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# Cell-specific immunomodulatory effects of snake venom-derived thrombin-like enzyme ancrod

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***Abstract***

*Systemic inflammatory response syndrome (SIRS) is recognized to be an exaggerated defense response to various stressors, including trauma. CD4$^+$ T-regulatory cells (CD4$^+$ Tregs) are key mediators in balancing inflammatory processes. Recent findings demonstrated that platelets and CD4$^+$ Tregs interact after injury and SIRS. Therapeutic strategies to modulate the activation of these and other immune cells in SIRS are currently lacking.*

*Ancrod has immunomodulatory effects on CD4$^+$ Tregs that could be beneficial in the treatment of SIRS. Therefore, we studied the impact of ancrod on activation levels of CD4$^+$ Tregs and CD4$^+$ non-Tregs, platelets, and antigen-presenting cells (APC) in vitro and in vivo. We tested the in vitro effect of ancrod (0-15 IU) and the in vivo effect of 8 IU ancrod vs. saline. After collection of spleens and platelet-rich plasma of male C57Bl/6N mice, cells were isolated and incubated with ancrod for 2 hours. Furthermore, we tested the effect of stimulation. CD4$^+$ Tregs, CD4$^+$ non-Tregs, APC and platelet activation were analyzed by flow cytometry.*

*Our results demonstrate that ancrod exerts selective effects on different cell populations. Ancrod affects the adaptive and innate immune responses. Furthermore, we identified a differential effect of ancrod on CD4$^+$ Tregs versus CD4$^+$ non-Tregs depending on the mode of cellular stimulation. Additionally, our findings suggest a dose-dependent role of ancrod in the modulation of platelet activation. Our findings provide the first evidence supporting the potential of ancrod in selectively modulating immune cells, highlighting ancrod as a promising candidate for further investigation.*

***Key words:*** *SIRS, CD4$^+$ regulatory T cells, platelets, immunomodulation, ancrod, CD4$^+$/CD8$^+$ cells, antigen-presenting cells.*

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## Introduction

Trauma-induced injury is still the leading cause of death for young patients up to the age of 45 years [https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/ghe-leading-causes-of-death]. These trauma-induced injuries possess the potential to lead to pro- and anti-inflammatory host responses. The two-hit model of injury, first proposed by Moore and Moore in 1993, describes injury itself as the first hit to the patient and surgical interventions or concomitant infections as the second hit [1]. The proinflammatory reaction following trauma has been determined as the systemic inflammatory response syndrome (SIRS), which can facilitate concomitant infections and lead to potentially fatal sepsis [2]. About 33% to 50% of hospital deaths are due to sepsis [3]. Matzinger and coworkers developed the 'danger hypothesis', which implies that after infection or injury, there is an increased release of alarm signals called alarmins [4]. These alarmins are referred to as either damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) [5]. According to this hypothesis, inflammation is mediated by pattern recognition receptors (PRRs) on cells of the innate and adaptive immune system [6].

Anti-inflammatory reactions are termed compensatory anti-inflammatory response syndrome (CARS), which can help resolve SIRS and prevent further damage such as multiple organ dysfunction syndrome (MODS). The adaptive immune system has been reported to be the most important part of CARS [7, 8]. CD4$^+$ T regulatory cells (CD4$^+$ Tregs)

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are suppressors of T cell activation [9], which are known to play a central role in autoimmune reactivity and disease. These suppressive and autoreactive qualities of $CD4^+$ Tregs led researchers to the idea of using this cell type as a potential immunomodulator after trauma and tissue injury [10]. The role of $CD4^+$ Tregs in trauma remains controversial. In a mouse model of CLP-challenged mice, the adoptive transfer of $CD4^+$ Tregs provided protective effects [11]. The hypothesis of immunomodulatory and thus protective $CD4^+$ Tregs was strengthened by studies with septic mice and patients [12, 13]. In addition, recently it was underlined that $CD4^+$ Tregs contribute to a positive outcome after early-phase sepsis, but the study does not support a significant role of $CD4^+$ Tregs in immune paralysis during late-phase sepsis [14].

We were able to determine the location and kinetics of $CD4^+$ Treg activation following injury [15] and showed that $CD4^+$ Tregs are activated early following trauma in local lymph nodes draining the injury site in a murine model of burn injury [16]. The mechanisms of $CD4^+$ Treg activation following trauma are not fully understood. Previously, we postulated a potential role for platelets to be involved in the activation of $CD4^+$ Tregs in this setting [16]. Our own recent research demonstrated that platelet depletion is associated with impaired $CD4^+$ Treg activation following trauma-induced injury [16]. Furthermore, thromboelastometry data (ROTEM) suggests reciprocal activation between $CD4^+$ Tregs and platelets following trauma [16]. Platelets are involved in a wide range of $CD4^+$ Treg interactivity. For example, platelets are involved in the regulation of $CD4^+$ T effector cell responses *via* constantly promoting $CD4^+$ Treg responses [17]. Furthermore, in an *in vitro* analysis, platelet factor 4 exerted differential effects on $CD4^+$ Tregs versus $CD4^+$ non-Tregs [18]. Unfortunately, pharmaceutical options are currently lacking to implement immunomodulatory strategies in the setting of SIRS, trauma, and sepsis. In particular, strategies to selectively modulate the cellular activity of $CD4^+$ Tregs are needed. In a former *in vivo* experiment of our group, the first indications for a cell-specific activating effect of ancrod on $CD4^+$ Tregs were detected [19].

Ancrod is a fibrinogen-splitting enzyme (fibrinogenase) derived from the snake venom of the Malayan pit viper (*Calloselasma rhodostoma*) [20]. As a proteolytic enzyme, it cleaves fibrinopeptide A from fibrinogen, but not fibrinopeptide B. This leads to abnormal fibrin polymerization, hypofibrinogenemia, hypoplasminogenemia, and increased levels of fibrin decay products [21, 22]. In addition, ancrod activates the fibrinolytic system by initiating the release of plasminogen activators from the vascular endothelium. However, there is no activation of factor XIII or other clotting factors by ancrod. The resulting clot is soluble, there is no cross-linking, and it degrades due to the progressive digestion of the A alpha chain. The soluble clot is then digested by the reticulo-endothelial system [23]. Defibrinogenation leads to a reduction in blood viscosity and improved vascular blood flow [24].

Due to its thrombin-like properties (protease), previous *in vivo* research on ancrod focused on various ischemic diseases (stroke, venous thrombosis, myocardial infarction, etc.), heparin resistance or contraindication, protamine sulfate allergies, and nephrological issues [25-30]. So far, it is known that ancrod can be used for vascular diseases and/or circulatory disorders.

Overall, there is currently a lack of pharmaceutical strategies to modulate the immune response following SIRS, trauma, and sepsis. In particular, the activation of $CD4^+$ Tregs, which are considered protective in this setting, cannot be modulated. For the first time, this experimental study systemically investigated the potential immunomodulatory effects of ancrod, and analyzed the underlying mechanisms of action. Using murine *in vitro* and *in vivo* approaches, we established that ancrod exerts selective effects on different cell (sub-) populations of the adaptive and innate immune system.

Furthermore, we observed a differential effect of ancrod on $CD4^+$ Tregs vs. $CD4^+$ non-Tregs in dependence of the mode of cellular stimulation. Additionally, our findings suggest a dose-dependent role of ancrod in the modulation of platelet activation. Our findings provide the first evidence underlining the potential of ancrod in selectively modulating immune cells, highlighting ancrod as a promising candidate for further investigation.

## Material and methods

### Animals

Male C57BL/6N mice (7-14 weeks) from Charles River Laboratories (Sulzfeld, Germany) were acclimatized for 1 week and maintained in the Center for Preclinical Studies (ZPF) of Klinikum rechts der Isar, Munich, Germany. Full supply (standardized feeding/water, controlled temperature and humidity) and a 12-h light/dark regimen were guaranteed in the virus antibody-free (VAF) animal facility. This study was carried out in strict accordance with the German legislation on protection of animals. Spleen and blood extraction after euthanizing the animals was possible without specific approval by the Committee on the Ethics of Animal Experiments in accordance with the German Protection of Animals Act (§4 Tierschutzgesetz). The protocol for animal experiments (§7 Tierschutzgesetz) was approved by the responsible Committee on the Ethics of Animal Experiments of the Technical University (Regierung von Oberbayern, Munich, Germany, Permit Number: 55.2-2532.Vet_02-20-57). Mice were randomized for the *in vivo* experimental groups ($n = 8$) according to the ARRIVE recommendations [31].

### Blood and spleen collection

Mice were euthanized by intraperitoneal injection of 400 mg/kg sodium pentobarbital (Narcoren, Boehringer Ingelheim, Germany) following isoflurane inhalation

(365 ml/min, 3.5%) before the spleens were harvested. Blood was collected by cardiac puncturing using a 14-gauge needle with a syringe containing 3.1% trisodium citrate (blood/citrate ratio 10 : 1).

## Rotational thromboelastometry (ROTEM)

Thromboelastometric analysis was performed using ROTEM delta (TEM International GmbH, Munich, Germany) for 90 minutes according to the manual. Platelet function was analyzed after extrinsic activation of the coagulation cascade by recalcification of the citrated blood with star-tem reagent. For examination of the extrinsic coagulation pathway, the ex-tem reagent was added afterwards to activate platelets.

We evaluated the parameters maximum clot firmness (MCF), which is the greatest amplitude of the reaction curve measured in millimeters (mm), and clot formation time (CFT), which is defined as the period from an amplitude of 2 mm to an amplitude of 20 mm.

## Reagents, cell preparation, and staining protocols

Cell preparation was conducted in C5 culture medium: RPMI 1640 supplemented with MEM, penicillin/streptomycin, L-glutamine 200 mM, HEPES buffer solution 1 M, 2-mercaptoethanol and 5% FBS from Sigma-Aldrich (St. Louis, MO). Harvested spleens were strained in C5 using stainless steel meshes and sieves with 70 µm pores. Blood was centrifuged at 200 × g and 21°C for 10 minutes, and platelet-rich plasma (PRP) was collected. PRP was then plated out and centrifuged.

The respective cell suspension was plated on 96-well plates at 400,000 cells/well (mixed culture) or 160,000 cells/well (monoculture) and rested in the incubator (37°C, 5% $CO_2$) for 1 hour.

Cell staining was performed in PBS and stopped after incubation time with PBA buffer: PBS supplemented with albumin (bovine serum) and sodium azide, from Sigma-Aldrich (St. Louis, MO).

Fc-block reagent (purified anti-mouse CD16/32) from BioLegend (San Diego, CA) prevented non-specific binding in the case of non-REA antibodies. A Zombie NIR Fixable Viability Kit from BioLegend (San Diego, CA) was used to distinguish dead from alive cells.

Surface staining of T cells was conducted with anti-CD4 (REA 604), anti-CD44 (REA 664), anti-CD62L (REA 828), anti-CD69 (REA 938), anti-CD8 (REA 865), anti-CD25 (REA 667), anti-CD38 (REA 616), and anti-aMHC Class II (REA 528) from Miltenyi Biotec (Bergisch Gladbach, Germany).

Surface staining of antigen-presenting cells was performed with anti-CD45RA (REA 639), anti-CD11b (REA 592), anti-CD11c (REA 754), anti-CD14 (REA 934), anti-CD19 (REA 749), anti-CD40 (REA 965), anti-CD69 (REA 938), anti-CD86 (REA 1190), and anti-F4/80 (REA 126) from Miltenyi Biotec (Bergisch Gladbach, Germany).

To identify FoxP3$^+$ cells, we used anti-FoxP3 from Miltenyi Biotec (Bergisch Gladbach, Germany). Primary antibodies included anti-ZAP-70 (99F2), anti-p-ZAP-70 (Tyr 493), anti-PKC-h (P632) and anti-p-PKC-h (Thr 538), all from Cell Signaling Technology (Danvers, MA). Secondary antibody use of Alexa Fluor 555 conjugated F(ab)′2 fragment of goat anti-rabbit IgG from Invitrogen (Carlsbad, CA) ensured detection.

For the staining of platelets, we used anti-CD41 (REA 1194), and anti-CD62P (REA 344), anti-CD63 (REA 563), anti-GARP (LRRC32) (REA 139), and anti-CD61 (REA 1192) from Miltenyi Biotec (Bergisch Gladbach, Germany).

## Flow cytometry

### *Extracellular phenotyping*

Conventional flow cytometry was used for extracellular phenotyping. CD4$^+$ identification was performed using anti-CD4 monoclonal antibodies, and CD8$^+$ identification using anti-CD8 monoclonal antibodies. Surface-staining antibodies for detection of T cell activation included anti-CD44, anti-MHC, anti-CD69, anti-CD25, anti-CD62L, and anti-CD38 monoclonal antibodies. Zombie NIR served as a viability dye.

Firstly, the single cells were selected from the remaining cell material in the FSC-A/FSC-H diagram. Cell aggregates have a significantly higher fluorescence intensity and differ from single cells both in their width (FSC-W) and in the area under the signal curve (FSC-A). This special feature is utilized in single-cell gating to exclude duplicates and cell debris.

The subsequent selection of viable cells was facilitated by using a viability dye (Zombie NIR). The lymphocyte population was then distinguished from the other cell groups (e.g. monocytes and granulocytes) in the FSC-H/SSC-H diagram based on their distinct light scattering characteristics. Subsequently, the CD4$^+$ cells were identified as PE-Vio770 positive cells, while the CD8$^+$ cells were identified as VioBlue positive cells.

The activity markers CD44, CD62L, CD69, CD25, CD38, and aMHC Class II were then determined in these CD4$^+$ and CD8$^+$ populations using fluorescence minus one (FMO) controls.

The parameters determined for each fluorescent dye were MFI (mean fluorescence intensity) and the percentage of positive cells.

B cell identification was conducted using anti-CD45RA and anti-CD19. Surface-staining antibodies for detection of B cell activation included anti-CD86 and anti-CD69. Dendritic cell identification was conducted using anti-CD45RA, anti-CD11b, and anti-CD14; activation markers used were anti-CD86 and anti-CD40. Macrophage identification markers included anti-CD45RA, anti-CD11c, and anti-F4/80; activation markers used were anti-CD86 and anti-CD40. Zombie NIR served as a viability dye.

Following washing, cells were fixed with 0.3% PFA, washed, and reconstituted in PBS for analysis.

For platelet staining, thrombocytes were stained using anti-CD41 and anti-CD61 for identification and anti-CD62P, anti-GARP, and anti-CD63 for visualizing platelet activity (45 min). The stained platelets were washed, fixed with 1% PFA (30 min) and resuspended with PBS for analysis.

### *Intracellular phenotyping*

For intracellular staining after isolation from the spleen, cells were dyed with Zombie NIR (20 min). After washing with PBA, the fixed cells were permeabilized with ice-cold methanol (10 min). Fc block was added. Staining antibodies, anti-CD4, and anti-FoxP3 were added to identify $CD4^+$ $FoxP3^+$ Treg cells. Cells were incubated with one of the following rabbit anti-mouse primary antibodies: anti-ZAP-70, anti-p-ZAP-70, anti-PKC-h, and anti-p-PKC-h. After washing, secondary goat anti-rabbit IgG (F(ab)′2 fragment) was added (30 min). Negative controls were generated by staining without the primary antibody. Cells were fixed with 0.3% PFA, washed, and reconstituted in PBS for analysis.

### *Controls*

Each staining panel included FMO controls to standardize gating and to be able to detect unspecific staining. Phospho-flow and conventional flow cytometry was conducted using MACSQuant 9 (extracellular, intracellular T cell and thrombocytes analysis) or MACSQuant 16 (APC analysis) from Miltenyi Biotec (Bergisch Gladbach, Germany). Calibration of the machine was done prior to each experiment using Calibration Beads from Miltenyi Biotec (Bergisch Gladbach, Germany). Automated multicolor compensation with single stained cells was conducted for each experiment to avoid fluorescence spillover. Regular bleaching and flushing of the instrument were performed to ensure that the tubes and flow chamber were clean.

Fluorescence intensity measurements were obtained using MACSQuant devices. The percentage of marker-expressing cells was determined from the gate defined for the respective marker using FMO controls. Mean fluorescence intensity (MFI) was calculated in the same gates as a relative measure of the amount of protein expressed by the cells.

## Experimental protocols

### *In vitro experiments*

In order to investigate the *in vitro* effect of different ancrod doses on expression of activity markers of $CD4^+$ and $CD8^+$ T cells (extra- and intracellular), on antigen-presenting cells (APC), B cells, macrophages, dendritic cells (DC), and on platelets, we incubated them with different doses of ancrod (0, 1, 5, 10 and 15 IU/ml) for 2 hours in the incubator (37°C, 5% $CO_2$). To determine whether the effect of ancrod is influenced by cell-specific stimulation, we compared activation levels of stimulated and unstimulated spleen cells and repeated these experiments with isolated cells to evaluate the effect of APC. To clarify whether the effect of ancrod on BC is influenced by immune cells other than APC, we compared activation levels of isolated and non-isolated BC. Regarding monocultures, the isolated spleen cells were separated using the Pan T or B Cell Isolation Kit from Miltenyi Biotec (Bergisch Gladbach, Germany). Before and after each separation, we performed quick stainings with anti-CD4 (T cells) or anti-CD45 (APC) antibodies to ensure the purity of the respective cell suspension.

PRP was also incubated with the same 5 doses of ancrod for 2 hours prior to staining.

After cell isolation and rest time, ancrod and stimulation reagents were diluted with C5 culture medium to obtain the desired concentrations. Stimulation of T cells was conducted using 1 µg/ml purified anti-mouse CD3ε from BioLegend (San Diego, CA) and 1 mg/ml LPS O26:B6 from Sigma Aldrich (St. Louis, MO) for antigen-presenting cells. Ancrod was provided by Nordmark Pharma GmbH (Uetersen, Germany). 100 µl of ancrod solution with or without stimulation reagent was added per well and kept in the incubator (37°C, 5% $CO_2$) for 2 hours. Prior to staining, plates were centrifuged at 1,200 rpm, 22°C for 10 minutes and supernatant was discarded afterwards.

### *In vivo experiments*

Mice (7-14 weeks) were injected intraperitoneally with 8 IU/ml ancrod mixed with 0.9% NaCl (total volume 50 µl) 2, 24, or 48 hours before euthanasia. This dose was found to be the most suitable during previous experiments (unpublished).

Spleen cells and platelets were plated out after isolation and immediately stained without ancrod incubation.

## Statistics

Statistical analysis was performed using Microsoft Excel 2016 and GraphPad Prism Software (version 9) from GraphPad Software, Inc. (La Jolla, CA, USA). Mean values ± SD or median values with 95% confidence interval (box plots) are given. We used one-way and two-way ANOVA (analysis of variance) and Šídák's multiple comparisons test to assess statistical significance. $p < 0.05$ was considered significant.

## Graphics

PowerPoint 2016 from Microsoft Corporation was used to create the figures and the graphical abstract.

## Results

### Different doses of ancrod significantly reduce activation of isolated and stimulated spleen-derived CD4+ cells *in vitro* and of CD4+ cells *in vivo* (after 24 h)

To investigate the *in vitro* effect of ancrod on murine spleen-derived CD4+ cell activation, we incubated them with 5 different doses of ancrod and measured the expression of cell surface markers CD44, CD62L, and CD69 *via* flow cytometry. To determine whether the effect of ancrod is influenced by stimulation, we compared activation levels of anti-CD3ε stimulated with unstimulated spleen cells and repeated these experiments with isolated T cells to evaluate the effect of antigen-presenting cells (APC). To determine the *in vivo* effect of ancrod, 8 IU/ml was injected 2, 24, or 48 hours prior to euthanasia. This dose was found to be the most suitable during previous experiments (unpublished data).

We were pleased to find a cell-specific and dose-dependent impact of ancrod on immune cells. In dependence of ancrod, significantly reduced CD44 MFI values of stimulated and monocultured CD4+ cells could be detected (Fig. 1D) (0 IU/ml vs. 10 IU/ml ancrod $p < 0.05$; 0 IU/ml vs. 15 IU/ml ancrod $p < 0.01$). *In vivo*, a significantly reduced CD44 percentage was observed 24 hours after injection of 8 IU/ml ancrod compared to saline (Fig. 1G) ($p < 0.05$).

Regardless of stimulation or monoculture, there was no significant effect of ancrod on CD4+ cell activation markers CD62L or CD69 *in vitro* and *in vivo*. However, there were nonsignificant tendencies for reduced activation in monocultured and stimulated CD4+ cells *in vitro* (CD69) and CD4+ cells *in vivo* (CD62L). The effect of stimulation itself was diminished in the monoculture setting; this applies in particular to CD44 and CD62L (Fig. 1A, B, D, E).

These findings suggest a potential differential impact of ancrod which depends *in vitro* on stimulation with anti-CD3ε and absence of APC. The *in vivo* effect is detectable only 24 h after injection of 8 IU/ml, suggesting a slightly delayed onset of drug action combined with a short-lasting effect.

### *In vitro*, activation of isolated and stimulated spleen-derived CD8+ cells showed a nonsignificant decrease with increasing doses of ancrod. Ancrod significantly reduced the percentage of CD8/CD44+ 24 hours after injection *in vivo*

To evaluate the *in vitro* and *in vivo* effects of ancrod on murine spleen derived CD8+ (cytotoxic) cell activation, we followed the same approach as for CD4+ cells.

Rising ancrod doses *in vitro* were associated with declining expression of CD44 and CD69, but only if CD8+ cells had been monocultured and stimulated (Fig. 2D, F; MFI). We found a similar reduced effect of anti-CD3ε in the monoculture setting, which applied in particular to CD44 and CD62L (Fig. 2A, B; % cells). *In vivo*, we observed a significantly reduced CD44 percentage 24 h after injection of 8 IU/ml ancrod compared to saline (Fig. 2G) ($p < 0.05$). No significant effect of 8 IU/ml ancrod on CD8+ cell activation markers CD62L and CD69 was observed *in vivo* (Fig. 2G).

This suggests that also for CD8+ cell activation, there was a differential impact of ancrod which depended on stimulation with anti-CD3ε and absence of APC. *In vivo*, CD8+ cells exhibited the same kinetics of ancrod effect as CD4+ cells.

### Increasing ancrod levels significantly and dose-dependently reduced the activation of spleen-derived CD4+ Tregs (T) and CD4+ non-Tregs (NT) *in vitro* in absence of anti-CD3+, and 8 IU/ml ancrod significantly enhanced activation of CD4+ Tregs after 24 hours *in vivo*

To analyze the *in vitro* and *in vivo* effects of ancrod on murine spleen-derived CD4+ FoxP3+ Treg and CD4+ FoxP3- non-Treg activation, we incubated them with different ancrod doses and with or without the stimulation reagent anti-CD3ε prior to staining with the primary antibodies anti-p-ZAP-70 or anti-p-PKC-h and the secondary IgG (F(ab)′2 fragment) Alexa. In order to analyze the early expression and phosphorylation of primary TCR signaling molecules, we used phospho-flow cytometry, as described before [15, 16, 19, 32-34]. Following TCR activation, the f-associated protein of 70 kDa (ZAP-70) is recruited to the TCR/CD3 complex. Protein kinase C-h (PKC-h) is part of NF-κB-dependent TCR signaling [35]. PKC-h activation is enhanced by CD28-B7 ligation [36], and it plays a role in regulating negative feedback within CD4+ Tregs. Flow cytometry gating for CD4+ FoxP3+ cells allowed for identification of CD4+ Tregs [15] and direct comparison of CD4+ Tregs with CD4+ non-Tregs.

Increasing ancrod levels are associated with a significant and dose-dependent reduction of pPKC and pZAP70 expression (% cells and MFI) of spleen-derived CD4+ Tregs and CD4+ non-Tregs *in vitro* in the absence of anti-CD3ε (Fig. 3A-F). In the presence of anti-CD3ε, this inhibitory effect is also noticeable in CD4+ non-Tregs, but it is not significant (Fig. 3A-D). Stimulated CD4+ Tregs behave similarly, but not as consistently as unstimulated CD4+ Tregs. Table 1 gives an overview of respective *p* values. A significant effect of 8 IU/ml ancrod on CD4+ Treg activation (pPKC and pZAP70) compared to saline injection was observed after 24 hours *in vivo* ($p < 0.05$).

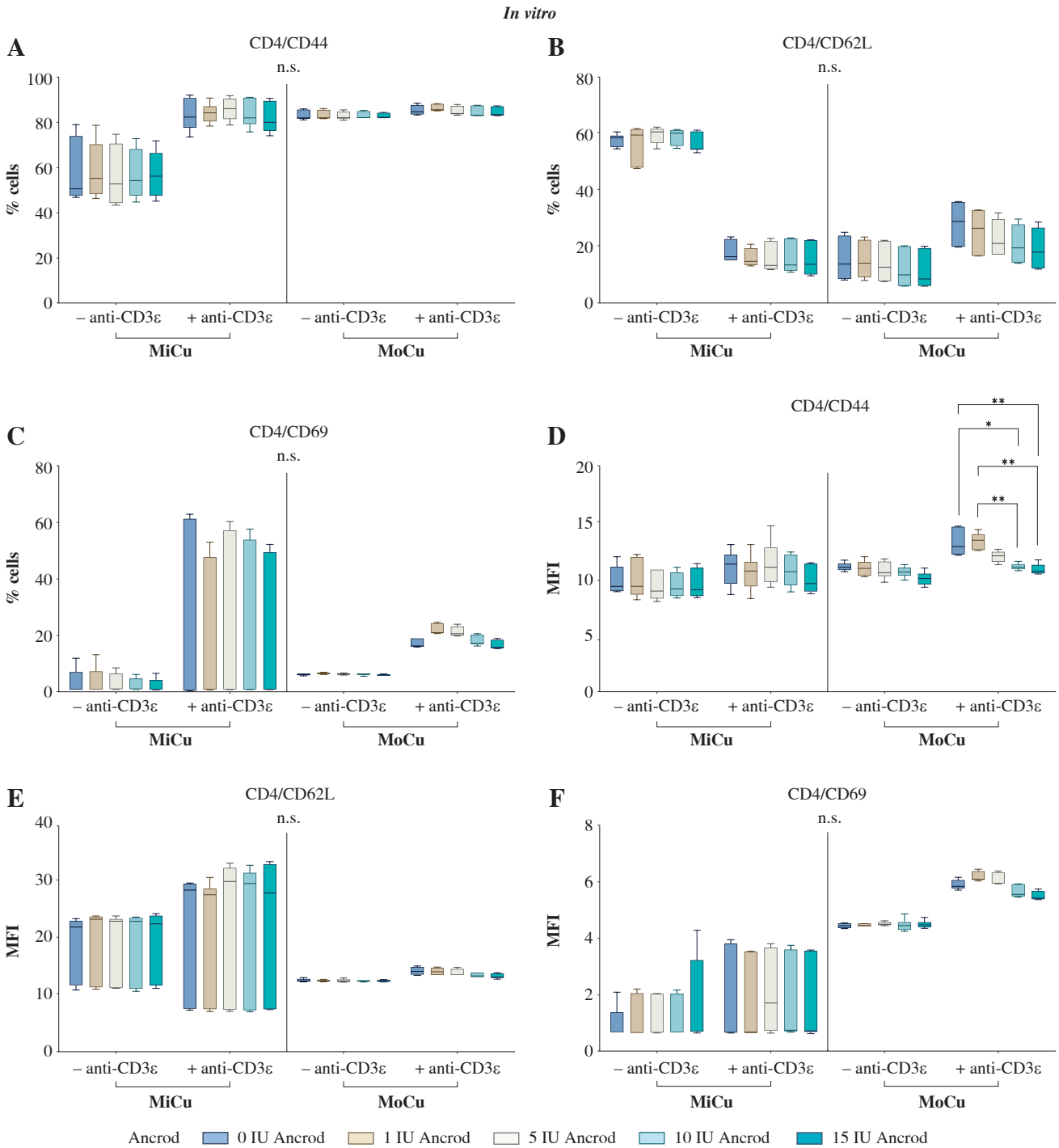


**Fig. 1.** Ancrod induces significantly reduced CD44 expression *in vitro* and *in vivo* after 24 h compared to NaCl = saline. No significant effect of different ancrod doses on CD4⁺ cell activation markers CD62L and CD69 was observed *in vitro*. To evaluate the influence of ancrod on the expression of CD44, CD62L, or CD69 on mouse spleen-derived CD4⁺ cells, we used flow cytometry after 2 hours of incubation *in vitro* or 2, 24, or 48 hours after injection *in vivo*. Percentages (**A-C**) and mean fluorescence intensity (MFI) (**D-F**) of mixed (MiCu) vs. monocultured (MoCu) mouse spleen CD4⁺ cells with and without stimulation (anti-CD3ε) and different doses of ancrod (0,1, 5, 10, 15 IU/ml) *in vitro* and percentages of mouse spleen CD4⁺ cells which were isolated after injection of ancrod (8 IU/ml) *in vivo* (**G**). $n = 3$ each group *in vitro*, 2 wells per condition; $n = 24$ each group *in vivo*, 3 wells per animal. Data are presented as boxplot (**A-G**) with median and 95% confidence interval

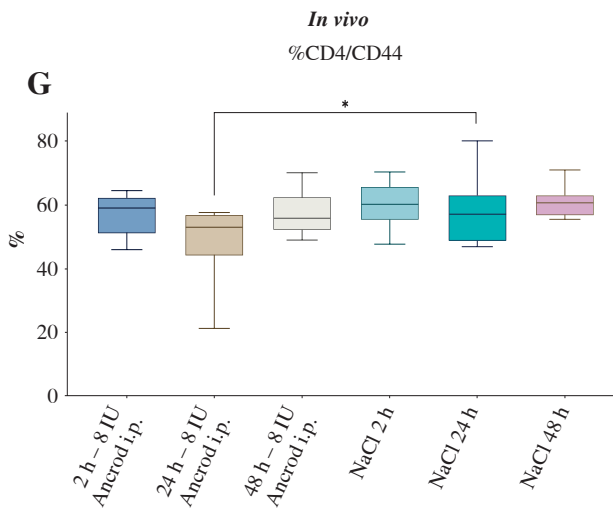


**Fig. 1.** Cont. Percentages of mouse spleen CD4⁺ cells which were isolated after injection of ancrod (8 IU/ml) *in vivo* (**G**). $n = 3$ each group *in vitro*, 2 wells per condition; $n = 24$ each group *in vivo*, 3 wells per animal. Data are presented as boxplot (**A-G**) with median and 95% confidence interval

Our results imply that ancrod exerts a differential impact for CD4⁺ Tregs and CD4⁺ non-Tregs activation, meaning that stimulation with anti-CD3ε reduces the activation-inhibiting effect of ancrod in CD4⁺ Tregs and non-Tregs *in vitro*. The fact that the same activation-inhibiting effect is not observed and even reversed *in vivo* suggests further factors influencing ancrod that are absent in the *in vitro* setting.

### Ancrod significantly enhances activation of B cells (BC) and macrophages (M) *in vivo*, but not *in vitro*

To evaluate the *in vitro* and *in vivo* effect of ancrod on murine spleen-derived B cell (BC), dendritic cell (DC) and macrophage activation, we incubated them with different ancrod doses and with or without the stimulation reagent LPS prior to staining with CD86 (all), CD40 (DC and macrophages), or CD69 (BC), and performed analysis via flow cytometry. To determine whether the effect of ancrod on BC is influenced by immune cells other than APC, we compared activation levels of isolated versus non-isolated BC. Increasing ancrod levels do not affect the expression of activation markers (% cells and MFI) of spleen-derived BC, DC, and macrophages *in vitro* in the presence and Wabsence of LPS (WA-H). This also applies to mixed and monocultured BC (Fig. 4A, E). Stimulation effects *via* LPS can be observed best in mixed-cultured BC (Fig. 4B, F). Interestingly, 8 IU ancrod *in vivo* highly significantly enhances the expression of the BC activation marker CD69 ($p < 0.0001$) and macrophage marker CD40 ($p < 0.0001$) compared to NaCl injections. CD40 expression of DC is non-significantly diminished by ancrod.

Our results suggest that the stimulating effect of ancrod on BC and macrophages, which occurs exclusively *in vivo*, depends on a third player which was not part of our *in vitro* setting. Furthermore, this implies that the differential *in vitro* impact of ancrod observed for CD4⁺, CD8⁺, and (non) Tregs depending on stimulation does not apply on antigen-presenting cells.

### Ancrod significantly and dose-dependently diminishes activation of platelets *in vitro* and significantly enhances activation, prolongs clot formation time, and reduces maximal clot firmness *in vivo*

To investigate the *in vitro* and *in vivo* effects of ancrod on cardiac blood-derived platelet activation, we incubated them with different ancrod doses prior to staining with the markers anti-GARP, anti-CD62P, and anti-CD63 in order to analyze cell activation *via* flow cytometry.

Increasing ancrod levels significantly and dose-dependently reduced CD62P expression (% cells, 0 vs. 5 IU/ml, $p < 0.01$; 0 vs. 10 and 15 IU/ml, $p < 0.0001$) of platelets *in vitro* (Fig. 5A). MFI values and anti-GARP and CD63 expression (% cells and MFI) showed similar trends, but were not statistically significant.

Interestingly, a contrary picture emerges *in vivo* as anti-GARP expression is significantly higher after 2 doses of 8 IU ancrod compared to NaCl ($p < 0.0001$). CD63 showed a similar trend, but the effect was not significant. CD62P expression is not influenced by 8 IU ancrod *in vivo*.

Our results indicate that also for platelets, there is a differential impact of ancrod which likely is influenced by other factors, as *in vitro* ancrod diminished activation and *in vivo* ancrod had an activating effect.

## Discussion

Trauma-induced injury is still the leading cause of death for young patients up to the age of 45 years (https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/ghe-leading-causes-of-death). These trauma-induced injuries often lead to pro- and anti-inflam-

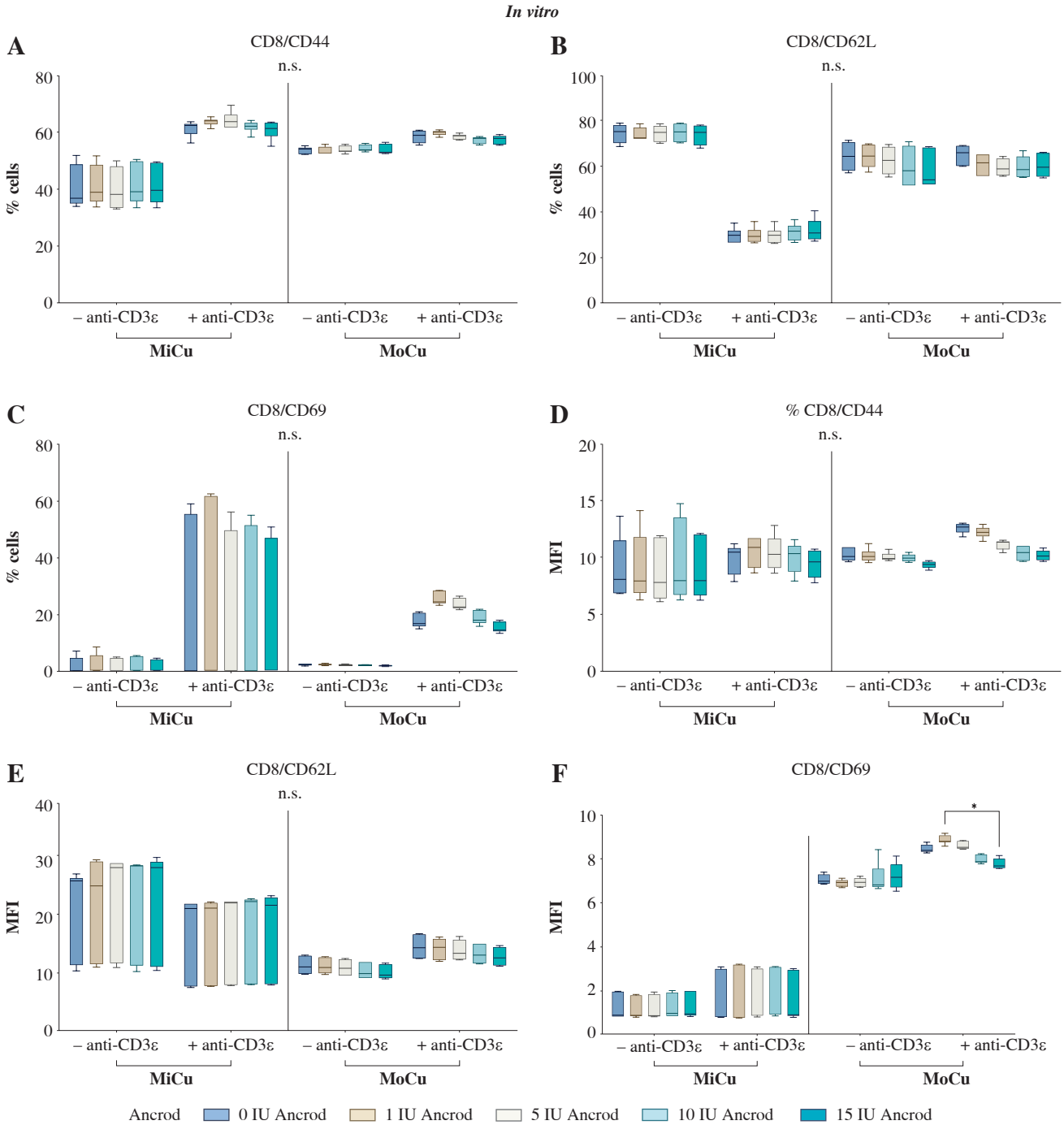


**Fig. 2.** Activation of isolated and stimulated CD8⁺ cells tended to decrease with rising ancrod doses *in vitro* and significantly reduced after 24 hours *in vivo*. No significant effect of different ancrod doses on CD8⁺ cell activation markers CD62L and CD69 was observed *in vivo*. To evaluate the influence of ancrod on the expression of CD44, CD62L, or CD69 on mouse spleen derived CD8⁺ cells, we used flow cytometry after 2 hours of incubation *in vitro* or 2, 24, or 48 hours after injection *in vivo*. Percentages (**A-C**) and mean fluorescence intensity (MFI) (**D-F**) of mixed (MiCu) vs. monocultured (MoCu) mouse spleen CD8⁺ cells with and without stimulation (anti-CD3ε) and different doses of ancrod (0, 1, 5, 10, 15 IU/ml) *in vitro* and percentage of mouse spleen CD8⁺ cells which were isolated after injection of ancrod (8 IU/ml) *in vivo* (**G**). $n = 3$ each group *in vitro*, 2 wells per condition; $n = 24$ each group *in vivo*, 3 wells per animal. Data are presented as boxplot (**A-G**) with median and 95% confidence interval

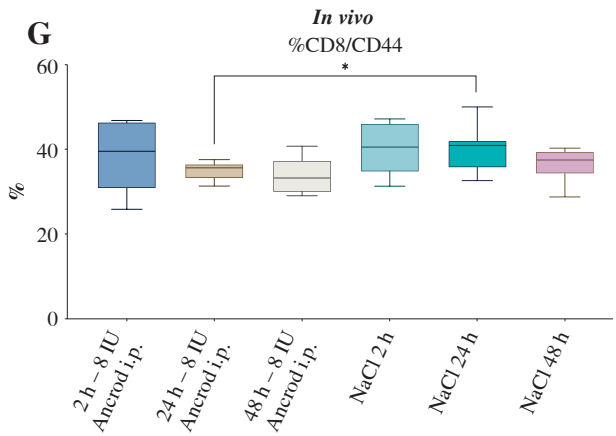


**Fig. 2.** Cont. Percentage of mouse spleen CD8⁺ cells which were isolated after injection of ancrod (8 IU/ml) *in vivo* (**G**). $n = 3$ each group *in vitro*, 2 wells per condition; $n = 24$ each group *in vivo*, 3 wells per animal. Data are presented as boxplot (**A-G**) with median and 95% confidence interval

matory host responses. The proinflammatory reaction SIRS occurs in up to 90% of trauma patients and often leads to concomitant infections and potentially fatal sepsis. About 33% to 50% of hospital deaths are due to sepsis [3].

Unfortunately, pharmaceutical options to perform immunomodulatory strategies in the setting of SIRS, trauma, and sepsis are currently lacking. In a former *in vivo* experiment of our group, the first indications for a cell-specific activating effect of ancrod on the trauma site draining lymph node-derived CD4⁺ Tregs were detected [19].

The present study is the first demonstration that ancrod is capable of selectively acting on different immune cell types. In addition, our results demonstrate that ancrod differentially affects CD4⁺ Tregs and CD4⁺ non-Tregs depending on the mode of cellular stimulation.

Furthermore, our findings suggest that ancrod modulates platelet activation in a dose-dependent manner.

## Previous applications of ancrod

Since ancrod was discovered and studied *in vitro* and *in vivo*, most applications have been focused on cardiovascular research areas due to its thrombin-like action. Various species-specific dosages were used [25-30]. For mice, intraperitoneal injection of 5-6 IU is suitable, given 18 and 2 hours before sample collection and subsequent euthanasia [37]. Injections of 8 IU have also been shown to be effective in previous experiments [33]. With regard to dosage, attention should be paid to the particular preparation and storage conditions, as ancrod consistently loses activity even when stored at +4°C. To address this fact, we measured enzyme activity regularly and the amount required for the experiment was adjusted respectively. The ancrod used in this study was derived from residual material from a human clinical trial and thus meets strict pharmacological safety requirements.

The overall question is: Which mechanisms might mediate the ancrod effect on immune cells? There is only little evidence of immunomodulatory effects of ancrod in the literature to date. Hypotheses of previous publications on mechanisms of action are mainly based on the effect ancrod has on fibrinogen and the reticuloendothelial system.

## Fibrinolytic system

Plasminogen circulating in the blood is converted into the active form by specific serine proteases called plasminogen activators. Specific inhibitors of plasminogen activators and plasmin circulating in the blood contribute to the prevention of excessive fibrinolysis and modulation of coagulation [38]. Plasminogen attaches itself to fibrin fibers during clotting and is only activated there. Both the soluble precursor of fibrin, fibrinogen, and fibrin itself are broken down into degradation products by plasmin. The fibrin-(ogen) degradation products in turn inhibit fibrin cross-linking. Serine proteases such as plasmin exert irreversible effects. Plasmin has an autocatalytic effect, i.e. it converts other molecules of plasminogen into active plasmin: The proenzyme is thus the substrate of the activated enzyme [39]. Different degradation products with different structure and mass are formed, which inhibit thrombin and thus slow down the conversion of fibrinogen into fibrin [38].

As ancrod is a proteolytic enzyme that cleaves fibrinopeptide A from fibrinogen, but not fibrinopeptide B; its administration results in abnormal fibrin polymerization, hypofibrinogenemia, hypoplasminogenemia, and elevated levels of fibrin degradation products [21-23]. In addition, ancrod activates the fibrinolytic system by initiating the release of plasminogen activators from the vascular endothelium [40]. The resulting clot is soluble, no cross-linking occurs, and it degrades due to progressive digestion of the A alpha chain. The soluble clot is then digested by the reticulo-endothelial system [22, 23].

Early studies of ancrod revealed that rapid defibrinogenation can lead to an overload of the fibrinolytic and reticulo-endothelial system, which leads to fibrin deposition and intravascular thrombosis [41]. Through the formation of a mesh-like network, fibrin contributes to injury by re-

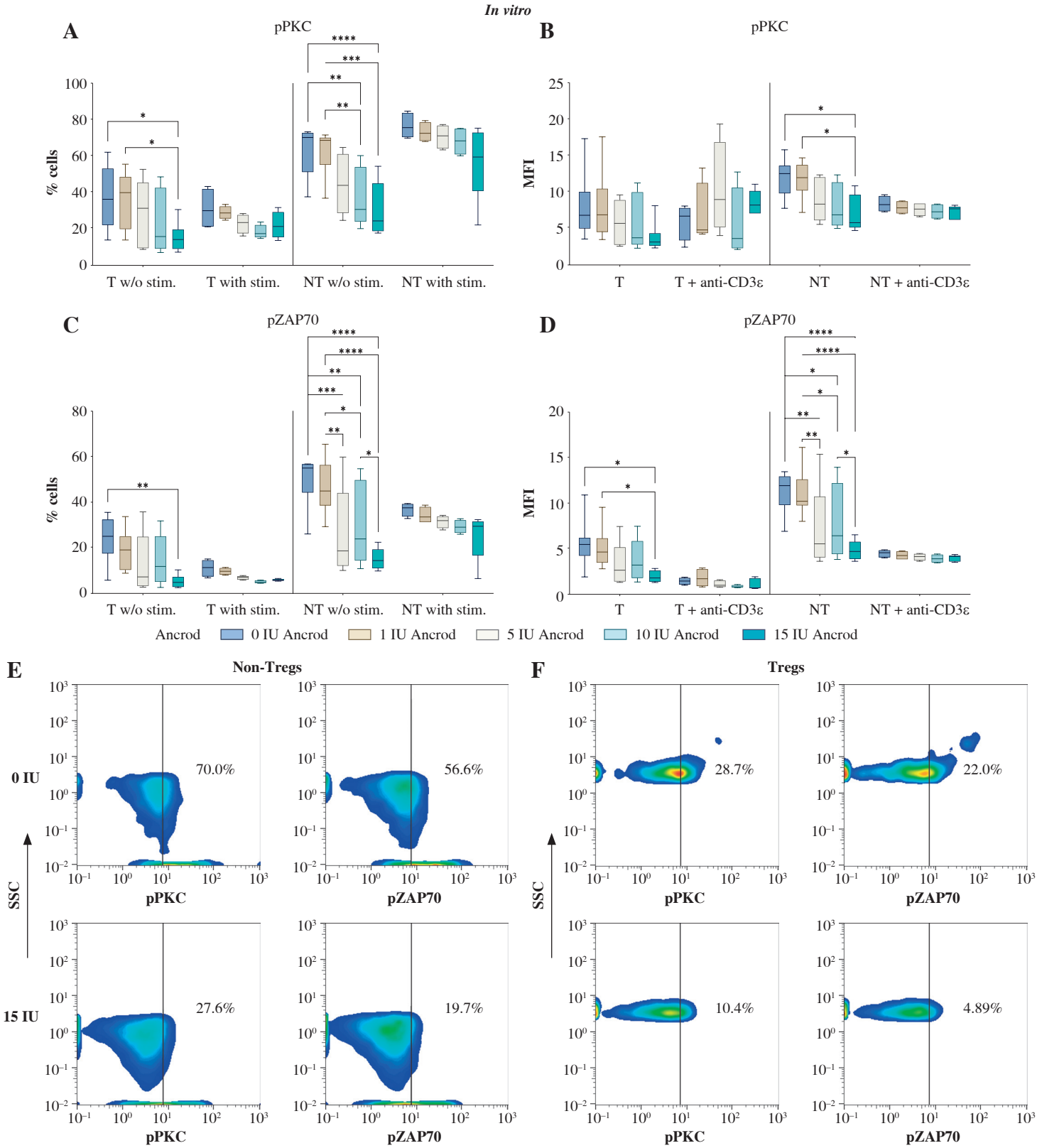


**Fig. 3.** Increasing ancrod levels significantly and dose-dependently reduced the activation of spleen-derived CD4⁺ Tregs (T) and CD4⁺ non-Tregs (NT) *in vitro* in absence of stimulation agent anti-CD3ε, and 8 IU/ml ancrod significantly enhanced activation of CD4⁺ Tregs after 24 hours *in vivo*. Percentages (**A, C**) and mean fluorescence intensity (MFI) (**B, D**) of mixed cultured mouse spleen-derived CD4⁺ Tregs and CD4⁺ non-Tregs after incubation with or without stimulation (anti-CD3ε) and different doses of ancrod (0, 1, 5, 10, 15 IU/ml) *in vitro* or MFI of mouse spleen-derived CD4⁺ Tregs and CD4⁺ non-Tregs which were isolated 2, 24, or 48 hours after injections of ancrod (8 IU/ml) *in vivo* (**G**). Phospho-flow cytometry was used to assess the intracellular expression and phosphorylation of the signaling molecules pPKC-θ and pZAP70, thereby measuring the activation of CD4⁺ Tregs and CD4⁺ non-Tregs following ancrod. Exemplary flow plots of *in vitro* pPKC-θ and pZAP70 expression of CD4⁺ Tregs and CD4⁺ non-Tregs incubated with 0 or 15 IU/ml ancrod are displayed in **E** and **F**. $n = 2$ (⁺anti-CD3ε) or $n = 4$ (-anti-CD3ε) *in vitro*, 2 wells per condition; $n = 24$ each group *in vivo*, 3 wells per animal. Data are presented as boxplot (**A-G**) with median and 95% confidence interval. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

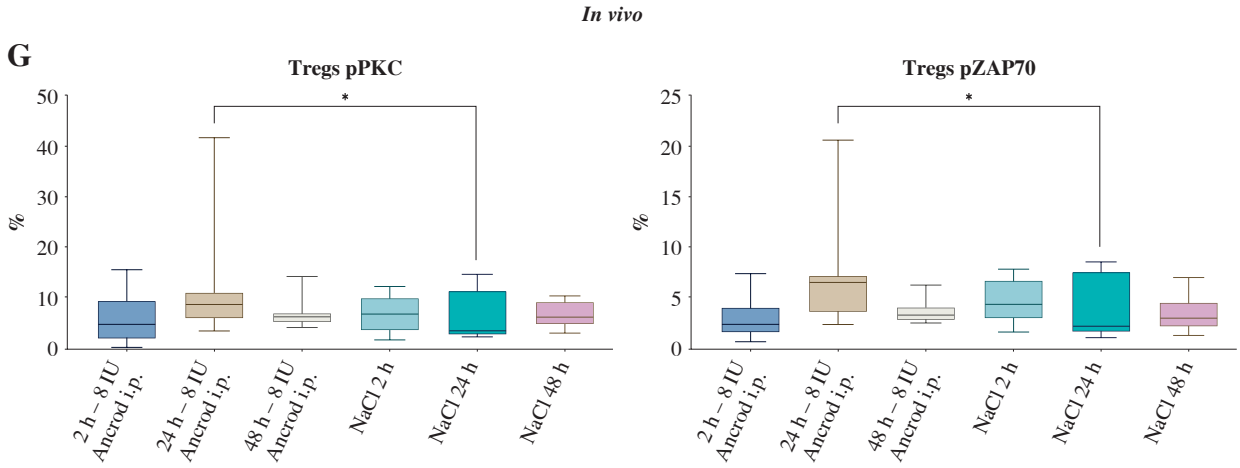


**Fig. 3.** Cont. Mouse spleen-derived CD4⁺ Tregs and CD4⁺ non-Tregs which were isolated 2, 24, or 48 hours after injections of ancrod (8 IU/ml) *in vivo* (**G**). Data are presented as boxplot (**A-G**) with median and 95% confidence interval. *$p < 0.05$

**Table 1.** *P* values of CD4⁺ Tregs (T) and CD4⁺ non-Tregs (NT) incubated with different doses of ancrod *in vitro* (compare Fig. 3); n.s. = non-significant

| Comparison | *p* % cells pPKC | *p* MFI pPKC | *p* % cells pZAP70 | *p* MFI pZAP70 |
|---|---|---|---|---|
| T w/o stim. 0 vs. 15 IU/ml | < 0.05 | n.s. | < 0.01 | < 0.05 |
| T w/o stim. 0 vs. 10 IU/ml | n.s. | n.s. | n.s. | n.s. |
| NT w/o stim. 0 vs. 15 IU/ml | < 0.0001 | < 0.05 | < 0.0001 | < 0.0001 |
| NT w/o stim. 0 vs. 10 IU/ml | < 0.01 | < 0.01 | n.s. | < 0.05 |
| NT w/o stim. 0 vs. 5 IU/ml | n.s. | n.s. | < 0.001 | < 0.05 |

ducing blood flow through capillaries and by recruiting inflammatory cells [42]. Lower fibrin levels were proposed to be associated with reduced monocytes and macrophage infiltration [43]. Toll-like receptor 4 mediates stimulation of macrophage chemokine secretion by fibrinogen [44]. Additionally, ancrod-generated fibrin induces morphological changes of endothelial cells [45] and prostacyclin synthesis [46].

Furthermore, the accumulation of fibrinogen degradation products (FDPs) as induced by ancrod were shown to suppress lymphocyte immune responses *in vitro* and *in vivo* [47-50] and may be harmful to endothelial cells [51] and mesangial cells [52]. This cell damage induces increased chemotaxis of monocytes [53], which in turn secrete a procoagulant factor [54].

These immunomodulating effects do not seem to be systemic, as wound healing [55] was not impaired and no wound or fracture complications in human patients were observed [56].

## Antigen-presenting cells

Since ancrod significantly enhances activation of B cells and macrophages only *in vivo* but not *in vitro*, at least one crucial component must be missing in our *in vitro* setting to achieve this activating effect. As the enhanced activation does not occur in the mixed culture experiments, which includes T cells and CD4⁺ Tregs, they cannot be the decisive component. Considering the previous findings mentioned in the literature, vascular endothelial cells and/or FDP present *in vivo* could contribute to the effect of ancrod on APC by induction of fibrin(ogen) formation [41], stimulation of plasminogen activation [40] and/or prostacyclin synthesis [46]. In addition, platelets could also play an important role in this context as they were also missing *in vitro*. This would be plausible as degradation products and platelets can only be present in sufficient amounts *in vivo*. In a direct co-culture experiment with platelets and lymphocytes, which were exposed to ancrod, we could observe that the inhibiting effect of ancrod on platelets is only present with mixed-cultured

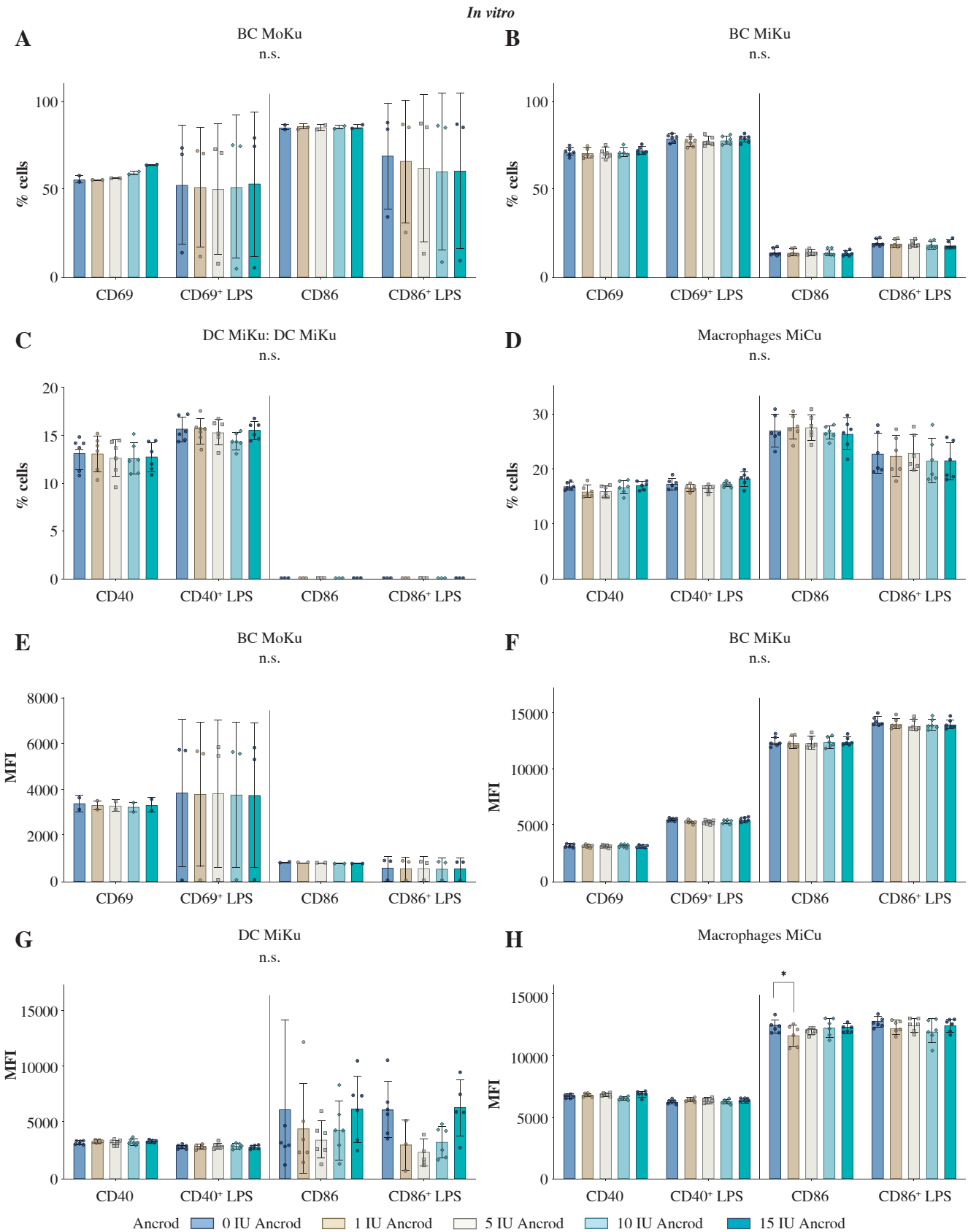


**Fig. 4.** Ancrod significantly enhances activation of B cells (BC) and macrophages (M) *in vivo*, but not *in vitro*. Percentages (**B-D**) and mean fluorescence intensity (MFI) (**F-H**) of mixed-cultured (MiCu) mouse spleen-derived B cells (BC), dendritic cells (DC) and macrophages or monocultured (MoCu) BC (**A + E**) after incubation with or without stimulation (LPS) and different doses of ancrod (0, 1, 5, 10, 15 IU/ml) *in vitro* or MFI of mouse spleen-derived antigen-presenting cells (**I**). Marker expression was assessed *via* flow cytometry after 2 hours of incubation *in vitro* or 2 hours after second injection (8 IU/ml) *in vivo*. Exemplary flow plots of *in vivo* BC and macrophages data are displayed in **J**. $n = 3$ each group *in vitro*, 2 wells per condition (MiCu) and 1 well per condition (MoCu); $n = 24$ each group *in vivo*, 3 wells per animal. Data are presented as mean with standard deviation. *$p < 0.05$, ****$p < 0.0001$

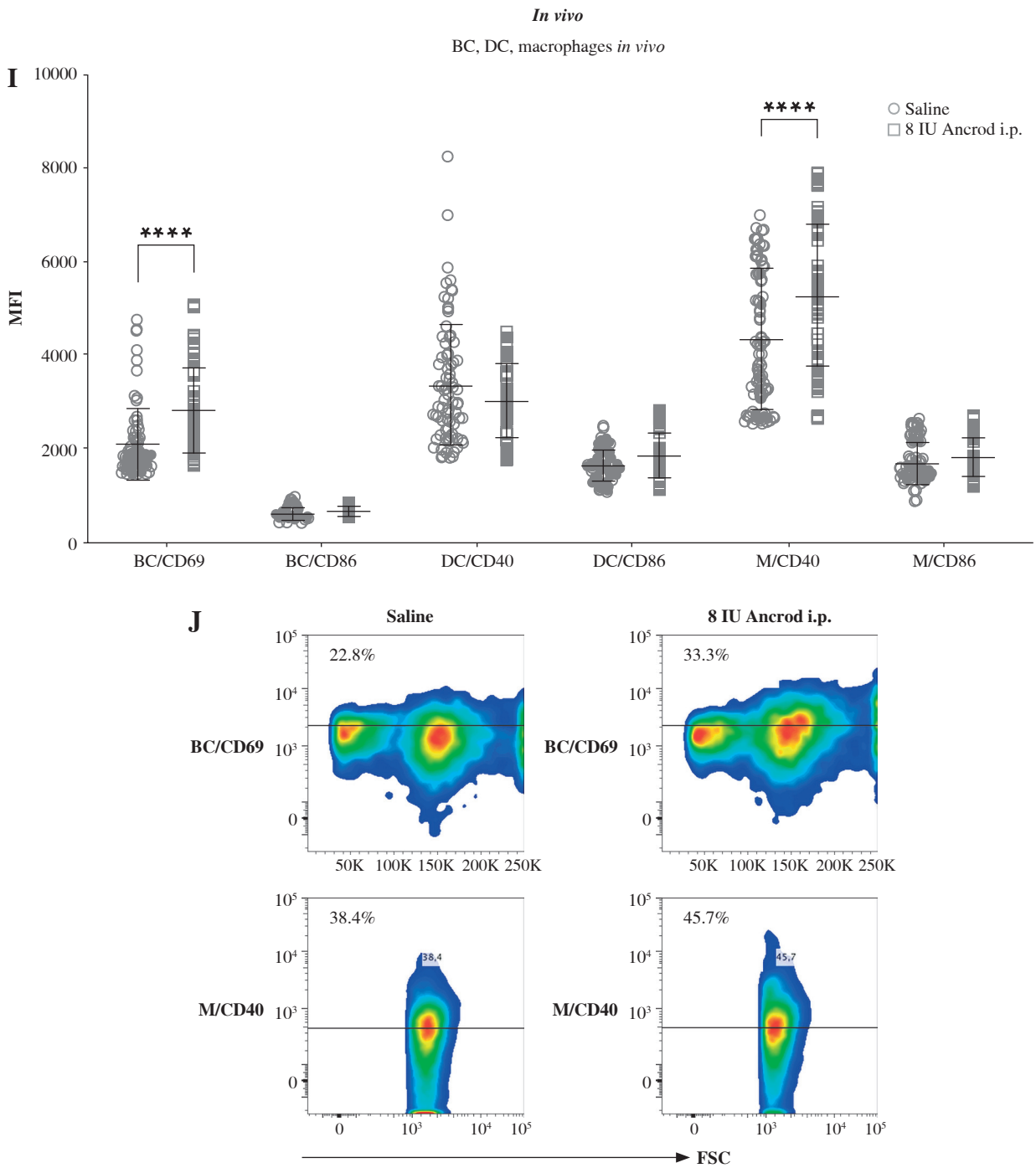


**Fig. 4.** Cont. MFI of mouse spleen-derived antigen-presenting cells (**I**). Marker expression was assessed *via* flow cytometry after 2 hours of incubation *in vitro* or 2 hours after second injection (8 IU/ml) *in vivo*. Exemplary flow plots of *in vivo* BC and macrophages data are displayed in **J**. $n = 3$ each group *in vitro*, 2 wells per condition (MiCu) and 1 well per condition (MoCu); $n = 24$ each group *in vivo*, 3 wells per animal. Data are presented as mean with standard deviation. *$p < 0.05$, ****$p < 0.0001$

lymphocytes which suggests that mono-cultured T-cells or B-cells somehow might interact differently with ancrod and/or platelets compared to co-culture where even an interaction between the two cell types may be possible (unpublished).

## CD4⁺ and CD8⁺ T cells, CD4⁺ Tregs and non-Tregs

CD4⁺ and CD8⁺ T cells, as well as CD4⁺ Tregs/non-Tregs, exhibit contrasting responses after exposure to an-

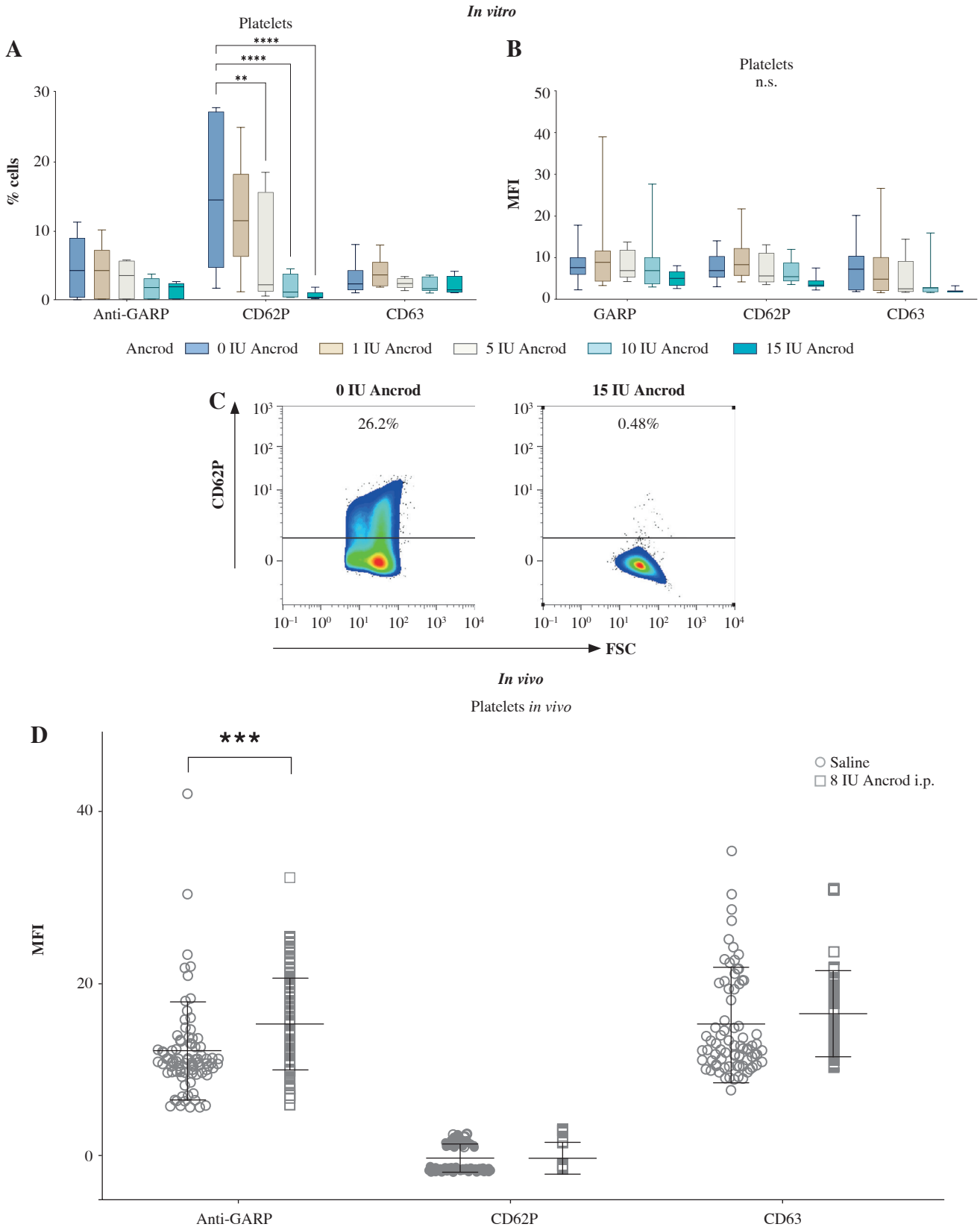


**Fig. 5.** Ancrod significantly and dose-dependently diminishes activation of platelets *in vitro* and significantly enhances activation, prolongs clot formation time, and reduces maximal clot firmness *in vivo*. Percentage (**A**) and mean fluorescence intensity (MFI) (**B**) of mouse cardiac blood-derived platelets after incubation with different doses of ancrod (0, 1, 5, 10, 15 IU/ml) *in vitro* or MFI of mouse cardiac blood-derived platelets which were isolated after 2 injections of ancrod (8 IU/ml) *in vivo* (**D**). Cells were assessed *via* flow cytometry after 2 hours of incubation *in vitro* or 2 hours after the last injection *in vivo*. Exemplary flow plots of *in vitro* platelet data are displayed in **C**, *in vivo* platelet data in **E**. $n = 3$ each group *in vitro*, 3 wells per condition; $n = 24$ each group *in vivo*, 3 wells per animal. Data are presented as bxplot with median and 95% confidence interval (**A**, **B**) or mean ± standard deviation (**D**, **F**). ROTEM data of mouse cardiac blood-derived platelets 2, 24, or 48 hours after injection of 8 IU/ml ancrod compared to saline (NaCl) are displayed in **F**) Clot formation time (CFT) and maximal clot firmness (MCF) of extrinsic coagulation pathway (extem). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

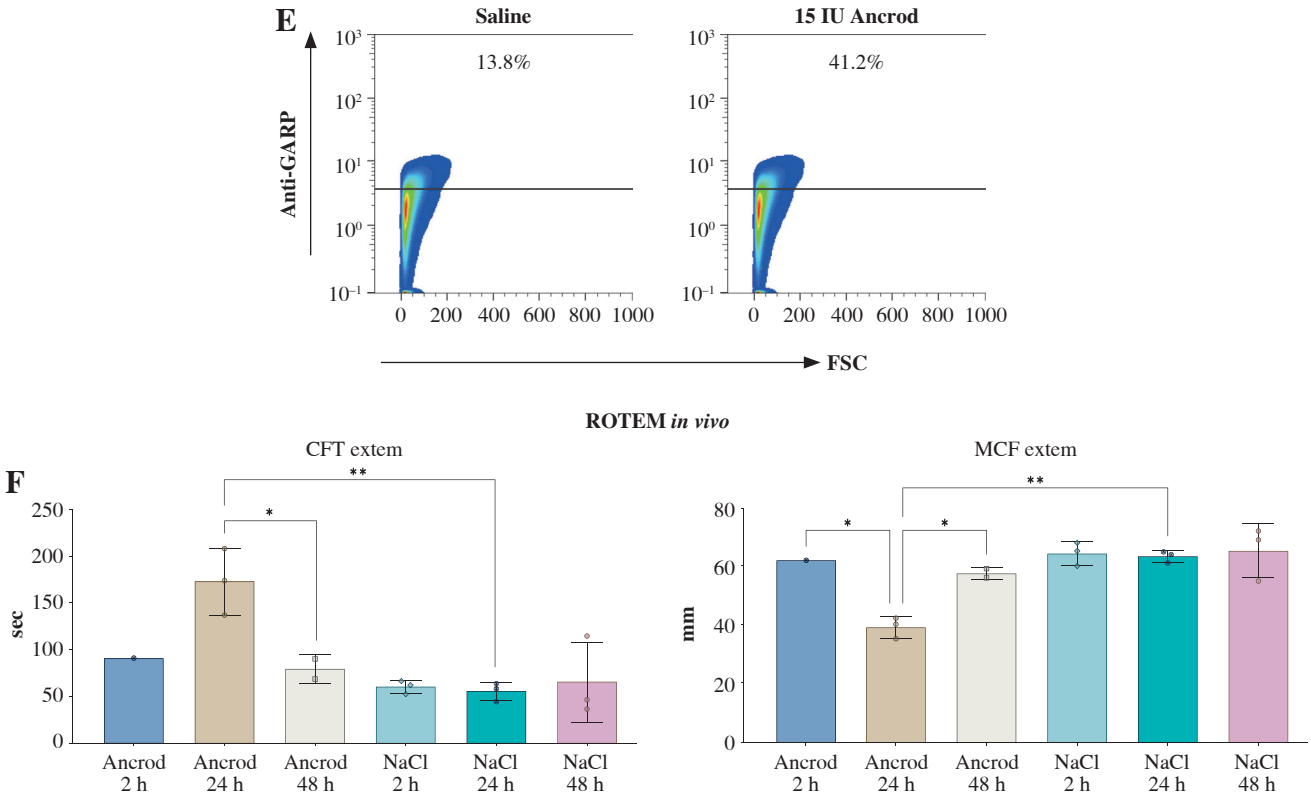


**Fig. 5.** Cont. Exemplary flow plots of *in vitro* platelet data are displayed in **C**, *in vivo* platelet data in **E**. $n = 3$ each group *in vitro*, 3 wells per condition; $n = 24$ each group *in vivo*, 3 wells per animal. Data are presented or mean ± standard deviation (**D**, **F**). ROTEM data of mouse cardiac blood-derived platelets 2, 24, or 48 hours after injection of 8 IU/ml ancrod compared to saline (NaCl) are displayed in **F**) Clot formation time (CFT) and maximal clot firmness (MCF) of extrinsic coagulation pathway (extem). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

crod. The activation-inhibiting effect was observed only for CD44 in the *in vitro* monoculture setting and *in vivo* after 24 hours. This observation makes the involvement of fibrin(ogen) and fibrin degradation products in the ancrod effect on T cells very unlikely, as no blood or plasma component was present in the *in vitro* setting. In the presence of APC (mixed culture), the effect was attenuated.

One possibility could be the involvement of PAR (protease-activated receptor) 4, one of two main receptors for thrombin [57, 58]. Ancrod is a thrombin-like enzyme, and both T cells and Tregs express PAR4 [59]. T cell recruitment is reduced with PAR4 inhibition [60].

In addition, a recent publication suggested that PAR4 signaling inhibits Treg function [61]. This could be confirmed by our group, as PAR4 activation prevented the posttraumatic activation of $CD4^+$ Tregs in the local draining lymph nodes [19].

We therefore hypothesize an ancrod-induced PAR4 signaling effect on $CD4^+$ and $CD8^+$ T cells and $CD4^+$ Tregs/non-Tregs, leading to diminished activation levels of these cells.

To determine why only CD44 shows a significant change after ancrod, one must differentiate between early and late activation markers. CD44 and CD69 are both early lymphocyte activation markers. We were surprised to observe that only CD44 responded significantly to ancrod.

CD44 is an important activation marker which plays a role in enhancing early T cell receptor signaling. After antigen encounter, T cells rapidly up-regulate CD44, and its expression is also maintained in memory T cells [62]. CD69 is a cell surface protein which is rapidly upregulated after lymphocyte activation and expressed on activated T cells, B cells, and NK cells, but not on resting lymphocytes [63]. On the other hand, CD62L (L-selectin) is considered a late activation marker and a key regulator of T cell trafficking as it acts as a homing receptor for lymphocytes to enter secondary lymphoid tissues [64]. Since the measurement was made after only a two-hour incubation with ancrod, the effects on the early activation markers CD44 (significant) and CD69 (nonsignificant) appear plausible to us. Differences between CD44 and CD69 could be based on the different expression patterns (existent on resting cells vs. non-existent).

But why is the *in vivo* effect of ancrod on $CD4^+$ and $CD8^+$ T cells and $CD4^+$ Tregs/non-Tregs inverse? For $CD4^+$ and $CD8^+$ T cells and $CD4^+$ Tregs, the stimulation

reagent anti-CD3ε influenced the impact of ancrod. For $CD4^+$ and $CD8^+$ T cells, the effect was more pronounced in the presence of stimulation, whereas for $CD4^+$ Tregs and non-Tregs it was exactly reversed. This observation may be due to the fact that the activation of T cells leads to a pro-inflammatory response [65] and the activation of immunosuppressive $CD4^+$ Tregs to an anti-inflammatory response [9], making the two cell types antagonists that are regulated via feedback mechanisms.

Ancrod has an overall inhibitory effect on $CD4^+$ and $CD8^+$ T cells; i.e. the actual effect of anti-CD3ε is reversed by ancrod, so it tends to lead to an anti-inflammatory reaction. With $CD4^+$ Tregs, on the other hand, a proinflammatory effect is achieved by inhibiting the immunosuppressive $CD4^+$ Tregs.

Another interesting observation is the different effect on the activation of spleen and lymph node-derived $CD4^+$ Tregs 2 hours after ancrod. For spleen-derived $CD4^+$ Tregs, no significant effect of ancrod was observed after 2 hours in our *in vivo* experiment, which is in line with previous findings of our group [19]. In contrast, basic $CD4^+$ Treg activity within draining lymph nodes was elevated after application of 8 IU of ancrod [19]. As suggested before, these results point more to a local than a systematic effect, in which the interaction between $CD4^+$ Tregs and platelets via PAR4 may play a significant role.

## Platelets

To ascertain why ancrod acts differently *in vitro* and *in vivo* on platelets, one must differentiate between the role of platelets in hemostasis and immunomodulation. Platelets release pro-inflammatory cytokines to communicate with the innate and the adaptive immune system [66] and directly modulate immune cell functions [67, 68].

There is some evidence in the literature concerning the effect of ancrod on platelets. Again, fibrin degradation products are reported to have an inhibitory impact on platelet function [69, 70]. Moreover, platelet aggregation requires fibrinogen [71], which binds to the GPIIb/IIIa complex of the platelet membrane [72]. Therefore, depleting fibrinogen may interfere with the formation of platelet thrombus (white clot) [25].

Also, PAR4 is expressed on mice platelets and is activated by high levels of proinflammatory thrombin, as well as through interaction with PAR3 at low levels [58, 73, 74], suggesting an immunomodulating role of activated platelets [19]. As an important player in immunothrombotic inflammatory interactions [60, 75], PAR4 regulates thrombin-dependent recruitment of pro-inflammatory leukocytes to the surface of platelets on thrombi *via* P-selectin (CD62P) [76]. The latest results of our group implied a platelet-dependent mechanism, as the activation of $CD4^+$ Tregs increases when PAR4 is blocked in the presence of platelet-rich plasma [19].

Our *in vitro* and *in vivo* settings cannot be compared as, for example, molecules originating during ancrod-induced fibrinolysis are reported to be immunomodulating *in vivo*. Furthermore, *in vitro* endothelial cells, which play a crucial role in the function of CD62P and the coagulation cascade, are lacking.

Ancrod does not activate coagulation *in vivo* (no effect on coagulation-activating CD62P within 2-48 hours after injection), as shown by ROTEM (coagulation even inhibited for the first hours after ancrod). However, apparently ancrod induces indirect modulation of $CD4^+$ Treg homeostasis and suppression function *via* increasing expression of GARP. The observed nonsignificant increase in CD63 expression, which occurred after platelet granule release, also suggests a specific immunomodulatory effect that does not affect coagulation. Such an increase in immunosuppression by Tregs could potentially be exploited in the therapy of exaggerated immune responses, such as those seen in SIRS.

This study showed that ancrod significantly and dose-dependently diminished activation (CD62P) of platelets *in vitro* and significantly enhanced activation (GARP) *in vivo.* Again, this suggests one or more components that are missing *in vitro* and present *in vivo.*

Since the plasma was discarded before addition of ancrod *in vitro*, it is unlikely that fibrin(ogen) or FDPs trigger the activation-inhibiting effect, even though this would be consistent with the existing literature [69, 70]. *In vivo*, these FDPs could be of importance.

Therefore, PAR4 involvement mediating platelet-leukocyte communication is more likely. Since any leukocyte and endothelial cell component that is present *in vivo* is absent *in vitro*, these cells could be the crucial factor causing the opposing observations. We observed that ancrod induced decreased CD62P expression *in vitro*, whereas *in vivo* no difference from saline (NaCl) control was observed. In this case, ancrod does not seem to act like thrombin, which upregulates CD62P expression *via* PAR4 for leukocyte recruitment [76]. Since CD62P is crucial for signaling of the $CD4^+$ Treg-platelet interaction [16, 34] and effective $CD4^+$ Treg development and suppressive function on $CD4^+$ non-Treg proliferation [77], $CD4^+$ Tregs could be the missing link in the *in vitro* setup.

Furthermore, glycoprotein-A repetition predominant (GARP) is constitutively expressed on activated platelets and Tregs [78], so platelet activation was induced by rising ancrod doses in our *in vivo* setting. GARP is necessary for platelet TGF-β release, which is crucial to suppress $CD4^+$ and $CD8^+$ T cells. Furthermore, platelet GARP activates the secretion of latent TGF-β1 by other cells [79]. For example, Tregs release TGF-β, and their GARP expression is essential for homeostasis and immunosuppression of Tregs [80, 81]. This also suggests an important role for $CD4^+$ Tregs in the action of ancrod on immune cells and opens the possibility that the effect on $CD4^+$ and $CD8^+$ cells could be indirectly mediated by enhanced ancrod-in-

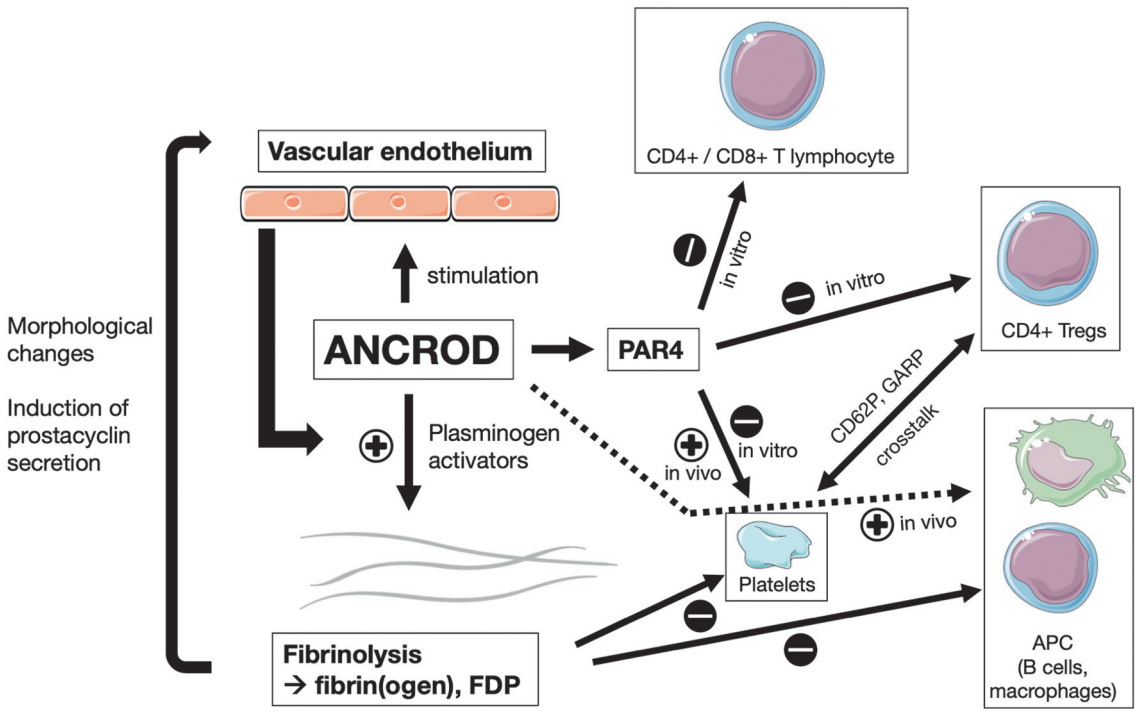


**Fig. 6.** Schematic summary. Scheme of potential effect mechanism of ancrod mediated by fibrin, fibrinogen and fibrin degradation products (FDP), protease-activated receptor (PAR) 4, and vascular endothelium. Ancrod induces elevated levels of fibrin degradation products and activates the fibrinolytic system by initiating the release of plasminogen activators from the vascular endothelium. *Via* PAR4, additional immunomodulating effects on CD4+, CD8+ T cells, CD4+ Tregs and non-Tregs, antigen-presenting cells (APC), and platelets could be conveyed. The figure contains adapted graphics from Les Laboratoirs Servier – Medical Art under the terms of the Creative Commons Attribution License (CC BY) for non-commercial use. The use, distribution, or reproduction in other forums is permitted: https://creativecommons.org/licenses/by/3.0/legalcode, last accessed March 4, 2022

duced CD4+ Treg suppression. Future experiments analyzing GARP expression following ancrod exposure would be interesting to elucidate this potential mechanism.

## Limitations

For this study, leukocytes from the spleen and not from lymph nodes were used, as only a significantly smaller number of cells could have been obtained with these than with spleens ($1 \times 10^6$ vs. $1 \times 10^8$). Thus, in line with the 3Rs principle (replace, reduce, refine), significantly fewer animals were needed. The same principle explains the relatively small *n* for the *in vitro* experiments. Cells used in these experiments were of murine origin, which raises the old question of transferability to human physiology and pathology. Since this study represents an initial screening, it was not justifiable for ethical reasons to conduct experiments directly on humans, especially for the *in vivo* experiments. Furthermore, it is very likely that the studies mentioned used the identical ancrod as our group, so the comparability of results is probably compromised to some extent. In particular, the progress in laboratory technology that has taken place since the 1960s must be taken into account here.

## Conclusions

Overall, the results of the present study imply cell-specific immunomodulatory effects of ancrod on different immune cells such as CD4+, CD8+ T cells, CD4+ Tregs and non-Tregs, APCs, and platelets, which could be mediated by a complex multifactorial mechanism.

We postulate and discuss different underlying mechanisms potentially associated with the described cell-specific immunomodulatory effects of ancrod. Firstly, molecules originating during ancrod-induced fibrinolysis are reported to be immunomodulating. Secondly, platelets and vascular endothelial cells, which are also modulated by fibrinolysis byproducts, do have an influence on this process themselves and could represent a key component as they were not included in our *in vitro* setting. Thirdly, we postulate and discuss PAR4 as an important receptor involved in ancrod signaling, since it is known to be expressed on all

of our investigated cells and exhibits immunomodulatory effects. The postulated underlying mechanisms associated with cell-specific immunomodulatory effects of ancrod need further studies. Results and potential postulated mechanisms are summarized in Figure 6.

These findings emphasize the potential of ancrod in selectively modulating immune cells, making it a suitable candidate for further investigation and potential therapeutic applications.

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## Disclosures

Approval of the Bioethics Committee was not required. The authors declare no conflict of interest.

### References


1. Moore FA, Moore E, Read R (1993): Postinjury multiple organ failure: role of extrathoracic injury and sepsis in adult respiratory distress syndrome. New Horiz 1: 538-549.
2. Lenz A, Franklin GA, Cheadle WG (2007): Systemic inflammation after trauma. Injury 38: 1336-1345.
3. Liu V, Escobar GJ, Greene JD, et al. (2014): Hospital deaths in patients with sepsis from 2 independent cohorts. JAMA 2014; 312: 90-92.
4. Matzinger P (1994): Tolerance, danger, and the extended family. Annu Rev Immunol 12: 991-1045.
5. Gallucci S, Matzinger P (2001): Danger signals: SOS to the immune system. Curr Opin Immunol 13: 114-119.
6. Raymond SL, Holden DC, Mira JC, et al. (2017): Microbial recognition and danger signals in sepsis and trauma. Biochim Biophys Acta Mol Basis Dis 1863: 2564-2573.
7. Venet F, Chung CS, Kherouf H, et al. (2009): Increased circulating regulatory T cells (CD4+ CD25+ CD127–) contribute to lymphocyte anergy in septic shock patients. Intensive Care Med 35: 678-686.
8. MacConmara MP, Maung AA, Fujimi S, et al. (2006): Increased CD4+ CD25+ T regulatory cell activity in trauma patients depresses protective Th1 immunity. Ann Surg 244: 514-523.
9. Sakaguchi S, Sakaguchi N, Asano M, et al. (1995): Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 155: 1151-1164.
10. Stoecklein VM, Osuka A, Lederer JA (2012): Trauma equals danger – damage control by the immune system. J Leukoc Biol 92: 539-551.
11. Heuer JG, Zhang T, Zhao J, et al. (2005): Adoptive transfer of in vitro-stimulated CD4+CD25+ regulatory T cells increases bacterial clearance and improves survival in polymicrobial sepsis. J Immunol 174: 7141-7146.
12. Venet F, Chung CS, Monneret G, et al. (2008): Regulatory T cell populations in sepsis and trauma. J Leukoc Biol 83: 523-535.
13. Murphy TJ, Choileain NN, Zang Y, et al. (2005): CD4+ CD25+ regulatory T cells control innate immune reactivity after injury. J Immunol 174: 2957-2963.
14. Tatura R, Zeschnigk M, Hansen W, et al. (2015): Relevance of Foxp3$^+$ regulatory T cells for early and late phases of murine sepsis. Immunology 146: 144-156.
15. Hanschen M, Tajima G, O'Leary F, et al. (2011): Injury induces early activation of T cell receptor signaling pathways in CD4+ regulatory T cells. Shock 35: 252-257.
16. Bergmann CB, Hafele F, Unger M, et al. (2016): Platelets modulate the immune response following trauma by interaction with CD4+ T regulatory cells in a mouse model. Immunol Res 64: 508-517.
17. Zhu L, Huang Z, Stĺlesen R (2014): Platelets provoke distinct dynamics of immune responses by differentially regulating CD4+ T-cell proliferation. J Thromb Haemost 12: 1156-1165.
18. Liu CY, Battaglia M, Lee SH, et al. (2005): Platelet factor 4 differentially modulates CD4+CD25+ (regulatory) versus CD4+CD25- (nonregulatory) T cells. J Immunol 174: 2680-2686.
19. Bock M, Bergmann CB, Jung S, et al. (2021): Platelets differentially modulate CD4+ Treg activation via GPIIa/IIIb-, fibrinogen-, and PAR4-dependent pathways. Immunol Res 70: 185-196.
20. Reid HA, Chan K, Thean P (1963): Prolonged coagulation defect (defibrination syndrome) in Malayan viper bite. Lancet 1: 621-626.
21. Dormandy JA, Reid HL (1978): Controlled defibrination in the treatment of peripheral vascular disease. Angiology 29: 80-88.
22. Bell W, Shapiro SS, Martinez J, et al. (1978): The effects of ancrod, the coagulating enzyme from the venom of Malayan pit viper (A. rhodostoma) on prothrombin and fibrinogen metabolism and fibrinopeptide A release in man. J Lab Clin Med 91: 592-604.
23. Pizzo SV, Schwartz ML, Hill RL, McKee PA (1972): Mechanism of ancrod anticoagulation: A direct proteolytic effect on fibrin. J Clin Invest 51: 2841-2850.
24. Lowe G, Morrice JJ, Forbes CD, et al. (1979): Subcutaneous ancrod therapy in peripheral arterial disease: Improvement in blood viscosity and nutritional blood flow. Angiology 30: 594-599.
25. Soutar R, Ginsberg J (1993): Anticoagulant therapy with ancrod. Crit Rev Oncol Hematol 15: 23-33.
26. Khan S, Gul A, Noreen R, et al. (2018): Potential applications of venom peptides as anti-thrombotic agents for management of arterial and deep-vein thrombosis. Protein Peptide Lett 25: 677-687.
27. Cole C, Fournier L, Bormanis J (1990): Heparin-associated thrombocytopenia and thrombosis: optimal therapy with ancrod. Can J Surg 33: 207-210.
28. Thomson NM, Moran J Simpson IJ, et al. (1976): Defibrination with ancrod in nephrotoxic nephritis in rabbits. Kidney Int 10: 343-347.
29. Cole EH, Glynn MF, Laskin CA, et al. (1990): Ancrod improves survival in murine systemic lupus erythematosus. Kidney Int 37: 29-35.
30. Lathan LO, Staggers SL (1996): Ancrod: The use of snake venom in the treatment of patients with heparin-induced thrombocytopenia and thrombosis undergoing coronary artery bypass grafting: Nursing management. Heart Lung 25: 451-460.
31. Kilkenny C, Browne WJ, Cuthill I, et al. (2010): Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol 8: e1000412.
32. Hanschen M, Tajima G, O'Leary F, et al. (2012): Phospho-flow cytometry based analysis of differences in T cell receptor signaling between regulatory T cells and CD4+ T cells. J Immunol Methods 376: 1-12.

33. Bock M, Bergmann CB, Jung S, et al. (2018): The posttraumatic activation of CD4+ T regulatory cells is modulated by TNFR2-and TLR4-dependent pathways, but not by IL-10. Cell Immunol 331: 137-145.
34. Rupp MC, Bergmann CB, Jung S, et al. (2021): The posttraumatic response of CD4+ regulatory T cells is modulated by direct cell-cell contact via CD40L-and P-selectin-dependent pathways. Cent Eur J Immunol 46: 283-294.
35. Thebault S, Ochoa-Garay J (2004): Characterization of TCR-induced phosphorylation of PKCθ in primary murine lymphocytes. Mol Immunol 40: 931-942.
36. Hayashi K, Altman A (2007): Protein kinase C theta (PKCθ): a key player in T cell life and death. Pharmacol Res 55: 537-544.
37. Rothfork JM, Dessus-Babus S, Van Wamel WJB, et al. (2003): Fibrinogen depletion attenuates Staphyloccocus aureus infection by preventing density-dependent virulence gene up-regulation. J Immunol 171: 5389-5395.
38. Aktories K, Flockerzi V, Förstermann U, et al. (2017): Allgemeine und spezielle Pharmakologie und Toxikologie: Begründet von W. Forth, D. Henschler, W. Rummel. Elsevier Health Sciences.
39. Waisman DM (2003): Plasminogen: Structure, activation, and regulation. Springer Science & Business Media.
40. Soszka T, Kirschbaum NE, Stewart GJ, Budzynski AZ (1988): Effect of fibrinogen-clotting enzymes on secretion of plasminogen activators from cultured human endothelial cells. Fibrinolysis 2: 49-57.
41. Ashford A, Ross J, Southgate P (1968): Pharmacology and toxicology of a defibrinating substance from Malayan pit viper venom. Lancet 1: 486-489.
42. Levy GA, MacPhee PJ, Fung LS, et al. (1983): The effect of mouse hepatitis virus infection on the microcirculation of the liver. Hepatology 3: 964-973.
43. Kim S, Wadhwa NK, Kant KS, et al. (1988): Fibrinolysis in glomerulonephritis treated with ancrod: renal functional, immunologic and histopathologic effects. Q J Med 69: 879-905.
44. Smiley ST, King JA, Hancock WW (2001): Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. J Immunol 167: 2887-2894.
45. Chang MC, Jeng JH, Cheong TC, et al. (1995): The morphologic change of endothelial cells by ancrod-generated fibrin is triggered by αv β3 integrin binding and the subsequent activation of a G-protein coupled phospholipase C. Biochim Biophys Acta 1269: 115-121.
46. Chang MC, Yang RS, Lin CH, et al. (1996): Integrin αvβ3 and phospholipase C regulate prostacyclin formation of endothelial cells caused by ancrod-generated fibrin. Eur J Pharmacol 297: 129-136.
47. Edgington T, Curtiss L, Plow E (1985): A linkage between the hemostatic and immune systems embodied in the fibrinolytic release of lymphocyte suppressive peptides. J Immunol 134: 471-477.
48. Girmann G, Pees H, Schwarze G, et al. (1976): Immunosuppression by micromolecular fibrinogen degradation products in cancer. Nature 259: 399-401.
49. Gerdin B, Saldeen T, Roszkowski W, et al. (1980): Immunosuppressive effect of vasoactive peptides derived from human fibrinogen. Thromb Res 18: 461-468.
50. Edgington T, Curtiss L, Plow E (1977): The immunosuppressive activity of plasmic degradation products of human fibrinogen. Thromb Haemostasis 38.
51. Dang CV, Bell WR, Kaiser D, et al. (1985): Disorganization of cultured vascular endothelial cell monolayers by fibrinogen fragment D. Science 227: 1487-1490.
52. Tsumagari T, Tanaka K (1984): Effects of fibrinogen degradation products on glomerular mesangial cells in culture. Kidney Int 26: 712-718.
53. Richardson D, Pepper D, Kay A (1976): Chemotaxis for human monocytes by fibrinogen-derived peptides. Br J Haematol 32: 507-514.
54. Cole E, Schulman J, Urowitz M, et al. (1985): Monocyte procoagulant activity in glomerulonephritis associated with systemic lupus erythematosus. J Clin Invest 75: 861-868.
55. Zulys VJ, Teasdale SJ, Michel ER, et al. (1989): Ancrod (Arvin) as an alternative to heparin anticoagulation for cardiopulmonary bypass. Anesthesiology 71: 870-877.
56. Lowe G (1984): Blood rheology and venous thrombosis. Clin Hemorheol Microcirc 4: 571-588.
57. Kahn ML, Zheng YW, Huang W, et al. (1998): A dual thrombin receptor system for platelet activation. Nature 394: 690-694.
58. Ma L, Dorling A (2012): The roles of thrombin and protease-activated receptors in inflammation. Semin Immunopathol 34: 63-72.
59. Shpacovitch V, Feld M, Hollenberg MD, et al. (2008): Role of protease-activated receptors in inflammatory responses, innate and adaptive immunity. J Leukoc Biol 83: 1309-1322.
60. Mende K, Reifart J, Rosentreter D, et al. (2014): Targeting platelet migration in the postischemic liver by blocking protease-activated receptor 4. Transplantation 97: 154-160.
61. Peng Q, Ratnasothy K, Boardman DA, et al. (2019): Protease activated receptor 4 as a novel modulator of regulatory T cell function. Front Immunol 10: 1311.
62. Schumann J, Stanko K, Schliesser U, et al. (2015): Differences in CD44 surface expression levels and function discriminates IL-17 and IFN-γ producing helper T cells. PLoS One 10: e0132479.
63. Sava F, Toldi G, Treszl A, et al. (2016): Expression of lymphocyte activation markers of preterm neonates is associated with perinatal complications. BMC Immunol 17: 19.
64. Cibrián D, Sánchez-Madrid F (2017): CD69: from activation marker to metabolic gatekeeper. Eur J Immunol 47: 946-953.
65. Brinkhoff A, Siebertchs A, Engler H, et al. (2018): Pro-inflammatory Th1 and Th17 cells are suppressed during human experimental endotoxemia whereas anti-inflammatory IL-10 producing T-cells are unaffected. Front Immunol 9: 1133.
66. Semple JW, Italiano JE, Freedman J (2011): Platelets and the immune continuum. Nat Rev Immunol 11: 264-274.
67. von Brühl ML, Stark K, Steinhart A, et al. (2012): Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med 209: 819-835.
68. Page C, Pitchford S (2013): Neutrophil and platelet complexes and their relevance to neutrophil recruitment and activation. Int Immunopharmacol 17: 1176-1184.
69. Kowalski E, Kopeć M, Wegrzynowicz Z (1963): Influence of fibrinogen degradation products (FDP) on platelet aggregation, adhesiveness and viscous metamorphosis. Thromb Haemost 10: 406-423.
70. Jerushalmy Z, Zucker M (1966): Some effects of fibrinogen degradation products (FDP) on blood platelets. Thromb Haemost 15: 413-419.
71. Niewiarowski S, Budzynski A, Lipinski B (1977): Significance of the intact polypeptide chains of human fibrinogen in ADP-induced platelet aggregation. Blood 49: 635-644.
72. Peerschke E (1985): The platelet fibrinogen receptor. Semin Hematol 22: 241-259.
73. Coughlin SR (2000): Thrombin signalling and protease-activated receptors. Nature 407: 258-264.
74. Estevez B, Du X (2017): New concepts and mechanisms of platelet activation signaling. Physiology 32: 162-177.

75. Fender AC, Rauch BH, Geisler T, et al. (2017): Protease-activated receptor PAR-4: an inducible switch between thrombosis and vascular inflammation? Thromb Haemost 117: 2013-2025.
76. Kaplan ZS, Zarpellon A, Alwis I, et al. (2015): Thrombin-dependent intravascular leukocyte trafficking regulated by fibrin and the platelet receptors GPIb and PAR4. Nat Commun 6: 7835.
77. Kohm AP, Miller SD (2003): Role of ICAM-1 and P-selectin expression in the development and effector function of CD4+ CD25+ regulatory T cells. J Autoimmun 21: 261-271.
78. Tran DQ, Andersson J, Wang R, et al. (2009): GARP (LRRC32) is essential for the surface expression of latent TGF-β on platelets and activated FOXP3+ regulatory T cells. Proc Natl Acad Sci U S A 106: 13445-13450.
79. Rachidi S, Metelli A, Riesenberg B, et al. (2017): Platelets subvert T cell immunity against cancer via GARP-TGFβ axis. Sci Immunol 2: eaai7911.
80. Shevach EM (2017): Garp as a therapeutic target for modulation of T regulatory cell function. Expert Opin Ther Targets 21: 191-200.
81. Wang R, Wan Q, Kozhaya L, et al. (2008): Identification of a regulatory T cell specific cell surface molecule that mediates suppressive signals and induces Foxp3 expression. PLoS One 3: e2705.