricted to the basal layer at all time points assessed, while  
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15 201 K10, loricrin and filaggrin were weakly expressed by RME at day 3. At day 5, RME exhibited  
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17 202 a strong K10 immunostaining throughout the suprabasal layer and both expressions of  
18  
19 203 filaggrin and loricrin in the granular and the corneous layers, respectively, were similar to  
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21 204 those observed in normal epidermis from adult mice. In conclusion, it appears that RME at  
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23 205 day 5 reproduces accurately the morphology of a normal epidermis and the expression profile  
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25 206 of epidermal markers.

### 207 **3.2. RME form a cohesive tissue and an efficient barrier.**

208 The appropriate cohesion of keratinocytes in a three-dimensional reconstituted epidermis is  
209 also dependent on the expression of proteins of adherent junctions and connexins (Cx)  
210 involved in GAP junctions. All over the epidermis, E-cadherin was expressed in the  
211 intercellular space of the RME from day 3 and was maintained up to day 8, like in a normal  
212 mouse epidermis (Figure 2A). Cx43 was found in the basal and spinous layers of a mature  
213 epidermis from day 3 to day 8, harboring the characteristic punctiform immunostaining  
214 observed in the normal epidermis (Figure 2A). RME highly expressed Cx26 at day 5 and 8,  
215 with a localization more restricted to the granular layer. Nonetheless, the expression seemed  
216 to be more sustained in the *in vitro* model compared to the normal epidermis (Figure 2A).

217 To confirm that RME acquired their natural barrier function, we have applied the hydrophilic  
218 fluorescent dye Lucifer Yellow (LY) on the top of each RME on day 0, 3, 5 and 8 for 6 h. The



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3 219 analysis of concentrations of LY that diffused through the RME showed that the dye start to  
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5 220 be retained at day 0 (~ 98% of retention), when keratinocytes form only a monolayer (Figure  
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7 221 2B). RME were effectively impermeable to LY from day 3 to day 8 (Figure 2B), LY being  
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9 222 mainly retained in the cornified layer as observed by confocal microscopy in RME sections at  
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11 223 day 5 and day 8 (Figure 2C). Whether gap junctions are involved in the barrier function has  
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13 224 been investigated by treating RME with a previously determined non-cytotoxic concentration  
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15 225 of 60  $\mu\text{M}$  of carbenoxolone (CBX), a commonly used blocker of gap junctions. Our results  
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17 226 showed that CBX had no direct impact on permeability to LY when RME at day 0 were  
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19 227 treated for 6 hours (Figure 2B). Similarly, RME correctly stratified at day 5 and treated with  
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21 228 CBX for 6 h retained their barrier property (not shown). However, when RME were treated  
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23 229 with CBX for the last 72 h during their development to day 5, the barrier function was  
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25 230 impaired, but this was mainly due to the altered differentiation/epidermal stratification, as  
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27 231 evidenced by histological studies showing unstructured RME (data not shown). Together,  
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29 232 these results confirmed that RME from day 3 to 8 formed a cohesive tissue and efficiently  
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31 233 acquired their natural property of impermeable barrier.  
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### 37 234 3.3. RME response to proinflammatory cytokines

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40 235 To complete the functional characterization of our RME model, we have investigated their  
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42 236 response to cytokines relevant to skin inflammation. Firstly, we analyzed the basal expression  
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44 237 of the different subunits of receptors for IL-22, OSM, IL-17A, TNF- $\alpha$  and IL-1 $\alpha/\beta$  by RT-  
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46 238 qPCR in RME at day 5 in comparison with normal epidermis. The expressions of TNFR1/R2,  
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48 239 IL-10RB, gp130 and OSMR $\beta$ , IL-17RC, IL-1R1 and IL-1RAcP were similar between RME  
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50 240 and normal epidermis, while IL-17RA and IL-22R1 exhibited a higher expression in the  
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52 241 epidermis (Figure 3).  
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3 242 Then, RME at day 5 were stimulated either with IL-22, OSM, IL-17A, TNF- $\alpha$  or IL-1 $\alpha$  at a  
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5 243 final concentration of 10 ng/ml or a mix of these five cytokines (M5) at both concentrations of  
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7 244 10 ng/ml and 1 ng/ml. Histological analyses of RME were performed after 72 h of stimulation  
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9 245 (Figure 4A). Stimulations with IL-1 $\alpha$ , TNF- $\alpha$  or IL-22 did not change the morphological  
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11 246 aspect of the epidermis. By contrast, IL-17A, OSM and M5 at the concentration of 1 ng/ml  
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13 247 induced a modest hyperplasia with a significant reduction of keratohyalin granules in the  
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15 248 granular layer, especially after OSM stimulation, while M5 stimulation at 10 ng/ml  
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17 249 completely disrupted the stratified aspect of the RME. Regarding innate immunity markers, a  
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19 250 strong synergistic induction of proinflammatory mediators including the antimicrobial  
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21 251 peptides S100A9, BD3 and the chemokine CXCL3 was observed at the transcriptional level  
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23 252 after RME stimulation with M5 at 10 ng/ml (Figure 4B). IL-1 $\alpha$ , TNF- $\alpha$  and IL-22 also  
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25 253 induced a modest expression of these proinflammatory markers, while IL-17A and OSM were  
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27 254 found to be the most active on RME. These inductions of S100A9 were confirmed at the  
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29 255 protein level by measuring S100A9 concentrations by ELISA in culture supernatants of RME  
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31 256 72 h after stimulation (Figure 4C). In correlation with the histological observations, the  
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33 257 analysis of the mRNA expression of differentiation markers showed a strong downregulation  
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35 258 of K10, filaggrin and involucrin 24 h after RME stimulation with OSM, IL-17A, M5 at 1  
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37 259 ng/ml and, to a lesser extent, with IL-1 $\alpha$ , TNF- $\alpha$  and IL-22 (Figure 4D). M5 at the highest  
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39 260 concentration strongly inhibited the expression of K10, loricrin and involucrin, 10-fold more  
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41 261 than IL-17A or OSM alone, confirming the synergistic proinflammatory activity of the  
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43 262 cytokine mix. A slight upregulation of K6 mRNA could also be observed in RME stimulated  
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45 263 with OSM, IL-17A and M5 (Figure 4D), which was confirmed by confocal microscopy  
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47 264 showing a positive immunostaining in the suprabasal layers of stimulated RME, including  
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49 265 TNF- $\alpha$ -stimulated RME as well (Figure 4E). RME stimulated with OSM, IL-17A and M5  
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51 266 presented a specific and strong Cx26 immunostaining close to keratinocyte nuclei compared  
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3 267 to unstimulated RME, while the cornified layer harbored a non-specific staining (Figure 4E).  
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5 268 No significant regulation of Cx43 expression by proinflammatory cytokines could be  
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7 269 observed (data not shown). Finally, we performed permeability assays by measuring the  
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9 270 concentrations of LY diffusing through RME 72 h after cytokine stimulation. In the non-  
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11 271 treated condition, RME permeability to LY was very low (< 1% of maximal permeability)  
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13 272 and the dye was retained in the cornified layer (Figures 4F and 4G). When RME were  
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15 273 stimulated either with OSM or IL-17A alone, the barrier function was altered as assessed by  
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17 274 the increased concentrations of LY in RME culture media reaching ~ 12% and 21% of  
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19 275 maximal permeability, respectively (Figure 4F). As expected, in RME stimulated with M5 at  
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21 276 the highest concentration, the structure of the epidermis was completely disrupted, allowing  
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23 277 the massive diffusion of the dye through RME, as evidenced by the observation of LY  
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25 278 staining under the cornified layer and the highest concentrations of LY in the lower chamber  
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27 279 (~ 64% of maximal permeability; Figure 4F and 4G). Together these results confirm that  
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29 280 RME are sensitive to proinflammatory cytokine stimulation and could be used to reproduce  
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31 281 some the features of psoriatic skin, such as the production of inflammatory molecules and the  
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33 282 altered epidermal differentiation.  
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#### 285 4. Discussion

286 Few models of *in vitro* reconstituted epidermis from primary mouse keratinocytes are  
287 described, mainly due to the difficulty to maintain primary keratinocyte cultures and to reach  
288 a complete epidermal stratification, even by using established cultures of keratinocyte  
289 progenitors. Here, we propose a new protocol for the rapid and convenient generation of RME  
290 from primary NMEK, isolated from newborn mouse skin, harboring the structural and  
291 functional features of a normal epidermis. Cultures of primary keratinocytes isolated from  
292 adult skin presented limited proliferative capacity and our attempts to generate RME were not  
293 fully satisfactory. The best results were obtained when keratinocytes were isolated from 1 to 3  
294 day-old mice. Indeed, primary mouse keratinocytes cultures from neonatal mice were shown  
295 to contain multiple progenitor cells, which are able to expand rapidly in few days (Kamimura,  
296 *et al.*, 1997). This method abrogates the use of murine keratinocyte progenitor cell lines,  
297 obtained after numerous passages as previously described (Segrelles, *et al.*, 2011, Vollmers, *et*  
298 *al.*, 2012), which need several weeks to reach a full epidermis-like structure. *In vitro* studies  
299 on mice skin equivalent models have also reported the dependence on the presence of a  
300 fibroblastic support or de-epidermised dermis for epidermis growth (Boehnke, *et al.*, 2007,  
301 Ikuta, *et al.*, 2006), thus complicating the protocol and introducing an additional inconstant  
302 parameter. In humans, the communication between fibroblasts and keratinocytes has been  
303 shown to be crucial for the formation of an efficient epidermis and a correct deposition of the  
304 basement membrane (El Ghalbzouri and Ponec, 2004). However, in human models, this  
305 fibroblastic dependence has been abrogated by introducing numerous and specific growth  
306 factors in keratinocyte culture media, including bovine pituitary extract, transferrin,  
307 hydrocortisone, L-ascorbic acid, CaCl<sub>2</sub> and KGF (Frankart, *et al.*, 2012). In our protocol, we  
308 have determined the optimal concentrations of these factors in the murine system to induce  
309 the differentiation and the complete stratification of keratinocytes without fibroblasts feeding.

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3 310 We further characterized our model of RME by analyzing the expression of a panel of  
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5 311 keratins and proteins involved in the formation of a stratified epidermis. Some studies  
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7 312 suggested that *in vitro* murine epidermis equivalents were thicker than a normal murine  
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9 313 epidermis (Ikuta, *et al.*, 2006). Our protocol generates RME at day 5 with a thickness  
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11 314 comparable to a normal mouse epidermis (~ 20-25  $\mu\text{m}$ ) with only two or three layers of  
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13 315 stacked keratinocytes. The expression of K14 was normally restricted to the basal layer, as in  
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15 316 normal mouse skin. As well, K6 expression was also found close the basement membrane,  
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17 317 with a high expression at days 3 and 5, but decreasing progressively from day 8 along with  
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19 318 the thickening of the cornified layer which precedes RME degeneration. This high expression  
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21 319 of K6 is a common feature of reconstituted epidermis models and it has been proposed that  
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23 320 the protocols mimic an *in vivo* wound healing process in which K6 is overexpressed  
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25 321 (Rosenberger, *et al.*, 2014, Segrelles, *et al.*, 2011, Stark, *et al.*, 1999). Furthermore, labeling of  
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27 322 K10, filaggrin and loricrin revealed their appropriate localization in the suprabasal and  
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29 323 granular layers of RME. As RME from day 8 change their morphology and their K6  
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31 324 expression profile, we propose to use RME at day 5 for functional studies. For further  
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33 325 characterization, we also analyzed the expression of connexins, which play an important role  
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35 326 in the coordination of keratinocyte proliferation/differentiation balance during epidermis  
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37 327 morphogenesis. A previous report described Cx43 localization in proliferating keratinocytes  
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39 328 and a Cx26 labeling in the granular layer (Kandyba, *et al.*, 2008). We found the same  
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41 329 localization in our model but Cx26 expression level seemed to be more elevated in RME than  
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43 330 in normal epidermis. Similarly to K6 expression, Cx26 has been reported to be upregulated in  
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45 331 a context of wound healing (Churko and Laird, 2013, Kretz, *et al.*, 2003), suggesting the  
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47 332 activated state of isolated primary keratinocytes engaged in a process of epidermis  
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49 333 reconstitution. Treatments of RME with the gap junction blocker CBX suggested that  
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51 334 connexins contribute indirectly to the barrier function of RME and are mostly involved in  
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3 335 epidermal differentiation and the complete stratification of RME. Finally, the cohesive  
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5 336 structure of RME was demonstrated by the homogenous expression of E-cadherin throughout  
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7 337 the structured epidermis and the constitution of a functional impermeable epidermal barrier.  
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10 338 As most of studies on mouse epidermis were carried out *in vivo*, how differentiated murine  
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12 339 keratinocytes respond individually to cytokine stimulation *in vitro* is poorly documented.  
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14 340 Thus, to complete the functional characterization of our RME, we analyzed their response to  
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16 341 proinflammatory cytokines previously described to target keratinocytes and to be involved in  
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18 342 psoriasis (Guilloteau, *et al.*, 2010). After showing that RME expressed all the subunits of  
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20 343 cytokine receptors studied in this work, we evaluated their individual and synergistic  
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22 344 activities, allowing a parallel with the human system. The mix of five cytokine, M5, induced a  
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24 345 complete disruption of the structure of the RME and a downregulation of the expression of  
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26 346 genes involved in epidermal differentiation. M5 stimulation reproduced also some features of  
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28 347 inflammatory keratinocytes through the secretion of antimicrobial peptides and chemokines.  
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30 348 These results were comparable to those observed in cytokine-stimulated RHE previously  
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32 349 described (Guilloteau *et al.*, 2010), highlighting similarities between human and mouse  
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34 350 systems. Nonetheless, some differences could be observed between the two species. In RME  
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36 351 stimulated by single cytokines, only IL-17A and OSM could disrupt the cornified layer by  
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38 352 inhibiting epidermal differentiation. These cytokines were also shown to be the most active to  
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40 353 induce a proinflammatory phenotype in murine keratinocytes (Guilloteau, *et al.*, 2010, Pohin,  
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42 354 *et al.*, 2016). Interestingly, IL-17 and OSM are the two cytokines that provoke a strong  
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44 355 induction of Cx26 expression and an alteration of the barrier property of RME. This  
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46 356 upregulation of Cx26 has been described in inflammatory conditions such as psoriatic skin  
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48 357 (Labarthe, *et al.*, 1998) , wound healing (Churko and Laird, 2013, Kretz, *et al.*, 2003), in a  
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50 358 model of RHE under stress conditions (Wiszniewski, *et al.*, 2000) and was shown to be  
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52 359 associated with profound barrier impairment in a model of transgenic mice overexpressing  
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3 360 Cx26 in the skin (Djalilian, *et al.*, 2006). By contrast, in humans, IL-22 and OSM were shown  
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5 361 to be the most potent inhibitors of epidermal differentiation, in particular by inducing STAT-3  
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7 362 activation pathway (Rabeony, *et al.*, 2014), whereas IL-1, IL-17A and TNF- $\alpha$  were mainly  
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9 363 involved in the induction of innate immunity mediators (Guilloteau, *et al.*, 2010). In our  
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11 364 hands, IL-22 was not as active as OSM on RME. This might be explained by the lowered  
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13 365 expression of the IL-22R1 chain in our RME compared to its expression in normal epidermis.  
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15 366 Finally, IL-1 $\alpha$  and TNF- $\alpha$  were also active on keratinocytes but failed to induce structural  
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17 367 changes in RME formation. The regulation of the inflammatory markers induced by these two  
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19 368 cytokines alone is limited but in a complex network involving multiple cytokines, such as in  
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21 369 atopic dermatitis or psoriasis, it is likely that they contribute to cytokine synergy increasing  
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23 370 disease severity.  
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28 371 In conclusion, we propose a new protocol for the formation of a complete RME in five days  
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30 372 which tends to reproduce closely the tissue architecture of a normal epidermis. These RME  
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32 373 have an intact barrier function and are able to respond to cytokines relevant to skin  
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34 374 inflammation, similarly to what has been described in the human system. This new model of  
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36 375 RME would greatly contribute to study the normal differentiation of epidermis or its alteration  
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38 376 in inflammatory conditions. Moreover, this model opens the possibility to generate RME from  
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40 377 genetically engineered mice to further dissect the role of the transgene (knock-in or knock-  
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42 378 down) on the physiology of the epidermis.  
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3 380 **Conflict of Interest**  
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6 381 The authors have declared that there is no conflict of interest.  
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12 383 **Acknowledgements**  
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15 384 We thank Dr. Anne Cantereau (ImageUP, Université de Poitiers) for technical assistance in  
16  
17 385 confocal microscopy, Dr. Michel Simon (INSERM U1056, Université de Toulouse) for the  
18  
19 386 protocol of Lucifer Yellow assay and Pr. Guylène Page (Université de Poitiers) for the use of  
20  
21 387 the Varioskan. This study was supported by grants from a clinical research program from  
22  
23 388 Poitiers University Hospital, « la Ligue contre le Cancer », « le Cancéropôle Grand Ouest »,  
24  
25 389 « l'Association Nationale de Recherche et de la Technologie », ECOS-Sud committee and  
26  
27 390 from "Le conseil régional de la région Poitou-Charentes". M. P. is supported by "Le conseil  
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29 391 régional de la région Poitou-Charentes".  
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37 393 **Author Contributions**  
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40 394 MP, CB, JG, JFJ designed protocols for RME generation. MP, CV, JG, CB, JFJ performed  
41  
42 395 the experiments. MP, CV, JG, CB, LC, VH, LF, JM, FXB, FM, JCL, JFJ analyzed and  
43  
44 396 interpreted the data. MP, JFJ wrote the manuscript. JFJ designed the research. All the authors  
45  
46 397 read and approved the final manuscript.  
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3 399 **Figure legends**  
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6 400 **Figure 1. RME reproduce the structure and the expression profiles of differentiation**  
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8 401 **markers of a normal mouse epidermis.**

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11 402 (A) Schematic representation of the main steps for RME formation. (B) H&E histology of  
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13 403 RME at days 3, 5 and 8 and normal mouse skin sections (upper panel). Scale bar, 20  $\mu\text{m}$ .  
14  
15 404 Immunofluorescent staining for K6, K14 and K10, loricrin and filaggrin (all in red) and  
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17 405 TOPRO-3 staining for cell nuclei (in blue). Original magnification:  $\times 600$ . The dotted white  
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19 406 line delineates the dermal-epidermal junction of mouse skin or the membrane of the insert for  
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21 407 RME. The solid white line marks the surface of the epidermis of mouse skin and RME.  
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23 408 Images are representative of three independent experiments.  
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31 410 **Figure 2. RME express GAP and adherence junctions and acquire their natural barrier**  
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33 411 **function.**

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36 412 (A) Immunofluorescent staining of RME at days 3, 5 and 8 and normal mouse skin sections  
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38 413 were performed with primary antibodies specific for E-cadherin (in red), Cx43 and Cx26  
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40 414 (both in green) and TOPRO-3 staining for cell nuclei (in blue). For details, see insets. The  
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42 415 dotted white line delineates the dermal-epidermal junction of mouse skin or the membrane of  
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44 416 the insert for RME. The solid white line marks the surface of the epidermis of mouse skin and  
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46 417 RME. Images are representative of three independent experiments. Original magnification:  
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48 418  $\times 600$ . (B) Quantification of RME permeability to Lucifer Yellow (LY). Concentrations of LY  
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50 419 passing through RME were measured in culture media, 6 h after dye deposition on the surface  
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52 420 of RME at days 0, 3, 5 and 8 ( $n=3$ ), or RME at day 0 in the presence of 60  $\mu\text{M}$  of  
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54 421 carbenoxolone (CBX). Data are expressed as mean  $\pm$  SEM percentage of the maximal  
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3 422 permeability for LY, previously determined by measuring LY concentrations that diffused  
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5 423 through the polycarbonate membrane insert without keratinocytes. Data are from three  
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7 424 independent experiments. (C) Lucifer Yellow staining (in green) by confocal microscopy, 6 h  
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9 425 after dye deposition on the surface of RME at day 5 and day 8. Cell nuclei were stained with  
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11 426 TOPRO-3 (in blue). The dotted white delineates the membrane of the insert. Original  
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14 427 magnification:  $\times 600$ .

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20 429 **Figure 3. RME express cytokine receptors similarly to a normal epidermis.**

23 430 Transcriptional analysis by RT-qPCR was performed for the indicated subunits of cytokine  
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25 431 receptors in RME at 5 day and in normal epidermis. Data represent mean  $\pm$  SEM expression  
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27 432 level of the gene of interest relative to the expression of the housekeeping gene GAPDH, from  
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29 433 4 independent experiments.  $*p < 0.05$ ; using two-way ANOVA followed by Bonferroni post-  
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32 434 test.

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38 436 **Figure 4. RME respond to skin-relevant proinflammatory cytokines by an altered**  
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40 437 **differentiation and barrier function.**

43 438 RME at day 5 were stimulated with the indicated single cytokines at the concentration of 10  
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45 439 ng/ml and the mix of 5 cytokines, M5, at 1 and 10 ng/ml, respectively. (A) After 72 h of  
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47 440 stimulation, RME histology was examined after H&E staining. NS, non-stimulated. Scale bar,  
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49 441 20  $\mu\text{m}$ . (B) After 24 h of stimulation, the expression of genes involved in the innate immune  
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51 442 response of keratinocytes including S100A9, CXCL-3 and BD3 was determined by RT-  
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53 443 qPCR. Data represent mean  $\pm$  SEM mRNA fold change above unstimulated RME, from 4  
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55 444 independent experiments. (C) Mean  $\pm$  SEM S100A9 concentrations measured by ELISA in

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3 445 culture media of RME 72 h after cytokine stimulation. Data are from three independent  
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5 446 experiments. (D) After 24 h of stimulation, the expression of the epidermal differentiation  
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7 447 genes K6, K10, filaggrin and involucrin was determined by RT-qPCR. Data represent mean  $\pm$   
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9 448 SEM mRNA fold change above unstimulated RME, from 4 independent experiments. (E)  
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11 449 Immunofluorescent staining of cytokine-stimulated RME was performed with primary  
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13 450 antibodies specific for Cx26 (in green) and K6 (in red). (F) Quantification of RME  
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15 451 permeability to LY after cytokine stimulation for 72 h. Data are expressed as mean  $\pm$  SEM  
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17 452 percentage of the maximal permeability for LY, from three independent experiments. (G)  
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19 453 Fluorescence detection by confocal microscopy of LY (in green) in sections of non-stimulated  
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21 454 (NS) and M5-stimulated RME, 6 h after dye deposition. Cell nuclei were stained with  
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23 455 TOPRO-3 (in blue) and the dotted white delineates the membrane of the insert. Original  
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27 456 magnification:  $\times 600$ .

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For Peer Review



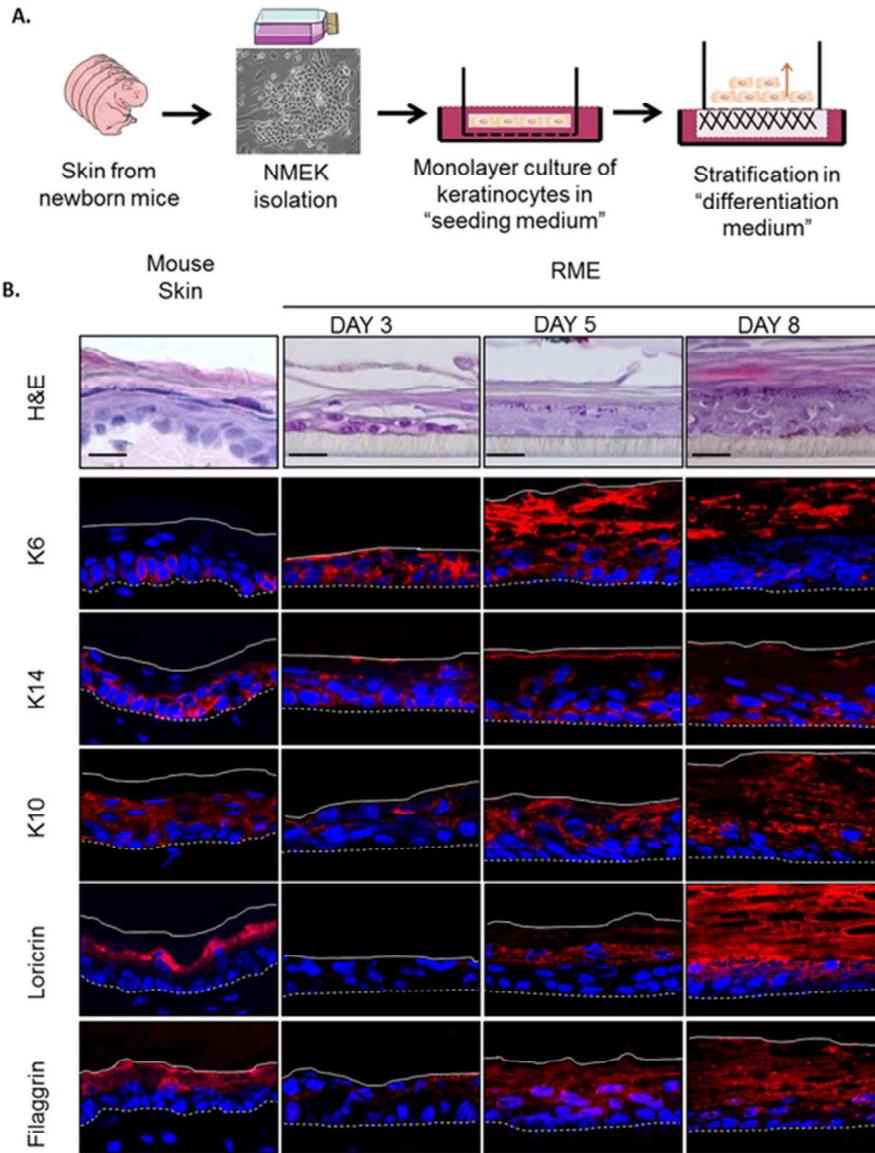


Figure 1

50x64mm (300 x 300 DPI)

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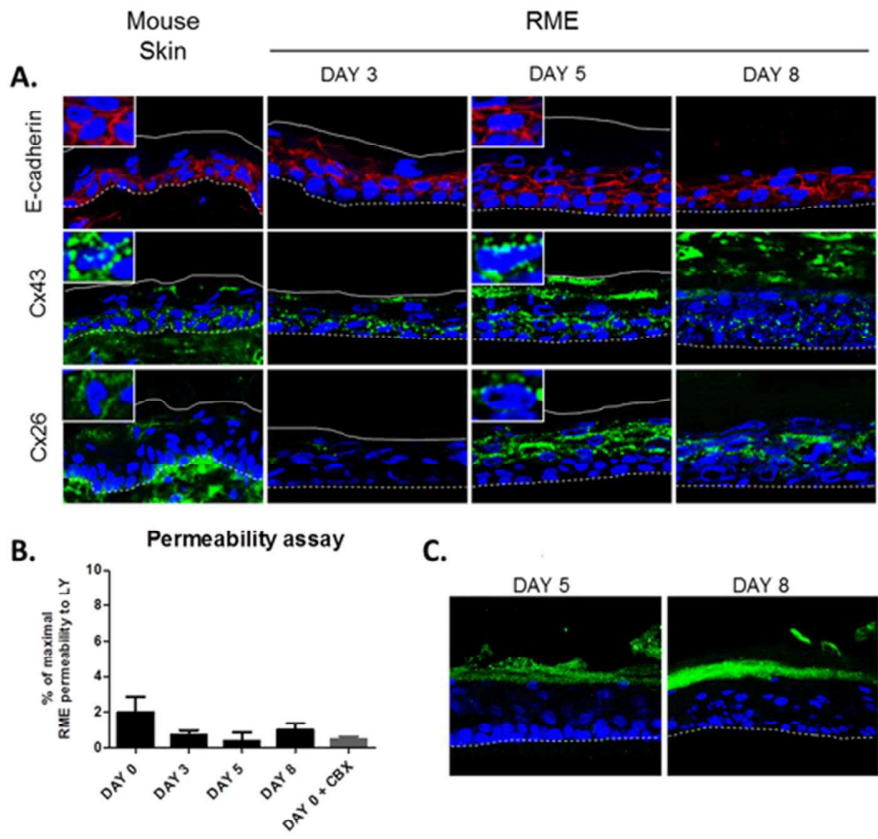


Figure 2

50x45mm (300 x 300 DPI)

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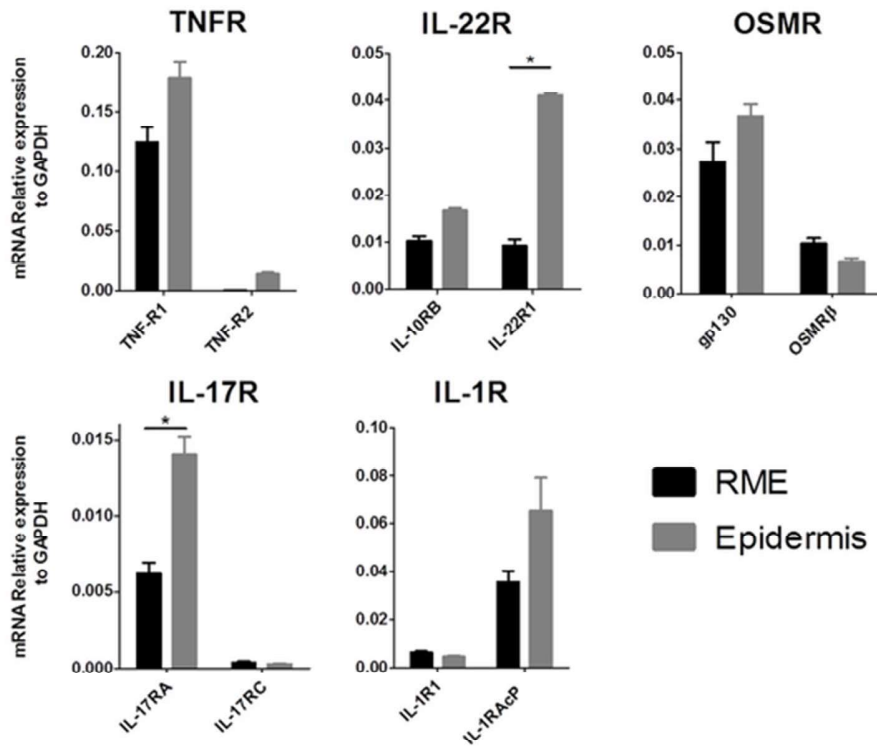


Figure 3

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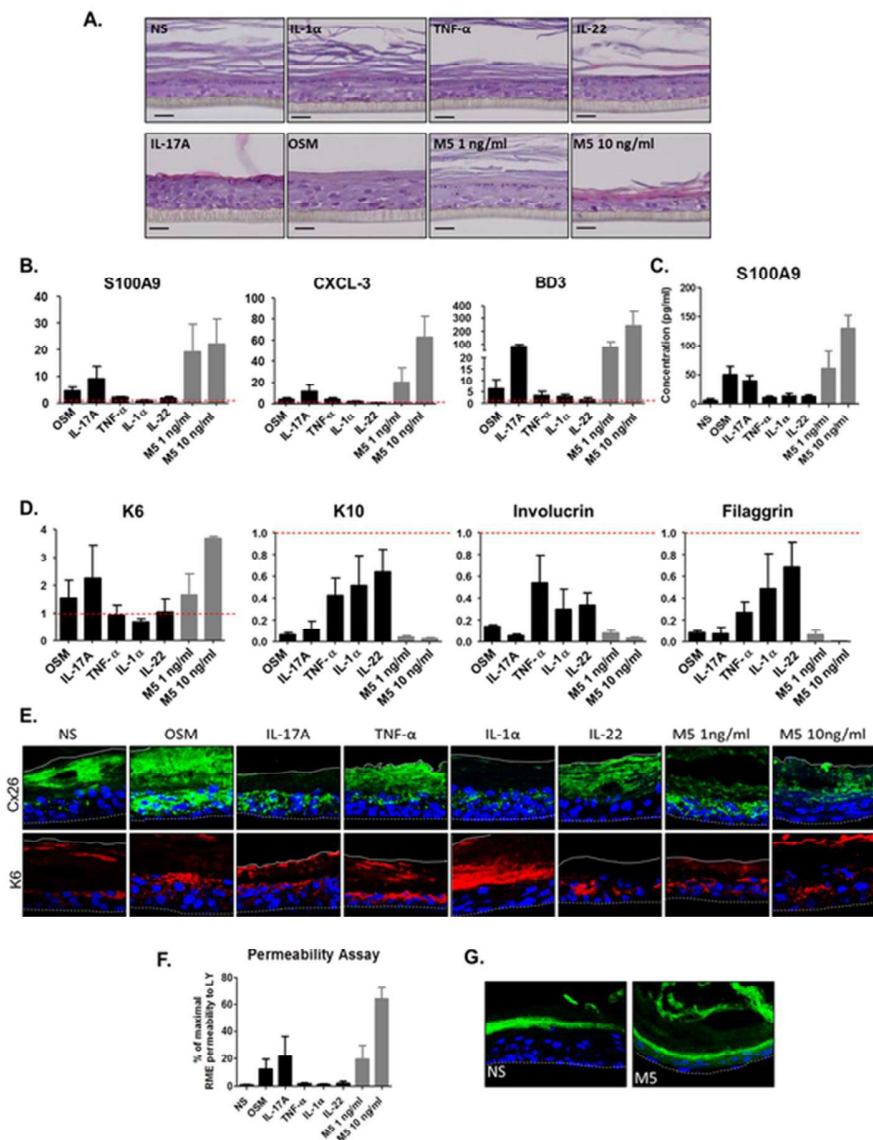


Figure 4

50x67mm (300 x 300 DPI)