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1 **Development and characterization of two porcine monocyte-derived macrophage cell lines**

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23 *Abbreviations:* ADCC, antibody-dependent cellular cytotoxicity; CD, cluster of
24 differentiation; E:T, effecter to target ratio; EDTA, ethylenediaminetetraacetic acid; FBS, fetal
25 bovine serum; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAPDH,
26 glyceraldehydes 3-phosphate dehydrogenase; IACUC, Institutional Animal Care and Use
27 Committee; IgG, immunoglobulin G; L-glut, L-glutamine; LPS, lipopolysaccharide; M-CSF,
28 macrophage colony-stimulating factor; MHC, major histocompatibility complex; NK, natural
29 killer; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PCR,
30 polymerase chain reaction; RPMI, Roswell Park Memorial Institute; TE, Trypsin-EDTA;
31 USMARC, U.S. Meat Animal Research Center
32

33 **Abstract**

34 Cell lines CΔ2+ and CΔ2- were developed from monocytes obtained from a 10-month-old,
35 crossbred, female pig. These cells morphologically resembled macrophages, stained positively
36 for α-naphthyl esterase and negatively for peroxidase. The cell lines were bactericidal and
37 highly phagocytic. Both cell lines expressed the porcine cell-surface molecules MHCI, CD11b,
38 CD14, CD16, CD172, and small amounts of CD2; however, only minimal amounts of CD163
39 were measured. The lines were negative for the mouse marker $H2K^k$, bovine CD2 control, and
40 secondary antibody control. Additionally, cells tested negative for Bovine Viral Diarrhea Virus
41 and Porcine Circovirus Type 2. Therefore, these cells resembled porcine macrophages based on
42 morphology, cell-surface marker phenotype, and function and will be useful tools for studying
43 porcine macrophage biology.

44

45 *Keywords:* Monocyte, Macrophage, Cytokine, Phagocytosis, Morphology, Cell surface
46 molecule

47

48 **1. Introduction**

49 Macrophages are an important component of the innate immune response against
50 pathogens. These cells are of myeloid origin and after circulating in the blood as monocytes,
51 differentiate into tissue macrophages. In addition to protecting the host, macrophages also
52 contribute to the infectious process by maintaining intracellular pathogens such as Porcine
53 Reproductive and Respiratory Disease Syndrome Virus (PRRSV; Van Reeth and Adair, 1997),
54 *Brucella* (Maria-Pilar et al., 2005), and *Salmonella* (Donné et al., 2005). Monocytes make up
55 only a small percentage of mononuclear cells in peripheral whole blood. In pigs, this value
56 ranges from 0-10% (The Merck Veterinary Manual, 1991). Isolating sufficient numbers of these
57 cells to perform *in vitro* experiments is time consuming and variation among animals in cell
58 numbers and activity level is high. Although numerous human and murine
59 monocytic/macrophage cell lines are publicly available, the same is not true for pigs. There are
60 only three pig monocytic/macrophage cell lines (CRL-2843, -2844, and -2845), (ATCC "Cell
61 Lines and Hybridomas" catalogue;
62 https://www.atcc.org/ATCCAdvancedCatalogSearch/tabid/112/Default.aspx). All of these are
63 virus transformed which can affect the function of the cells (Beharka et al., 1998). Other porcine
64 cell lines of monocyte lineage have been described; however, these are not available in a public
65 repository (Wardley et al., 1980; Kadoi et al., 2001; Weingartl et al., 2002; Lee et al., 2010).
66 Therefore, there is a strong need for available, non-transformed, porcine monocyte-derived cell
67 lines for agricultural research. These cells would allow for the completion of "proof of concept
68 studies" and drug development work requiring macrophages without the time and expense (i.e.,
69 Institutional Animal Care and Use Committee [IACUC] approval and monitoring) of obtaining
70 whole blood from experimental animals. We describe the development of porcine monocyte-

71 derived cell lines with the characteristics of macrophages that will be deposited in a cell
72 repository for public access.

73 **2. Materials and methods**

74 *2.1. Culture of LM-929 cells for supernatant*

75 LM-929 cells (ATCC CCL 1.2) were used as the source of macrophage colony-
76 stimulating factor (M-CSF; Beharka et al., 1998). LM-929 cells were grown to confluency in
77 tissue culture flasks in Roswell Park Memorial Institute (RPMI) medium (Invitrogen, Carlsbad,
78 CA) supplemented with 5% fetal bovine serum (FBS; HyClone), Antibiotic/Antimycotic (A/A;
79 Invitrogen), and L-glutamine (L-glut; Invitrogen). Supernatants were stored at -80°C, and then
80 filter sterilized prior to use.

81 *2.2. Isolation of porcine monocytes and generation of cell lines*

82 Whole blood was obtained with IACUC approval in accordance with USDA animal care
83 guidelines from a 10-week-old, mixed-breed, female pig housed at the U.S. Meat Animal
84 Research Center (USMARC) swine facility. Approximately, 70 ml whole blood was obtained
85 via jugular venapuncture, into 35-ml syringes containing 0.1 M EDTA. Peripheral blood
86 mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-Paque
87 Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden), as previously described (Chitko-
88 McKown et al., 2004). Purified PBMC were counted, cytocentrifuged, and stained to
89 differentiate between monocytes and lymphocytes. Cells were resuspended at 1 X 10^6
90 monocytes/ml RPMI without serum and 11 ml were placed into 25-cm^2 tissue culture flasks and
91 allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. Medium was
92 then replaced with RPMI containing 5% FBS, A/A, and L-glut (complete RPMI) to remove the
93 lymphocyte population. After culturing under these conditions for 17 days, cells were cultured

94 in medium containing 10% LM-929 supernatant as indicated by "+" in the cell line
95 nomenclature. After 5 months in culture, a subculture of these cells was reintroduced to medium
96 without LM-929 supernatant (CΔ2-). Culture medium was changed once per week until the cells
97 formed a confluent monolayer stage. Cells were then passaged and replated or frozen.
98 *2.3. Cell dispersal and freezing*
99 Adherent cell monolayers were dispersed by treatment with Trypsin-EDTA (TE;
100 Invitrogen; Helgason, 2005). Cell preparations used for cell-surface phenotyping were dispersed
101 using 0.2% EDTA without trypsin. 1 - 5 X 10^6 cells/vial/ml were prepared for storage in liquid
102 nitrogen. They were suspended in freezing medium consisting of 10% dimethyl sulfoxide in
103 FBS (Yokoyama, 1997).
104 *2.4. Karyotype analysis*
105 Cell lines were subcultured 1:2 for karyotyping at passages 20 to 24. Briefly, cells were
106 grown to confluence in 75-cm^2 flasks, trypsinized and transferred to new flasks in culture
107 medium containing 5-bromo-2'-deoxyuridine (BrdU) to a final concentration of 25 μg/ml
108 (Sigma; Riggs, et al., 1997). After 20 h, medium was replaced with fresh culture medium
109 lacking BrdU. Cultures were incubated for an additional 4 h, then medium was replaced with
110 0.075 M KCl. Mitotic cells were shaken from the flask into the hypotonic solution and incubated
111 for 20 min, then fixed with multiple changes of a solution of 3:1 methanol: glacial acetic acid.
112 Chromosomes were stained in 4% Giemsa (Life Technologies) or banded as described by Rønne
113 (1985), and karyotyped according to international convention (Committee for the Standardized
114 Karyotype of the Domestic Pig, 1988). Fluorescence in situ hybridization (FISH) to identify the
115 sex chromosomes was conducted with bacterial artificial chromosome probes for *KAL1* and
116 *CSF2RA* genes as previously described (Raudsepp et al., 2008).

117

118 *2.5. Phenotypic and immunophenotypic analysis*

119 Cytospin preparations of the cell lines were stained as per the manufacturer's directions

120 using HEMA3 differential stain (Fisher Scientific Company, Kalamazoo, MI), the leukocyte

121 peroxidase kit (Sigma-Aldrich, St. Louis, MO) and the α-naphthyl esterase kit (Sigma-Aldrich).

122 Cells were stained for flow cytometric analysis of cell surface determinants essentially as

123 described (Potts et al., 2008). Primary antibodies against Mouse $H\text{-}2K^k$, CD172, CD16, CD11b

124 (BD Pharmingen, San Jose, CA), MHC Class I, CD14, and bovine CD2 (VMRD, Inc., Pullman,

125 WA), were added for a final concentration of 1:50. FITC-Streptavidin (KPL, Gaithersburg, MD)

126 was added to cells stained with anti-mouse $H\text{-}2K^k$, and FITC-labeled anti-mouse IgG (KPL) was

127 added to all other cells. Fixed samples were stored at 4°C in the dark until assayed.

128 *2.6. LPS stimulation of cell cultures*

129 Cells were cultured in medium containing 1 μg/ml LPS (Laegreid et al., 1998). To obtain

130 RNA for the measure of cytokine expression, cells were cultured in either 25-cm^2 tissue culture

131 flasks or 24-well tissue culture plates for 0 - 48 h.

132 *2.7. Nitrite production*

133 A colorimetric assay (as described by Stuehr et al., 1989) was used to determine the

134 amount of nitrite (NO_2^-) present in LPS-stimulated cell supernatants. A sodium nitrite ($NaNO_2$)

135 standard was assayed concurrently with the samples, and medium was used as a negative control.

136 Quantity of NO_2^- present in the samples was determined by regression analysis.

137 *2.8. Bactericidal assay*

138 Colorimetric bactericidal assays using *Escherichia. coli* O157 and *Staphylococcus aureus*

139 as targets were performed essentially as described by Stevens, et al. (1991). Bacteria were

140 opsonized by incubation at 37°C with heat-inactivated bovine serum previously determined to
141 have high antibody titers against *E. coli* O157. Non-opsonized bacteria were incubated in
142 medium without serum. Cells (3 X 10^4) were placed into 96-well tissue culture plates with
143 either opsonized on non-opsonized bacteria at an effecter to target ratio (E:T) of 1:100 for *E. coli*
144 O157 and 1:10 for *S. aureus.* MTT (Sigma-Aldrich)
145 *2.9. Phagocytosis assay*
146 Phagocytosis was measured by the uptake of fluorescent microspheres as previously
147 described, with some modifications (Potts and Chapes, 2008). Flow cytometric analysis to
148 calculate microsphere uptake was performed on a Becton Dickinson FACSCalibur flow
149 cytometer.
150 *2.10. RNA isolation and cytokine expression*
151 Total RNA was extracted from LPS-treated cells by acid guanidine phenol extraction
152 (Chomczynski and Sacchi, 1987). First strand cDNA synthesis was performed on 1 μg total
153 RNA using the SuperScript™ III Platinum® Two Step qRT-PCR Kit (Invitrogen) as per the
154 manufacturer's instructions. Cytokine PCR was performed using a quantitative simultaneous
155 multiplex real-time assay (Duvigneau et al., 2005). Three multiplexed reactions were run:
156 Primer/Probe Set 1 assayed for the lymphokines IL-2, IL-4, and IFN-γ; Primer/Probe Set 2
157 assayed for the proinflammatory cytokines IL-1α, IL-6, and IL-10; and Primer/Probe Set 3
158 assayed for the housekeeping genes β-actin, GAPDH, and cyclophilin. Resulting values for
159 cytokine Cts were normalized against the numerical average of the three housekeeping gene Cts.
160 *2.11. Virus infection*
161 Cells were tested by PCR for bovine viral diarrhea virus (BVDV) infection using the
162 primer set F5'-CATGCCCATAGTAGGAC-3' and R5'-CCATGTGCCATGTACAG-3' for first

163 round PCR amplification and cycle sequencing. This primer set amplifies sequences from the
164 genomic 5' untranslated region of type 1 and type 2 BVDV, but does not appear to amplify
165 sequences from BVDV (Bolin et al., 1994; Ridpath and Bolin., 1998). Additionally, aliquots of
166 CΔ2+ and CΔ2- lysates were mixed 1:1 with Minimum Essential Medium (MEM, Invitrogen)
167 and inoculated onto bovine turbinate (BT) cells that had been seeded into a 24-well plate. After
168 14 days of incubation at 37°C, the BT cell lysates were tested by PCR for propagation of BVDV.
169 Cell lines were tested for PCV2 by real-time PCR as described by Opriessnig et al. (2003).

170 **3. Results and discussion**

171 We isolated monocytes from the peripheral blood of a crossbred pig in order to develop
172 porcine monocyte-derived macrophage cell lines. The peripheral blood mononuclear cell
173 population was isolated over a density gradient, and monocytes were obtained by removal of
174 non-adherent cells from cultures. After two weeks in culture, the monocytes were adhered to the
175 culture flasks and were considered monocyte-derived macrophages. At this time, LM929
176 supernatant was added to the cultures to provide a source of M-CSF to stimulate cell
177 proliferation. Monolayers soon formed and cells morphologically resembled cultured
178 macrophages (Figure 1A). The addition of LPS to the medium caused the cells to develop the
179 "fried egg" appearance of activated macrophages (Figure 1B). This cell line was named CΔ2+,
180 the "+" denoting the inclusion of LM929 supernatant in the culture medium. After several
181 months in culture, the LM929 supernatant was removed from the medium of a subculture of the
182 CΔ2+ cell line, and these cells continued to proliferate and this subculture was named CΔ2- to
183 designate the absence of supernatant in the medium (Figure 1A). The CΔ2- line responded to the
184 addition of LPS to the medium similarly to the CΔ2+ cells (Figure 1B). The cell lines were
185 characterized both earlier and later than ten passages, and were found to be stable.

186 Monocytes stain weakly for the enzyme myeloperoxidase found only in their lysozomal
187 vacuoles, and staine diffusely for α-naphthyl-esterase (Van Furth, 1988). Resident and resident-
188 exudate macrophages have peroxidase-positive nuclear envelopes (Van Furth, 1988). In the
189 mouse, 95% of blood monocytes are positive for esterase activity as are 99% of resident
190 peritoneal macrophages (Van Furth, 1988). In contrast, only 60% of blood monocytes and 0% of
191 resident peritoneal macrophages stain positively for peroxidase (Van Furth, 1988). The CΔ2+
192 cell line stained strongly positively for α-naphthyl-esterase and diffusely for myeloperoxidase
193 (Figure 1C), in comparison, the CΔ2- cells stained diffusely for peroxidase, and not as strong as
194 the CΔ2+ cell line for α-naphthyl-esterase (Figure 1C). These results suggest that these cell lines
195 are in the macrophage lineage beyond the monocyte stage since they had properties that were
196 consistent with those described between monocyte and resident macrophage stages (Van Furth,
197 1988).

198 Cytogenetic analysis indicated that both cell lines are consistent with a diploid female pig
199 karyotype of 38,XX. The CΔ2+ cell line contains a large metacentric chromosome derived
200 from a reciprocal translocation of pig chromosomes SSC 8 and SSC 16 (Figure 2A). The large
201 derivative chromosome was observed in all metaphase nuclei examined and is stably maintained.
202 In contrast, the translocated chromosome is not found in the CΔ2- cell line. This cell line
203 appears to be near normal in the majority of cells, but some cells were observed to be
204 aneuploidy, with chromosome numbers from 36 to 38. Sex chromosome identification was
205 verified by FISH of probes for *KAL1* and *CSF2RA,* both typically located in the
206 pseudoautosomal region at the distal p-arm of the X chromosome (SSC X). Interestingly, a
207 small rearrangement was observed in the CΔ2- cell line. In these cells, *CSF2RA* is translocated to

208 from the tip of SSC X to a submetacentric chromosome tentatively identified as SSC 5
209 (supplemental figure). This region of SSC X remains intact in the CΔ2+ cells (not shown).
210 We stained the cell lines with a panel of antibodies against porcine cluster of
211 differentiation (CD) markers normally found on cells of monocyte lineage, as well as several
212 controls (Figure 3, Table 1). Both cell lines were negative (≤2%) for murine $H2K^k$ indicating
213 that the lines were not contaminated by the murine LM-929 cells used as the source of M-CSF.
214 They were also negative for the bovine CD2 marker. CΔ2- cells had a lower level of staining for
215 CD2 (6%), CD11b (4%), CD14 (12%) and CD16 (5%) and MHCI, whereas in all cases CΔ2+
216 had a higher level of expression than the CΔ2- cells for these same cell surface markers (Table
217 1). In the presence of antibody, the low affinity Fcγ receptor, CD16, can facilitate phagocytosis
218 and antibody-dependent cellular cytotoxicity (ADCC; Ravetch and Kinet, 1991). The low levels
219 of CD11b, CD14 and CD16 would be consistent with the hypothesis that CΔ2- cells were less
220 differentiated than CΔ2+ cells (Fleit and Kobasiuk, 1991; Leenen et al., 1990; Leenen et al.,
221 1994). Lastly, CD172, also known as SIRPα, is found on cells of monocyte/macrophage lineage
222 (Ezquerra et al., 2009) and was the highest expressed marker tested, and it too was expressed in
223 greater numbers on the CΔ2+ cells (Table 1). CD2, normally found of the surface of T and NK
224 cells and a sub-population of macrophages, was also present in low amounts on both CΔ2+ and
225 CΔ2- cells (6 and 8%, respectively). Since we thought that CΔ2+ and CΔ2- might be useful
226 tools for the study of PRRSV, we also examined the expression of CD163 (one of the described
227 receptors for the virus; Calvert et al., 2007; Van Gorp et al., 2008). We found that CΔ2- cells
228 had only 2% expression above background compared to 7% expression on CΔ2+ cells. It is not
229 clear if this level of expression will allow for virus entry and replication. However, the cells may
230 serve as a suitable host even if they have to be transfected with CD163 to improve the expression

231 level since they can provide a suitable porcine macrophage environment necessary for virus
232 growth.

233 Although both cell lines have undergone chromosomal rearrangements, they appear to be
234 fairly stable cytogenetically. Each cell line is karyotypically distinct with two derivative
235 chromosomes maintained in CΔ2+ cell line, and a rearrangement involving the X chromosome in
236 the CΔ2- cells.

237 The expression of iNOS and production of nitrite/nitrate by porcine
238 monocytes/macrophages are under debate (Zelnickova et al., 2008). We used the Griess reagent
239 to measure nitrite production by the cell lines after exposure to LPS. In our hands, this assay
240 reliably measures nitrite production by murine (Fleming et al., 1991) and bovine
241 monocytes/macrophages (Chitko-McKown et al., 1992). However, no nitrite production was
242 measurable from either control or LPS-treated cell line supernatants (data not shown). The
243 absence of a nitric oxide response could be because of the relative immature stages of both the
244 CΔ2+ and CΔ2- cells. Alternatively, this may reflect the fact that porcine macrophages have
245 poor *nos2* expression and nitric oxide responses and the cell lines parallel primary porcine
246 monocyte/macrophages (Pampusch et al., 1998; Kapetanovic et al., 2012).

247 When we measured the bactericidal activity of the CΔ2+ and CΔ2- cells, we found that
248 both cell lines were bactericidal against Gram$^-$ (*E. coli*) and Gram$^+$ (*S. aureus*) organisms (Table
249 2). Differences were observed in the levels of killing between opsonized and non-opsonized
250 bacteria were no statistically significant. This may be a result of the efficiency of direct
251 bactericidal killing of bacteria, which didn't leave room for significant enhancement with
252 opsonization. Alternatively, the serum used for opsonization may have included some factors
253 which interfered with the bactericidal activity of the cell lines.

254 The efficient bactericidal activity of the CΔ2+ and CΔ2- cells was consistent with the
255 observation that both cell lines were highly phagocytic. However, the CΔ2+ cells were more
256 efficient phagocytes compared to the CΔ2- cells based on the speed that they phagocytosed latex
257 beads (Figure 4). Although 97% of the CΔ2+ cells ultimately phagocytosed beads by 18 h,
258 compared to 85% for CΔ2-, at 3 h, over 75% of the CΔ2+ cells had phagocytosed beads
259 compared to less that 25% of the CΔ2- cells. This difference in phagocytosis efficiency is
260 consistent with the hypothesis that CΔ2- cells were less differentiated as CΔ2+ cells.
261 To determine if the cell lines expressed cytokines normally attributed to cells of
262 monocyte/macrophage lineage, we used sets of multiplexed assays for real-time PCR analysis.
263 Cell lines were stimulated with LPS and compared to non-stimulated cultures over time ranging
264 from 0-24 h (Figure 5). Both cell lines expressed mRNA for the housekeeping genes tested, as
265 well as the proinflammatory cytokines IL-1α and IL-6, but not for the cytokines IL-2, IL-4, and
266 IFNγ, which are normally produced by cells of lymphocyte lineage. IL-1α was expressed in
267 CΔ2- by 4 h with or without LPS treatment. However, it was only expressed by CΔ2+ at 24 and
268 48 h and not earlier time-points. IL-6 was measured in both LPS and non-treated CΔ2+ and
269 CΔ2- lines, with CΔ2- expressing the most at all but the 48 h time-points.
270 Contamination of banked cell lines with Bovine Viral Diarrhea Virus (BVDV) through
271 the use of contaminated FBS/FCS in culture medium is of great concern (Cobo et al., 2005).
272 Therefore, we tested the cell lines for BVDV. Both cell lines were negative for BVDV, as
273 determined by PCR analysis using a positive control 10^6 virions per ml (Figure 6). Additionally,
274 the cell lines were tested for the presence of the porcine respiratory pathogen PCV2 by real-time
275 PCR. No endogenous virus was found at a minimum detection level of 20 copies per well in
276 either line.

In conclusion, both porcine monocyte-derived macrophage cells CΔ2+ and CΔ2- closely mimic the morphology and activity of primary monocyte/macrophage cultures. Their relative ease of culture renders them useful tools for the in vitro study of porcine monocyte/macrophage biology.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table

Table 1.
Cell surface staining/flow cytometry.

	CΔ2+	CΔ2-
Antigen	% positive	% positive
Murine $H2K^k$	---	2
Bovine CD2	---	---
CD2	8	6
CD11b	29	4
CD14	14	12
CD16	11	5
MHCI	16	5
CD172	55	35
CD163	7	2

Cells were stained for flow cytomeric analysis essentially as described in Materials and Methods. % positive are the percent positive cells after the isotype control background fluorescence was removed (Figure 3). All markers are porcine-specific unless noted.

Table 2.
Bactericidal activity of CD2+ and CD2- on *E. coli* and *S. aureus*.

	E. coli (1:100)*		S. aureus (1:10)*	
	Nonopsonized	Opsonized	Nonopsonized	Opsonized
CΔ2+	62 ± 3	35 ± 16	56 ± 15	50 ± 20
CΔ2-	58 ± 23	36 ± 10	78 ± 24	53 ± 2

Values are expressed as % killed mean ± std of 2 experiments.
*Effector to target cell ratio.

Figure
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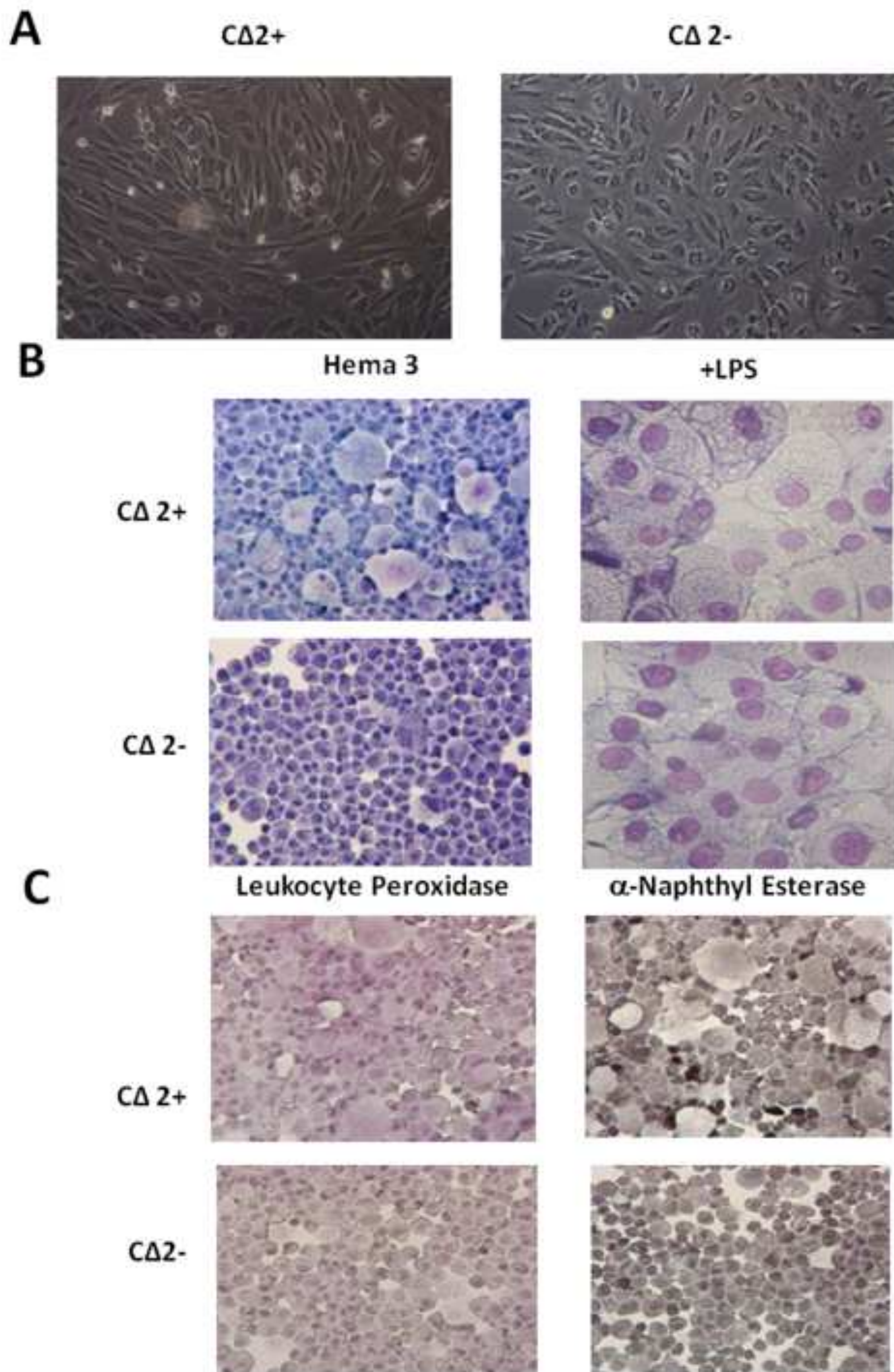

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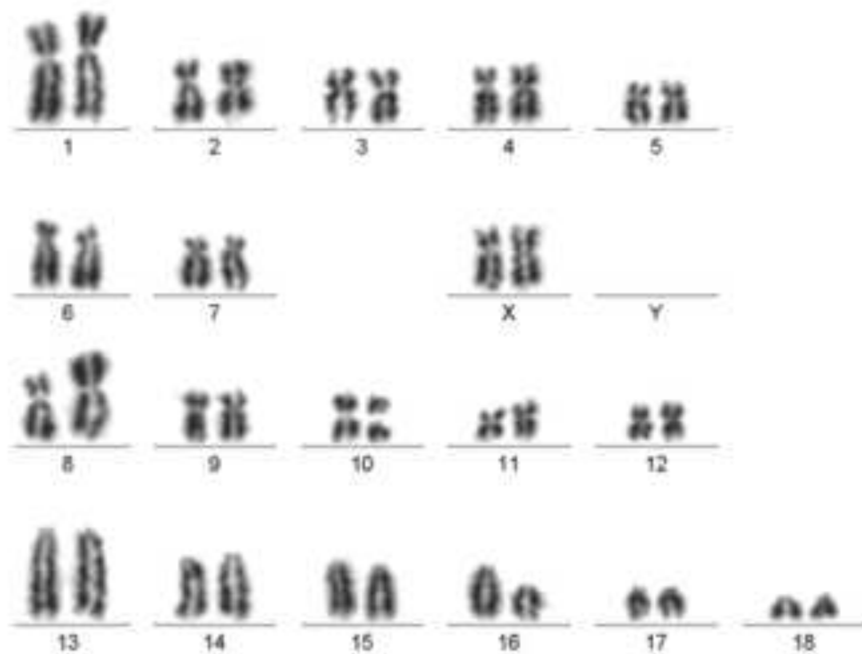

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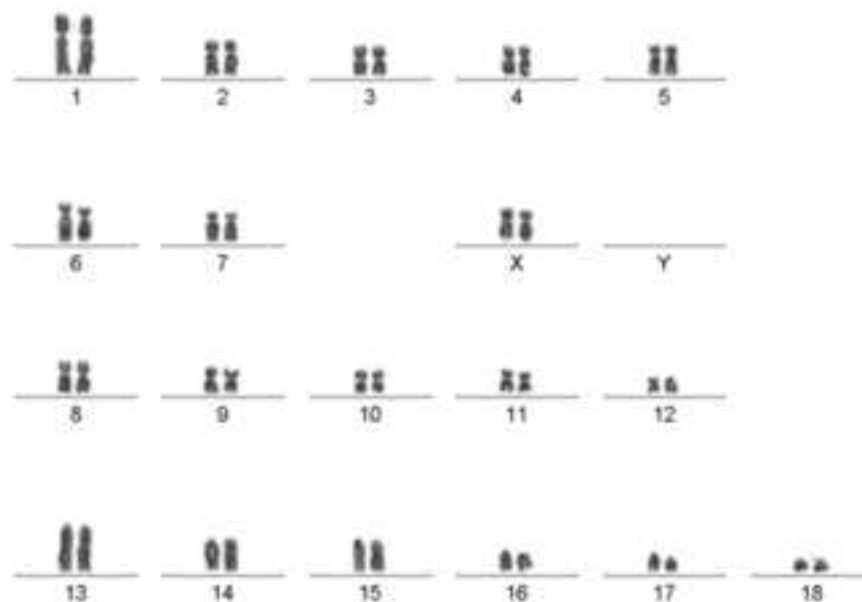

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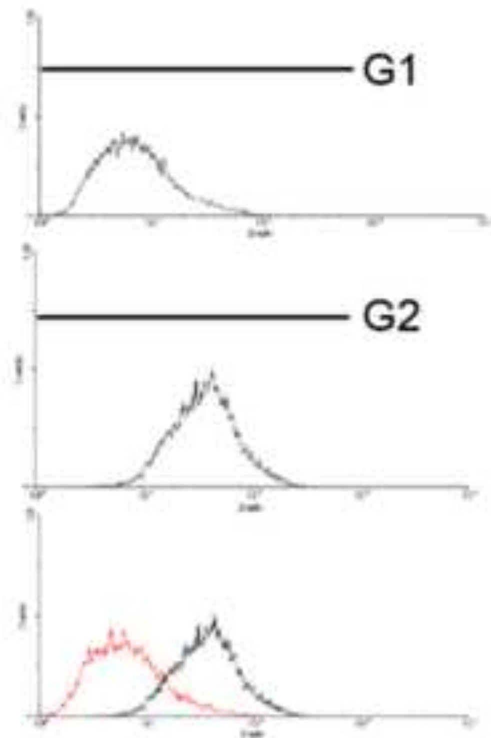

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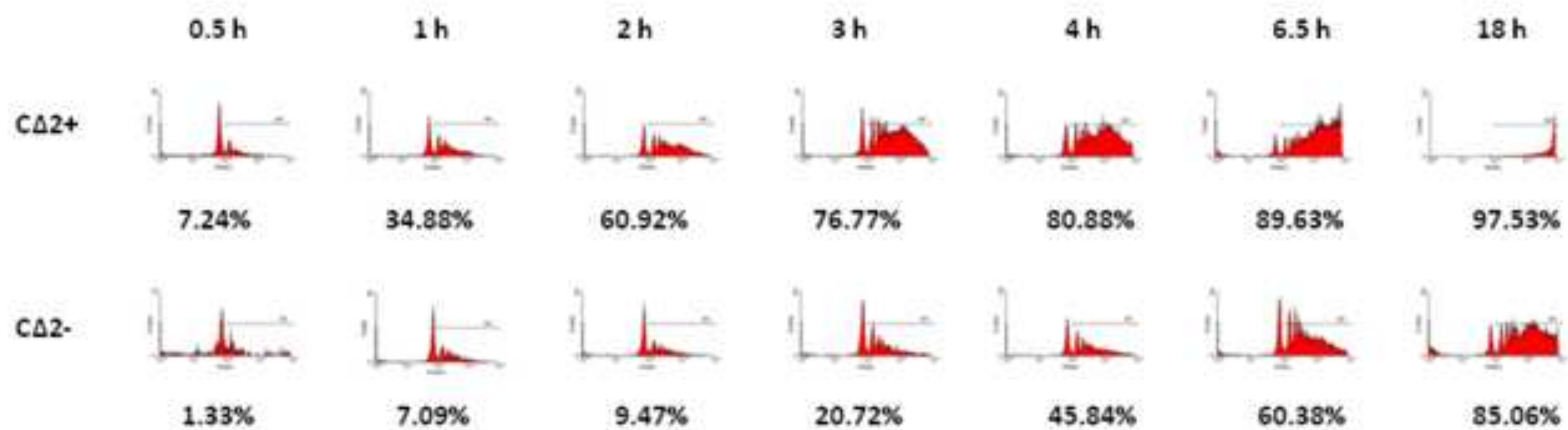

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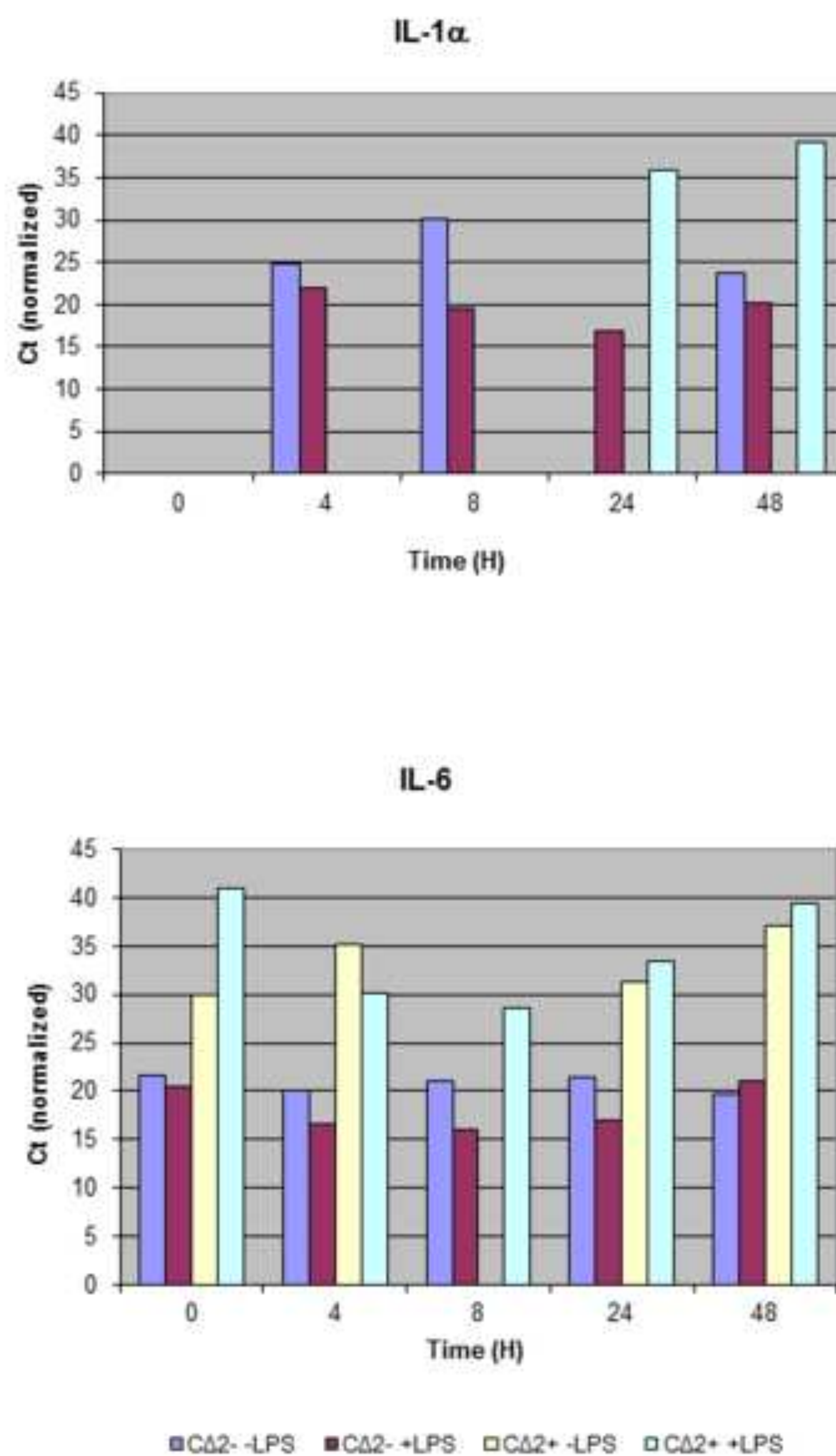

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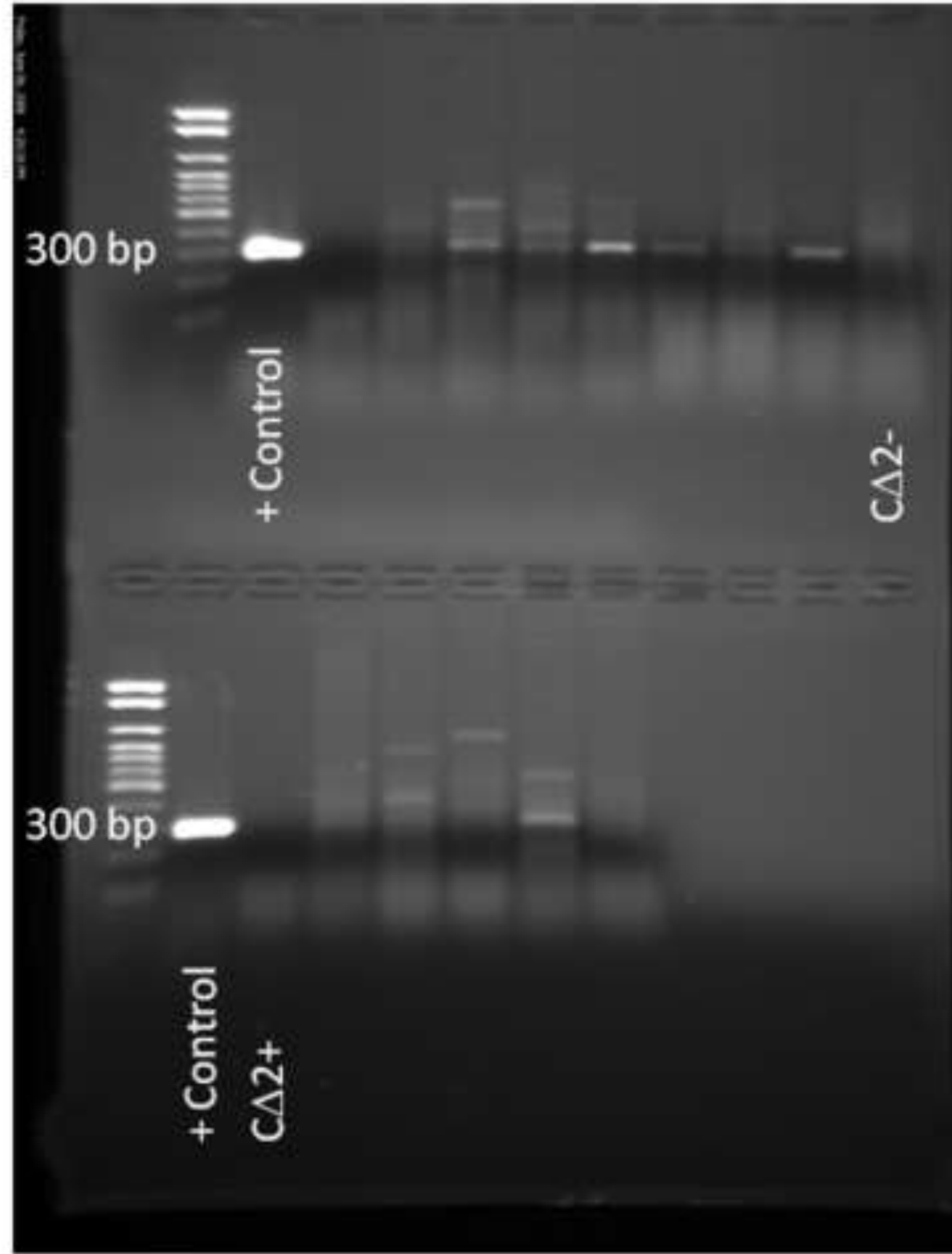

1 **Supplemental figure**. Fluorescence in situ hybridization to CΔ2- cell line of KAL1 (green) and
2 CSF2RA (red). The two red and green signals are together on the normal X chromosome, while
3 the CSF2RA region (red) has been translocated from the second X chromosome SSC X to the
4 smaller submetacentric chromosome.
5

Supplementary Information
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