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Polyunsaturated fatty acids in cod evoke chemotaxis and mobilize intracellular calcium in human eosinophils in part *via* FFAR4

To the Editor

The eosinophilic granulocyte is a nearly ubiquitous feature of allergies. This tissue-bound cell has a predilection for the gastrointestinal tract, where the first encounter between food antigens and the immune system normally occurs. Why the cell is involved in the misdirected immune reactivity to innocuous inhaled environmental antigens or ingested foods is unknown. The purpose of this study was to determine if cod could directly activate human eosinophils to migrate and/or evoke intracellular calcium fluxes, and to elucidate the receptors and signaling pathways employed by cod. The capacity of eosinophils to migrate and mobilize intracellular calcium in response to cod, the identity of the bioactive substances in cod, and the signal pathways and receptors involved, were studied using blood eosinophils derived from healthy, non-allergic individuals.

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Cod was found to be an eosinophil chemoattractant, giving rise to mobilization of intracellular calcium and eosinophil migration (Sup. Fig. 1). Moreover, cod completely desensitized the effects of the eosinophil chemoattractants fMLF, CCL11, and PGD_2 (Sup. Fig 2), but was not desensitized by these same chemoattractants, suggestive of common intracellular pathways but separate receptors (Sup. Fig 3). The major fish allergen, parvalbumin, was not responsible for eosinophil migration. Boiling or proteinase digestion of the cod extract only had a minimal effect on its capacity to evoke eosinophil calcium mobilization and migration (Sup. Fig. 4). This indicated that the bioactive substances were not likely to be proteins. Instead we investigated the ability of fatty acids of various chain length present in cod to evoke calcium transients in eosinophils. The polyunsaturated acids of the *n*-6 series arachidonic acid (AA), as well as the *n*-3 series eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), were strong activators of intracellular calcium transients in human eosinophils (Fig. 1A).

We next investigated the ability of eosinophils to migrate towards AA, EPA and DHA. AA was a particularly strong chemoattractant, triggering migration of >65% of the eosinophils, double that of cod (Fig. 1B). EPA triggered a migratory response of 35% similar to cod (Fig. 1B). In contrast, only a discrete migratory response of 3% was elicited by DHA (Fig. 1B). The next step was to see if these long-chain fatty acids could abolish or diminish the chemotactic response of eosinophils to cod. It was found that eosinophils preincubated with cod were unable to migrate towards AA and EPA but no such desensitizing effect was seen when eosinophils were preincubated with either AA or EPA and then exposed to cod. As expected, cod could desensitize itself regarding migratory capacity (Fig. 1C). The interpretation of these results was that the fatty acids with chemoattractant activity present in cod acted *via* more than one receptor on human eosinophils.

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To test the hypothesis of the existence of more than one receptor, we proceeded to block receptors used by long-chain fatty acids, the free fatty acid receptors-1 and -4 (FFAR1 and FFAR4). The receptor agonist GW9508, which binds to both FFAR1 and FFAR4, was tested. Pretreatment of eosinophils with GW9508 almost entirely abolished calcium responses engendered by cod (Fig. 2A). These results were indeed specific and not due to toxic effects of the receptor agonists on the eosinophils, as shown by staining eosinophils with the viability stain 7AAD (Sup. Fig. 5). Blocking these receptors not only decreased the ability of cod to evoke calcium fluxes in eosinophils, but also substantially diminished the chemotactic response of eosinophils to cod and EPA, but less so to AA (Fig. 2B). AA and its metabolites presumably act via other receptors such as the OXE-receptors.[1] In view of the recent report that human eosinophils mainly express FFAR4 (mRNA as well as protein) but no or only negligible amounts of FFAR1, we conclude that the cod-mediated activation occurred chiefly through FFAR4 and not FFAR1.[2]

Besides being a putative food allergen, fish is also considered to be part of a healthy food diet. Our findings that fish fatty acids strongly activate human eosinophils, the prototype cells of allergic reactions may seem incompatible with the notion of fish being a healthy food. However, it is far from clear what function eosinophils have in food allergies. In fact, a series of studies indicate that eosinophils may have immune suppressive and anti-inflammatory functions. We have previously reported that eosinophils can inhibit T cell proliferation.[3] An anti-inflammatory mechanism that is pertinent to this study is the ability of eosinophils to convert EPA to resolvins and DHA to protectins, lipid mediators which are key to the resolution of inflammatory processes.[4] Eosinophils have this ability thanks to the 12/15-LOX enzymatic system,[5] and one theory holds that if this enzymatic function is not working properly, it may result in severe allergic asthma.[6] On the other hand it has also been reported

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that eosinophils incubated with AA produce eoxin (EX)C4 rather than leukotriene (LT)C4, EXC4 is an eosinophil metabolite that can contribute to a number of proinflammatory processes.[7]

There are contradictory reports as to whether omega-3 fatty acids are beneficial to human health. One study found that high levels of EPA in serum was associated with a lowered risk of allergy[8], whereas another study reported that high levels of fatty acids in cord blood was coupled to an increased risk of allergy.[9] In addition, studies have questioned the benefits of eating fish or fatty acid supplements during pregnancy and the subsequent likelihood of the child to become allergic.[10, 11] Whether AA and EPA and other cod-derived fatty acids result in eosinophil-mediated reactivity that is beneficial or not to the host cannot be answered by this study, but our findings indicate that human eosinophils are highly reactive to the long-chain fatty acids contained in healthy foods such as fish.

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Short title: Fatty acids from fish activate eosinophils

Conflict of interest

The authors declare no conflicts of interest.

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Author contributions

C.L. participated in the performance and design of the experiments and the analysis, as well as the writing of the manuscript. E.R and K.A participated in the performance and design of the experiments and the analysis. C.W. participated in the design of the experiments and the writing of the manuscript and supervised the entire study.

Ethical approvement

This study was approved by the Regional Ethics Committee in Gothenburg.

Correspondence

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Figure legends

Figure 1. (A) Intracellular calcium release in eosinophils evoked by AA, EPA and DHA. Calcium fluxes were measured by loading cells with the two calcium-binding fluorochromes Fluo-3 and Fura red and continuous measurement of emitted light by using flow cytometry. The ratio of the two fluorochromes is indicated on the Y-axis, time on the X-axis. One representative experiment is shown (n=6-16). (B) Chemotaxis of eosinophils towards cod, AA, EPA and DHA (n=6). (C) Chemotaxis of eosinophils prestimulated by AA and EPA and then exposed to cod ,as well as, eosinophils prestimulated by cod and then exposed to AA and EPA (n=4).

Figure 2. (A) Intracellular calcium release in eosinophils preincubated with either medium, the FFAR1 and FFAR4 agonist GW9508 and then exposed to cod (n=2). Calcium fluxes were measured by loading cells with the two calcium-binding fluorochromes Fluo-3 and Fura red and continuous measurement of emitted light by using flow cytometry. The ratio of the two fluorochromes is indicated on the Y-axis, time on the X-axis. (B) Chemotaxis of eosinophils prestimulated with either medium or the FFAR1 and FFAR4 agonist GW9508 and then exposed to cod, EPA or AA (n=2-4).

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Fig. 1

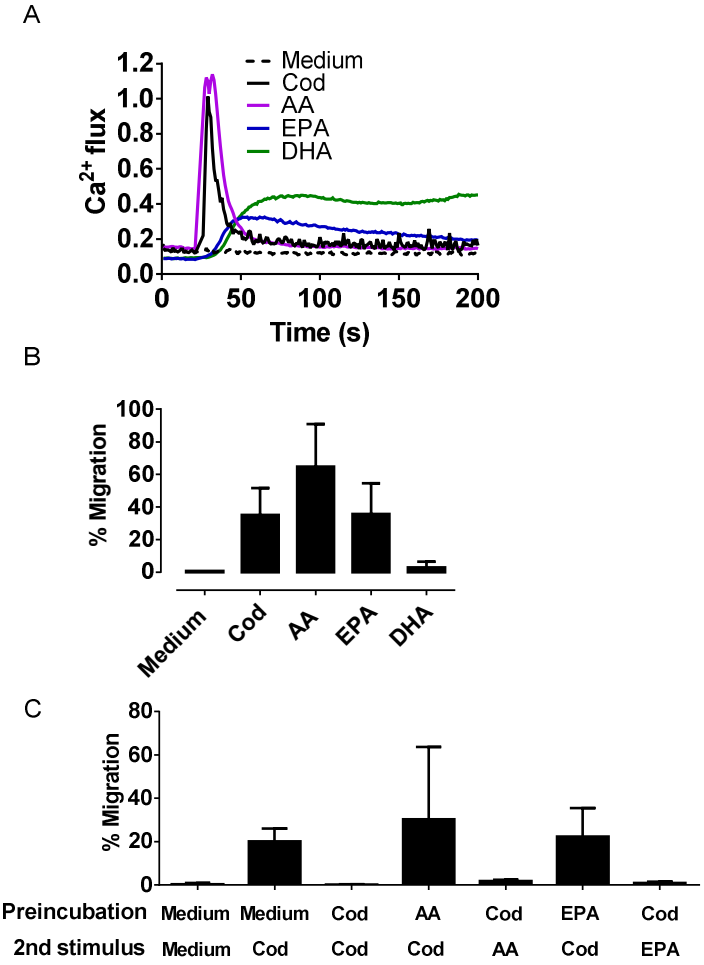

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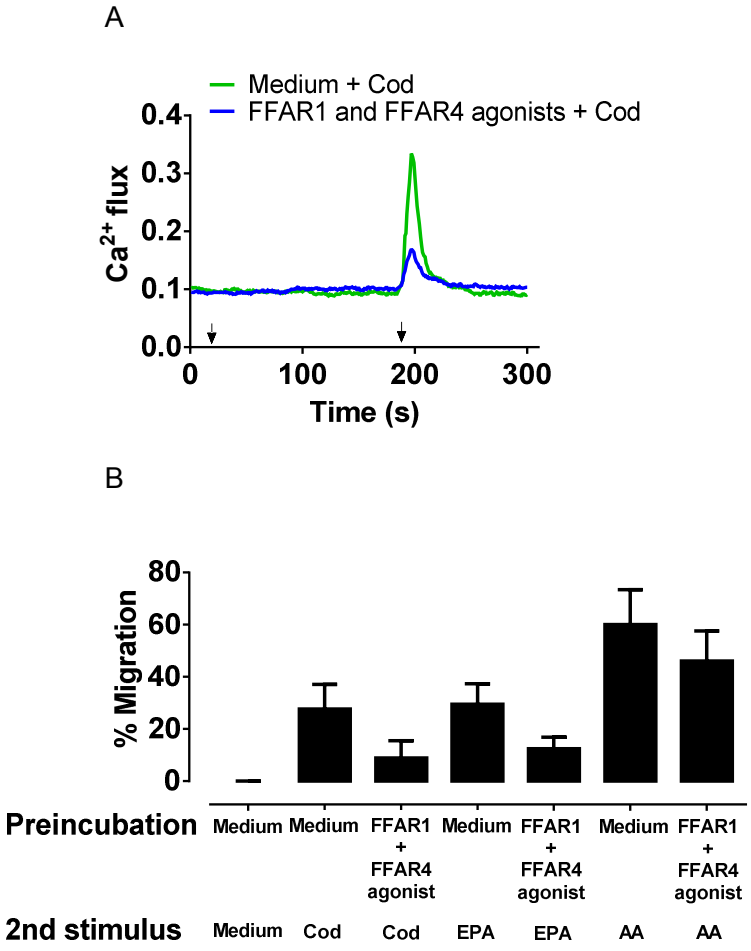
Fig. 2



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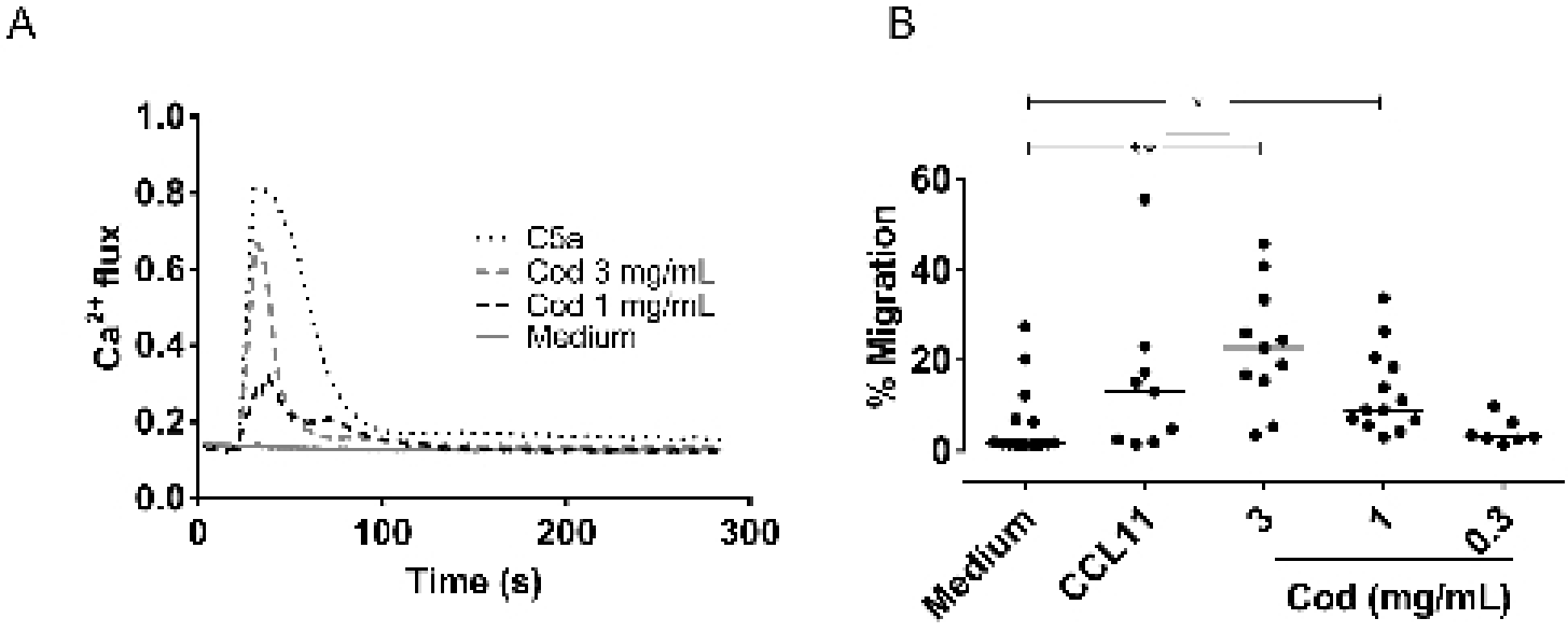
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Cod 3 mg/mL
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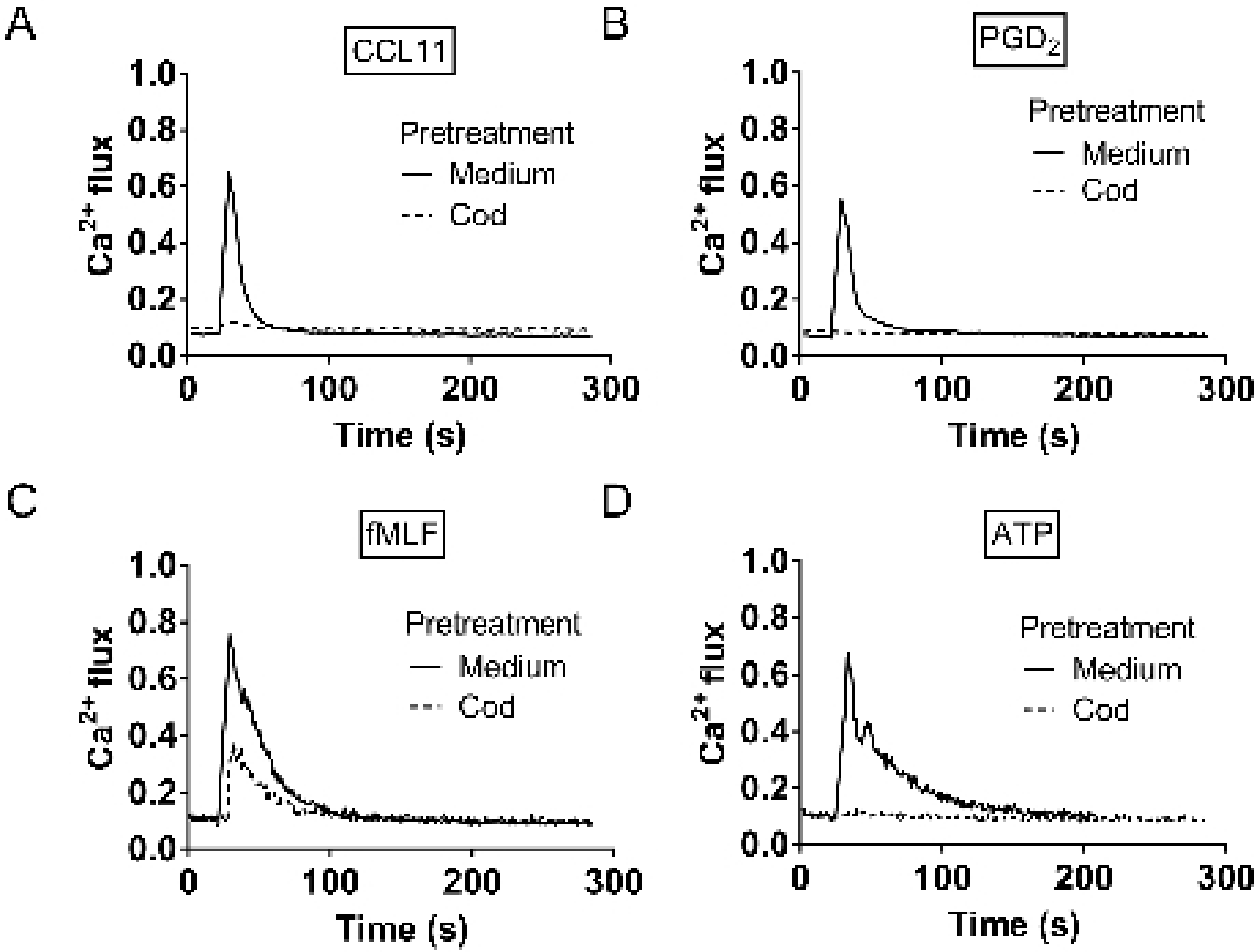
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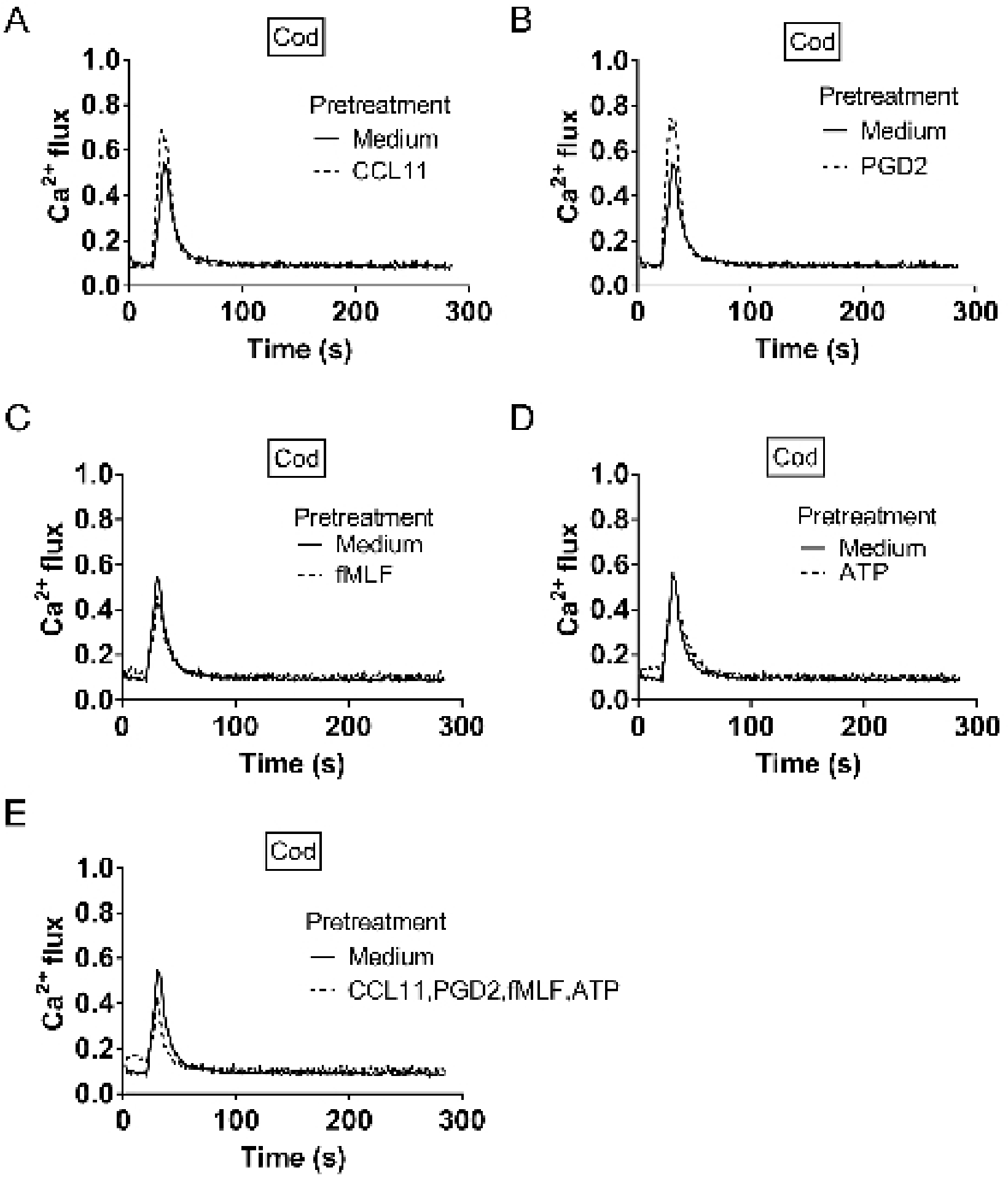
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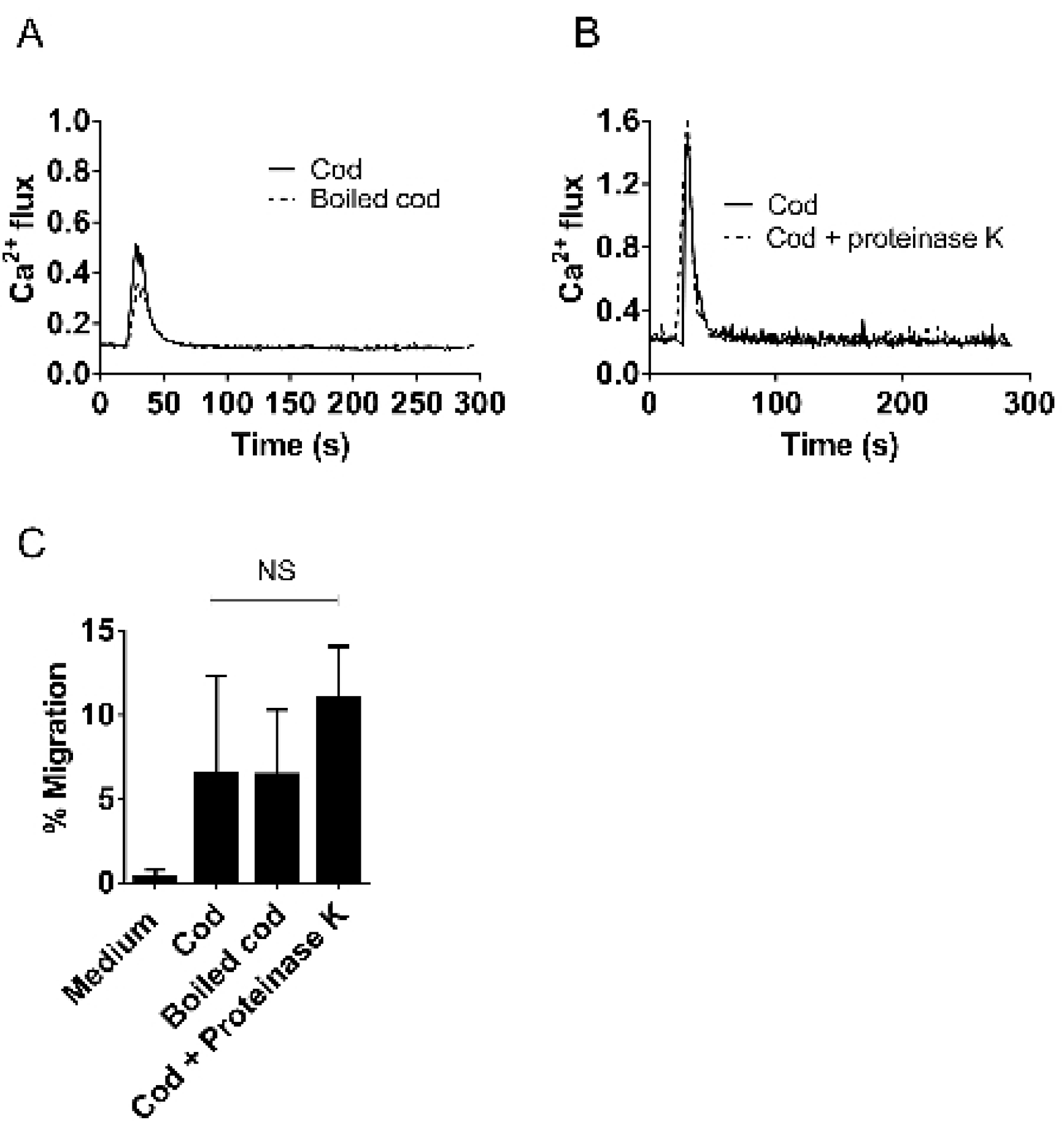
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Time (s)
B
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Cod + proteinase K
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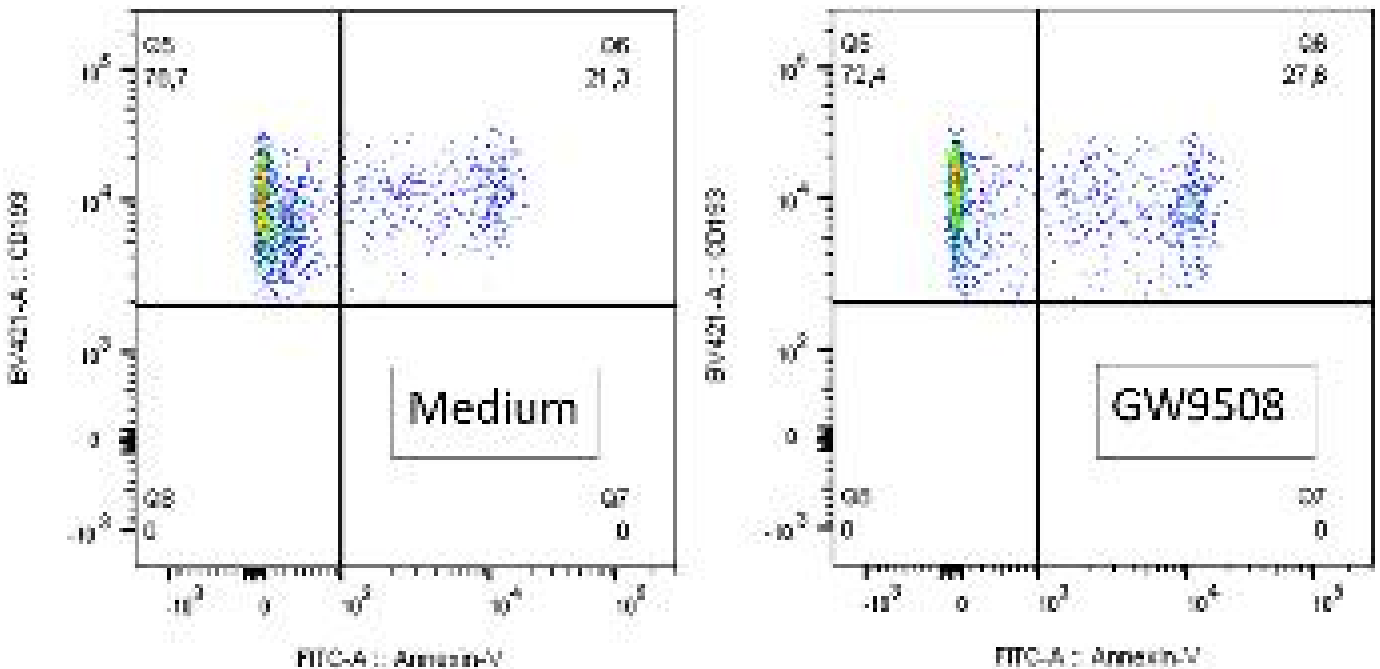
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Q6
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Q8
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Q7
0
GW9508
BV421-A :: CD193
FITC-A :: Annexin-V

Supplementary material

Materials and methods

Food allergen extracts

Extract from Atlantic cod (*Gadus morhua*) was reconstituted (ThermoFisher, Eugene, OR, USA) in PBS to 3 mg/mL. The protein content of the cod extract was determined by photometric absorbance (NanoDrop Technologies, Inc., Wilmington, DE, USA) based on a standard curve using BSA (Fluka, Buchs, Switzerland).

Reagents

C5a, prostaglandin D_2, fMLF, ATP, proteinase K (Sigma-Aldrich, Stockholm, Sweden), CCL11 (eotaxin-1) (R&D Systems, Minneapolis, MN, USA), parvalbumin (Innovest AS, Medilab, Haukeland University Hospital, Bergen, Norway), GW9508, DHA, EPA, ALA (Cayman Chemical Company, Ann Arbor, MI, USA) and Pefabloc (Roche Applied Science, Mannheim, Germany).

Eosinophil purification

Eosinophils were freshly isolated from heparinized venous blood from healthy volunteers by negative immune depletion of CD16-expressing granulocytes as previously described.[20] Exclusion criteria were any past or current allergic conditions. Eosinophils were routinely > 97% pure as determined by Diff-quick staining (Dade Behring AG, Eschborn, Germany) and > 99% viable as determined by Trypan blue exclusion.

Chemotaxis

Eosinophil migration was determined using 30 µL-volume 96-well cell migration plates covered by a hydrophobic filter with 3 µm-pores (ChemoTx; Neuro Probe Inc., Gaithersburg, MD) as previously described.[21] In short, 30,000 eosinophils were allowed to migrate towards the tested stimuli and positive control CCL11 [10^{-7} M] for 90 minutes at 37° C. For the desensitization and blocking experiments, the cells were preincubated with medium, cod

extract, fatty acids [$5x10^{-5}$ M] or FFAR1 and FFAR4 agonist GW9508 on ice for 10 minutes prior to the cells being loaded onto the filter. All cells and stimuli were diluted in KRG buffer with 0.3% BSA. Eosinophils were stained with CD193 and 7AAD and analyzed with a FACS Canto II flow cytometer (BD Bioscience) and FACS Diva Software and were analyzed with FlowJo software (TreeStar) for viability evaluation.

Measurement of intracellular calcium transients

Intracellular calcium mobilization was measured as previously described.[20] Six million eosinophils were stained with Fluo-3 AM (4 μg/mL) and Fura Red AM (10 μg/mL) (ThermoFisher) for 30 min at 37° C in cell-loading medium (Krebs Ringer glucose with 1% FCS). Excess dye was washed away and the cells were placed on ice. Five minutes prior to analysis, the cells were preincubated with medium, stimulus or antagonists at 37° C and a baseline was established in the flow cytometer FACSCalibur (BD Biosciences, San Jose, CA, USA).

Digestion of proteins in cod extract

Cod extract (3 mg/mL) was heated to 100°C for 5 min or digested by exposure to Proteinase K (Fermentas International Inc., Burlington, Canada) for 10 min at 56°C. Proteinase K was first adjusted to pH 8.0 with Tris buffer and then added to the lyophilized cod, before the mixture was adjusted to pH 7.0 by the addition of HCl. The enzymatic reaction was stopped by addition of Pefabloc (Sigma-Aldrich).

Figures

Supplementary Figure 1. (A) Cod evokes intracellular calcium release in eosinophils. Calcium fluxes were measured by loading cells with the two calcium-binding fluorochromes Fluo-3 and Fura red and continuous measurement of emitted light by using flow cytometry. The ratio of the two fluorochromes is indicated on the Y-axis, time on the X-axis. Two concentrations of cod were tested (n=8). One representative experiment is shown. (B)

Chemotaxis of eosinophils towards cod. Each dot represents the median of triplicates from one individual. n = 7-13. Solid lines indicate the median for each group, the dotted line depicts the median spontaneous migration of eosinophils towards medium. CCL11 (eotaxin-1) at [10^{-7} M] was used as a positive control. * = $P < 0.05$, **= $P < 0.01$. One representative experiment is shown.

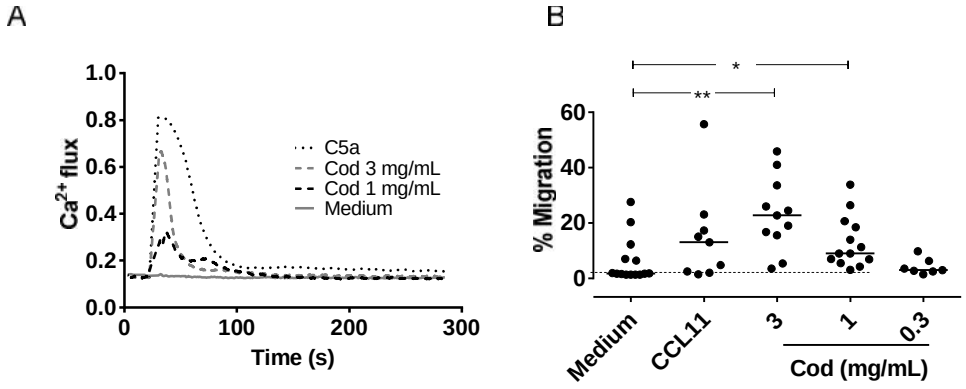
Supplementary Figure 2. Desensitization of intracellular calcium release evoked by cod in human eosinophils. Calcium release is inhibited in eosinophils prestimulated with cod upon addition of the second stimulus. Eosinophils were prestimulated with either medium or cod and thereafter exposed to (A) CCL11 (eotaxin-1), (B) PGD_2, (C) fMLF or (D) ATP. Calcium fluxes were measured by loading cells with the two calcium-binding fluorochromes Fluo-3 and Fura red and continuous measurement of emitted light by using flow cytometry. The ratio of the two fluorochromes is indicated on the Y-axis, time on the X-axis (n = 3-6).

Supplementary Figure 3. Eosinophils resistant to desensitization of intracellular calcium release when prestimulated with (A) CCL11 (eotaxin-1), (B) PGD_2, (C) fMLF, (D) ATP or (E) a mixture of all the stimuli and thereafter exposed to cod (n=2). Calcium fluxes were measured by loading cells with the two calcium-binding fluorochromes Fluo-3 and Fura red and continuous measurement of emitted light by using flow cytometry. The ratio of the two fluorochromes is indicated on the Y-axis, time on the X-axis (n = 3-8).

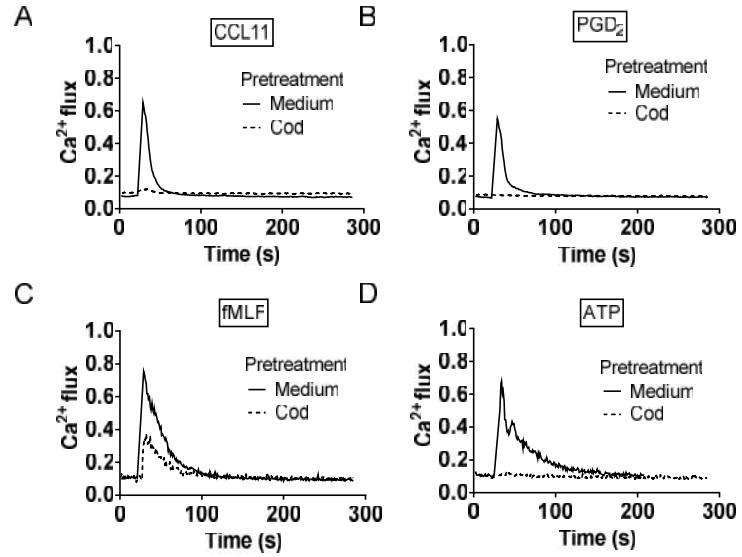
Supplementary Figure 4. Intracellular calcium release in eosinophils evoked by cod after (A) boiling or (B) proteinase digestion (n=2). (C) Chemotaxis of eosinophils towards cod after boiling or proteinase digestion (n=2). Calcium fluxes were measured by loading cells with the two calcium-binding fluorochromes Fluo-3 and Fura red and continuous measurement of emitted light by using flow cytometry. The ratio of the two fluorochromes is indicated on the Y-axis, time on the X-axis.

Supplementary Figure 5. Flow cytometry analysis showing a representative dotplot demonstrating eosinophils expressing CD193 and 7AAD.

Supplementary Fig. 1



Supplementary Fig. 2



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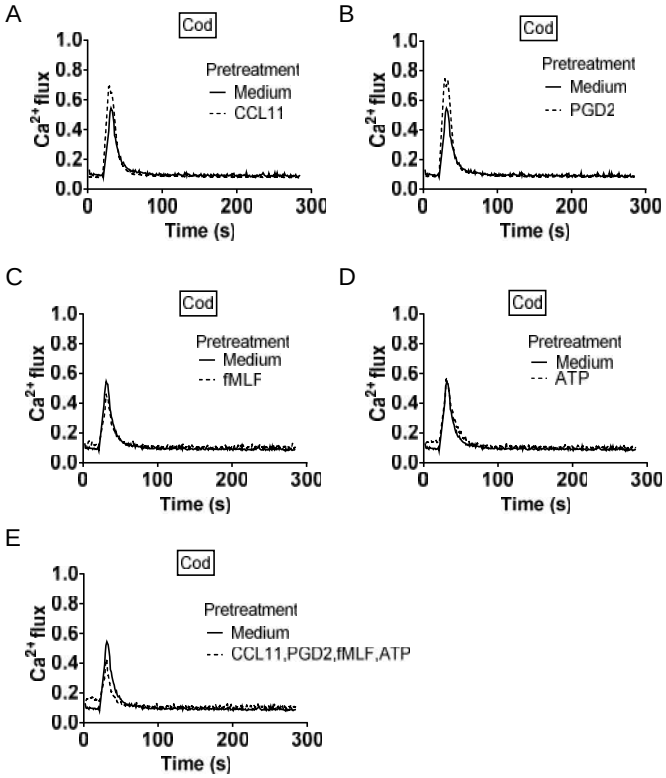

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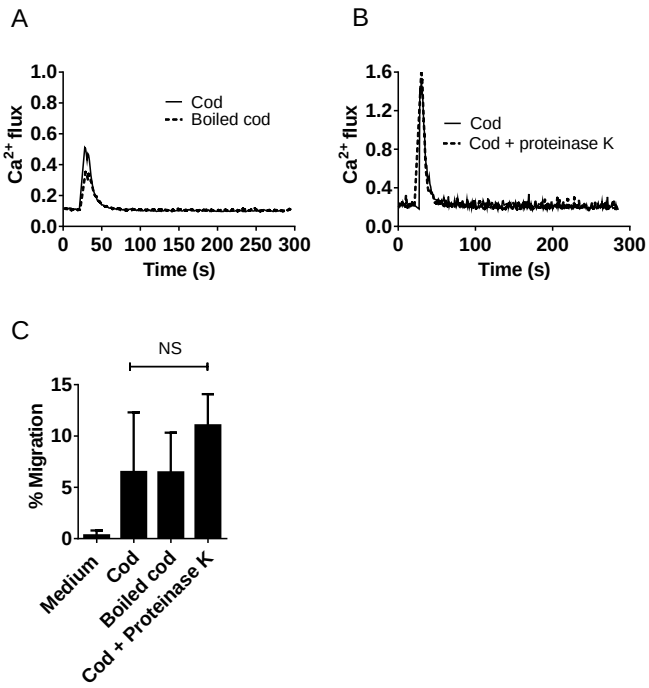

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96 **Supplementary Fig. 5**

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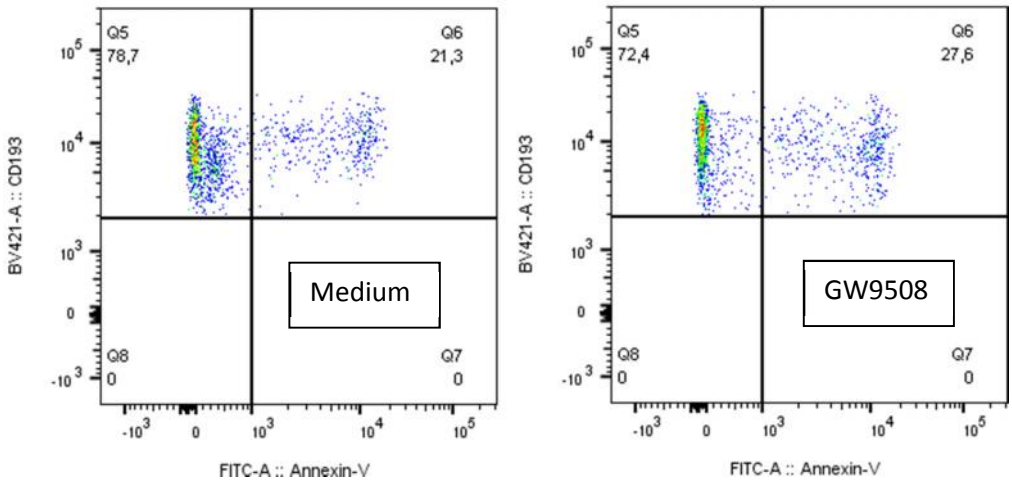
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