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A Ca^{2+} concentration of 1.5 mM, as present in IMDM but not in RPMI, is critical for maximal response of Th cells to PMA/ionomycin

In the original activation of T lymphocytes, distinct cytokine genes and genes for other functionally relevant proteins are imprinted for reexpression upon secondary activation by antigen [1, 2]. To measure this imprinting independently of a particular antigen, T cells can be stimulated with PMA and ionomycin, mimicking TCR stimulation [3]. PMA directly activates protein kinase C and hence leads to the nuclear translocation of NF- κ B via phosphorylation of its antagonist I κ B [4–6]. Ionomycin induces increased cytoplasmic Ca^{2+} concentrations that activate calcineurin [7, 8]. Calcineurin dephosphorylates NFAT and thus enables its translocation to the nucleus [9–11]. NFAT, NF- κ B, and AP-1, which are also activated by PMA/ionomycin, induce the transcription of imprinted genes [1, 12, 13]. The nuclear translocation of NFAT occurs in an all-or-none fashion, due to the multiple dephosphorylation events required, a process mediated by the Ca^{2+} -dependent phosphatase calcineurin [11]. Thus, when a population of T cells is restimulated, calcineurin activity

determines the frequency of cytokine-producing cells in that population, rather than the amount of cytokine expressed per cell [14, 15].

When addressing the functional potential of Th cells in murine T cell transfer-induced colitis [16], we compared the frequencies of cytokine-producing mucosal Th cells, isolated *ex vivo* and restimulated with PMA and ionomycin in RPMI (Roswell Park Memorial Institute 1640) or in IMDM (Iscove's modified Dulbecco's medium). In IMDM, the frequencies of Th cells expressing IFN- γ , IL-17, IL-10, IL-22, or TNF were consistently higher than in RPMI. The difference in frequencies ranged from 1.4-fold for IFN- γ to more than threefold for TNF (Fig. 1 and Supporting Information Fig. 1). This effect was also observed for Th cells from spleen and mesenteric LNs of colitic mice (data not shown).

One major difference in the formulations of RPMI versus IMDM of potential relevance for PMA/ionomycin stimulation is the concentration of Ca^{2+} . While RPMI contains 0.42 mM Ca^{2+} , IMDM contains 1.49 mM. Indeed, supplementation of RPMI with Ca^{2+} to a total concentration of 1.5 mM was sufficient to trigger frequencies of cytokine-producing cells in RPMI comparable to IMDM (Fig. 1). Increasing the Ca^{2+} concentration of RPMI even further, from 1.5 to 2.5 and 3.5 mM, had no significant effect on the frequencies of IL-17- or IL-22-expressing Th cells (Fig. 2A). For IFN- γ , we observed a minor increase upon restimulation with 2.5 mM of Ca^{2+} compared with that at 1.5 mM (Fig. 2A, middle). Taken together, a Ca^{2+} concentration of the medium of at least 1.5 mM reveals the maximal frequencies

of cytokine-expressing Th cells upon PMA/ionomycin stimulation.

Conversely, lowering the concentration of available Ca^{2+} of IMDM by the Ca/Mg-chelating reagent EDTA, resulted in a dose-dependent reduction in the frequencies of cytokine-expressing cells (Fig. 2B). Since EDTA binds Ca^{2+} in an equimolar fashion and with 100 \times higher affinity than Mg^{2+} [17], 1 mM of EDTA reduced the concentration of available Ca^{2+} from 1.49 mM to approximately 0.5 mM, comparable to the Ca^{2+} concentration of RPMI ($[\text{Ca}^{2+}] = 0.42$ mM). Indeed, addition of 1.0 mM EDTA to IMDM reduced the frequencies of cytokine-producing cells to those obtained in RPMI, as shown for IL-17, IFN- γ , and IL-22 in Figure 2B.

Maximizing the PMA/ionomycin stimulation by Ca^{2+} supplementation of RPMI had minimal impact on the viability of the activated Th cells, nor did it change the kinetics of cytokine expression. The viability of the Th cells 4 h after onset of PMA/ionomycin treatment was 78.6 ± 1.8 in RPMI, 76.7 ± 1.8 in IMDM, and 77.3 ± 2.1 in CaCl_2 -supplemented RPMI (data not shown). The frequencies of cytokine-expressing Th cells differed between media, but were comparable at 4, 6, and 8 h after onset of each stimulation, as shown for IL-17-, IFN- γ -, and IL-22-expressing Th cells in Figure 2C. In particular, the frequencies of cytokine-producing Th cells in RPMI did not increase at 6 or 8 h to frequencies in IMDM or in CaCl_2 -supplemented RPMI (Fig. 2C).

We show here that conventional RPMI, routinely used for the activation of Th lymphocytes with PMA and ionomycin, contains too little Ca^{2+} for maximal ionomycin stimulation. A significant underestimation

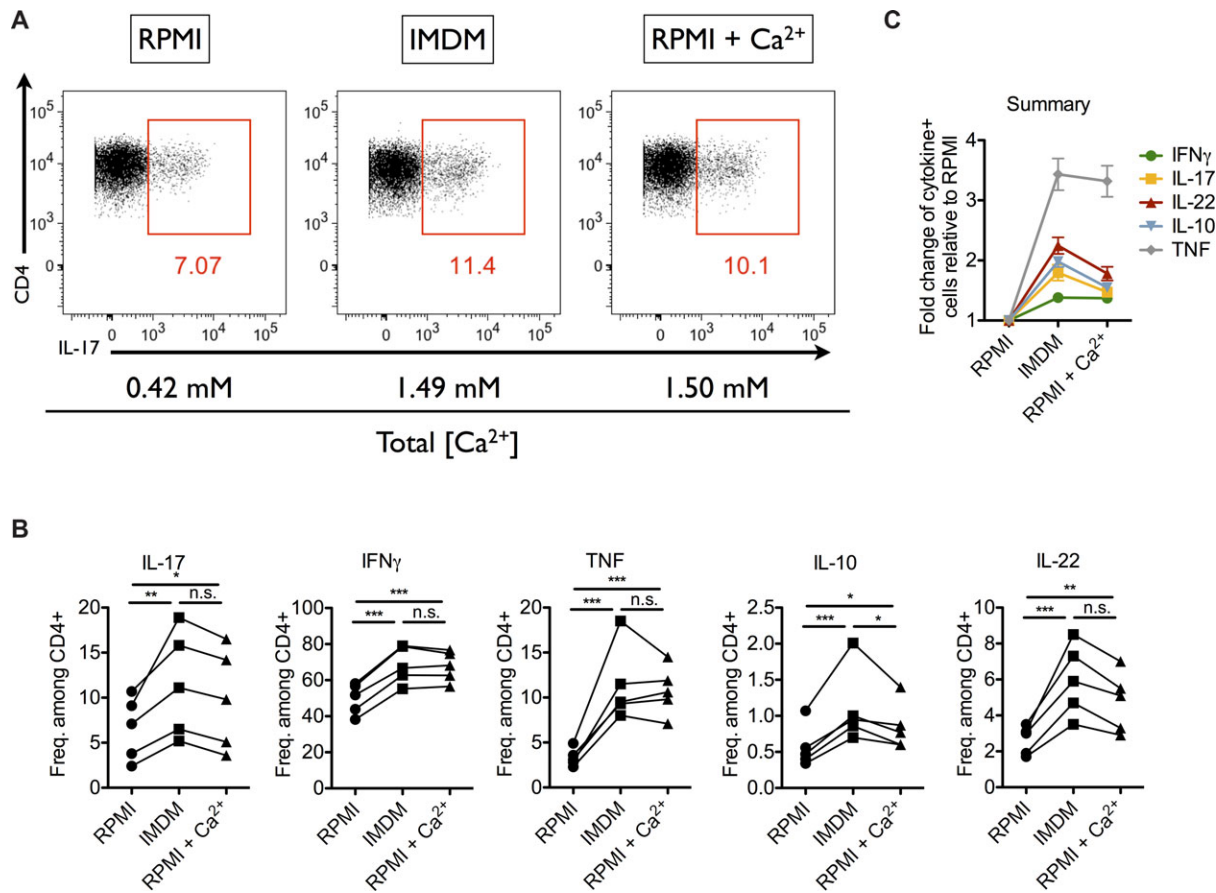


Figure 1. Frequencies of cytokine-expressing Th cells upon restimulation with PMA/ionomycin in RPMI, IMDM, and Ca²⁺-supplemented RPMI. Small intestine lamina propria (SI LP) cells of colitic mice were stimulated with PMA/ionomycin in RPMI, IMDM, or Ca²⁺-supplemented RPMI. (A) Intracellular staining for IL-17 in SI LP Th cells. (B) Frequencies of IL-17, IFN-γ, TNF, IL-10, and IL-22-expressing cells among SI LP Th cells stimulated in RPMI, IMDM, and Ca²⁺-supplemented RPMI, as determined by flow cytometry. Lines represent cells from individual mice. (C) Fold change in frequencies of cytokine-expressing cells, stimulated in IMDM or Ca²⁺-supplemented RPMI compared to RPMI, as determined by flow cytometry. Data are shown as mean ± SEM, *n* = 5 mice/group and are from one experiment representative of four independent experiments with similar results. (B) **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 by one-way ANOVA for repeated measurements and Tukey's post hoc test.

of antigen-experienced Th cells imprinted for expression of distinct cytokines is the consequence, as demonstrated here for Th cells isolated ex vivo from inflamed tissue or secondary lymphoid organs of mice with colitis. Increasing the Ca²⁺ concentration of RPMI from the regular 0.42 to ≥1.5 mM is sufficient to correct this effect and obtain a more accurate estimation of the functional potential of polyclonal Th cell populations.

Methods

Mice

Specific pathogen free C57BL/6J and C57BL/6 *Rag1*^{-/-} mice were obtained

from Charles River (Sulzfeld, Germany). All animal experiments were performed in accordance with institutional, state, and federal guidelines.

T cell transfer colitis

Colitis was induced as described before [16]. Briefly, CD4⁺ T cells from spleen and LNs of C57BL/6J donors were purified by MACS using mouse CD4 microbeads (L3T4, Miltenyi Biotec). Viable CD4⁺CD45RB^{hi}CD25⁻ cells were isolated by FACS. A total of 4 × 10⁵ cells were injected i.v. into each of the C57BL/6 *Rag1*^{-/-} recipients. Mice were sacrificed 2–3 weeks after transfer, when signs of diarrhea and weight loss became apparent.

Isolation of lamina propria (LP) mononuclear cells

LP mononuclear cells were isolated from colon and small intestine (SI) as described before [18]. In brief, fat was removed from colon and SI. They were then opened longitudinally and washed with PBS. The epithelial layer was stripped off by two rounds of incubation in calcium/magnesium-free HBSS with 5 mM EDTA and 10 mM HEPES for 20 min at 37°C with 100 RPM shaking. To obtain a single cell suspension of the LP, colons and SIs were minced into small pieces and incubated three times for 20 min with 0.5 mg/mL Collagenase D (Roche), 0.5 mg/mL DNase I (Sigma), and 0.05 U/mL Dispase (BD)

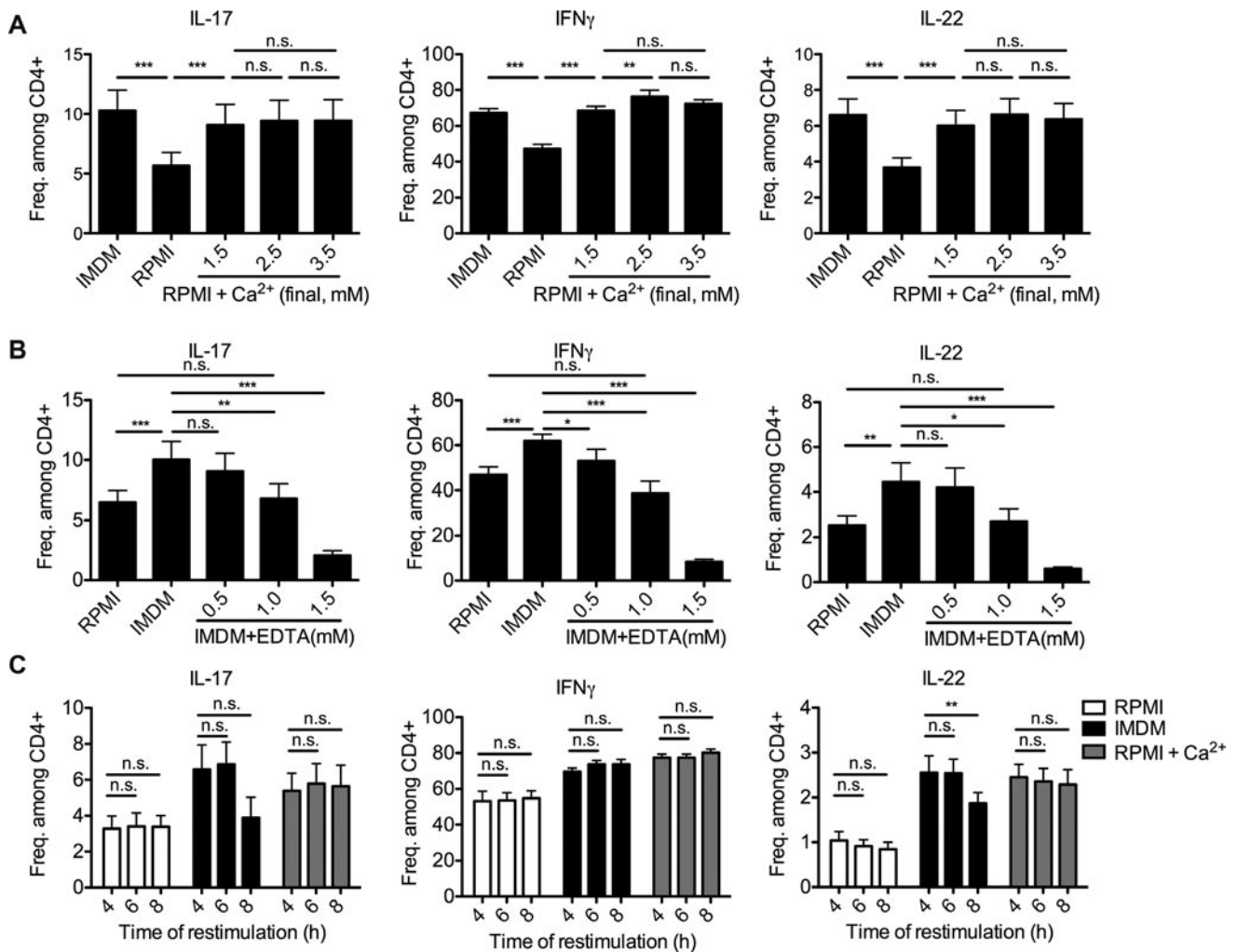


Figure 2. Quantitative and kinetic analysis of cytokine expression of Th cells restimulated with PMA/ionomycin in different Ca²⁺ concentrations. (A, B) SI LP cells of colitic mice were stimulated with PMA/ionomycin in IMDM, RPMI, or (A) Ca²⁺-supplemented RPMI or (B) IMDM supplemented with EDTA. (A) The frequencies of Th cells expressing IL-17, IFN- γ , and IL-22 were measured by flow cytometry. Data are shown as mean + SEM of 9 mice/group, pooled from two independent experiments. (B) The frequencies of Th cells expressing IL-17, IFN- γ , and IL-22 were measured by flow cytometry. Data are shown as mean + SEM of 9 mice/group, pooled from two independent experiments. (C) LP Th cells of colitic mice were stimulated in RPMI, IMDM, or Ca²⁺-supplemented RPMI for 4, 6, or 8 h. The frequencies of Th cells expressing IL-17, IFN- γ , and IL-22 were measured by flow cytometry. Data are shown as mean + SEM of 4 mice/group, from one experiment. (A–C) **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 by one-way ANOVA for repeated measurements and Tukey's post hoc test.

in calcium/magnesium-containing HBSS with 10 mM HEPES at 37°C with 100 RPM shaking. For the SI, LP mononuclear cells were separated from debris by centrifugation over a Percoll gradient (GE Healthcare).

Restimulation and flow cytometry

Ca²⁺-supplemented RPMI was obtained by adding 1.08 mM of CaCl₂ to

RPMI ([Ca²⁺_{final}] = 1.5 mM). IMDM ([Ca²⁺] = 1.49 mM), RPMI (Life Technologies, [Ca²⁺] = 0.42 mM), and Ca²⁺-supplemented RPMI ([Ca²⁺] = 1.50 mM) were supplemented with 10% FCS and contained 25 mM HEPES. For intracellular cytokine staining, 1–2 × 10⁶ cells were restimulated with 10 ng/mL PMA (Sigma) and 1 μg/mL ionomycin (Santa Cruz) in the respective media at 5 × 10⁶–1 × 10⁷ cells/mL for a total of 4 h. After 1 h, brefeldin A (BioLegend)

was added to a final concentration of 5 μg/mL. Following washing with PBS, cells were stained with a fixable live/dead staining (pacific orange succinimidyl ester, Life Technologies) for 20 min on ice. After 20 min fixation using the BD Cytofix/Cytoperm buffer, cells were washed with and stained in 0.5% w/v Saponin (Sigma) for 20 min on ice. A total of 1–2 × 10⁵ cells were measured with a FACSCanto II (BD).

Antibodies

Epitope	Color	Clone	Manufacturer
CD3	Allophycocyanin-eFluor 780	145–2C11	eBioscience
CD4	Pe-Cy7	RM4–5	eBioscience
IFN- γ	PerCP-Cy5.5	XMG1.2	eBioscience
IL-17	FITC	TC11–18H10	BioLegend
IL-10	Pe	JES5–16e3	eBioscience
IL-22	Allophycocyanin	IL22JOP	eBioscience
TNF	AlexaFluor405	MP6–XT22	Custom

Data presentation and statistics

Individual data points refer to Th cells isolated from individual colitic mice and are presented as mean + SEM unless stated otherwise. Multiple comparisons were tested for significant differences by one-way ANOVA for repeated measurements followed by Tukey's post hoc test with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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Abbreviations: IMDM: Iscove's modified Dulbecco's medium · LP: lamina propria · RPMI: Roswell Park Memorial Institute 1640 · SI: small intestine

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The detailed *Materials and methods* for Technical comments are available online in the Supporting information