Università degli Studi del Piemonte Orientale "Amedeo Avogadro" and RBM/Merck Serono International S.A

Dottorato di Ricerca
in
Medicina Molecolare
Ciclo XX

Relazione IV° anno

TITOLO:

Characterization of PTPH1 knockout mice in animal models of pain and inflammation

Candidato: Claudia Patrignani
MerckSerono Tutor: Valeria Muzio
Academic Tutor: Andrea Graziani

SEZIONE 1
RISULTATI SCIENTIFICI

INTRODUCTION

Tyrosine phosphorylation plays an important role in several signaling pathways regulating cell growth, differentiation, cell cycle, apoptosis, inflammation and pain [1,2]. The phosphorylation/dephosphorylation balance is controlled by protein tyrosine kinases and phosphatases. PTPs can be distinguished into four classes: 1) classical PTPs that can be subdivided into transmembrane, receptor-like enzymes, and the intracellular, nonreceptor PTPs, 2) dual-specificity PTPs (Ser and Tyr phosphatases), 3) low molecular weight PTP and 4) the Asp-based PTPs (Tyr/Ser phosphatase activity) [3].

During my 4th year PhD I focused my attention on a NRPTP, PTPH1, and on its possible role in animal models of pain and inflammation. PTPH1 (also called PTPN3) belongs to a sub-family of non receptor cytosolic PTPs characterized by the presence of a FERM domain (band 4.1, ezrin, radixin, moesin) at its N-terminus, responsible for the interaction with transmembrane proteins and/or phospholipids in the cell membrane [4-6]. In addition PTPH1 has a PDZ domain in the central part responsible for the interaction with other proteins, whereas the single catalytic domain is located at the C-terminus.

PTPH1 activity has been involved in a variety of functions including cell cycle regulation [7-9], endoplasmic reticulum assembly [10], cardiac sodium channel modulation [11] and TNFα converting enzyme inhibition [12]. Our group recently demonstrated PTPH1 functionality in cognitive functions. Indeed PTPH1 is expressed hippocampus, cerebellum, thalamus and retrosplenial cortex, key regions for cognition, and PTPH1-KO mice display altered rotarod and Y-maze performances, possibly due to a deregulation of the GH-IGF1 axis[13].

PTPH1 is also known to be involved in the inflammatory response and in particular in the TCR-mediated response. Overexpression of PTPH1 in the Jurkat T cell leukemia cell line inhibits TCR signal transduction leading to activation of the promoter for the T cell growth-promoting cytokine, IL-2[14]. Moreover the FERM domain-deleted

mutant of PTPH1 is impaired in its ability to inhibit TCR-induced IL-2 promoter activity coincident with an inability of this mutant to localize to the plasma membrane[15]. This finding suggests that PTPH1 inhibits TCR signaling in Jurkat by dephosphorylating a plasma membrane-localized substrate. A recent study indicates that one important target of PTPH1 is the TCRζ chain. In this regard, PTPH1 was identified as the only PTP able of interacting physically with TCRζ and dephosphorylating TCRζ ITAMs in vitro[16]. This finding has contributed to the notion that PTPH1 acts as negative regulator of TCR signaling by dephosphorylating the TCRζ chain in vitro. A recent study on *ex vivo* samples from PTPH1-KO mice, lacking the phosphatase domain, demonstrates no alteration in the TCR-induced signal transduction, cytokine production, and proliferation [17].

To better characterize *in vivo* PTPH1 role in TCR signaling and inflammation, the present study investigates two different animal models (carrageenan-induced and a LPS-induced inflammation) in PTPH1-WT and KO female mice.

MATERIAL AND METHODS

Animals

PTPH1-KO mice were generated as described in detail elsewhere[18]. The experiments were performed on adult female mice PTPH1-WT and KO individually housed in top filter cages with free access to food and water, under controlled temperature (22±2°C), and relative humidity (55±10%), on a 12:12 h light-dark cycle. Animals were allowed to acclimate for 1 week before the beginning of the experiments. All behavioral tests were performed during the light phase and animals were allowed 1-hour habituation to the test room, if different from the holding room, before testing. Testing sequence was randomized between KO and WT animals, and all apparatus were thoroughly cleaned between two consecutive test sections.

Carrageenan-induced inflammation

Female PTPH1-KO and WT mice (3 months old) were tested for inflammation-induced edema and hyperalgesia/allodynia. On the test day n=7-8 animals per genotype were injected subcutaneously in the right hind paw plantar surface with 30μL of a solution of 2% carrageenan λ (Sigma, Germany) freshly prepared in saline. 30μL of saline was injected as control in the controlateral paw. Animals were tested at Hargreaves and automated Von Frey apparatus, to evaluate respectively thermal and tactile hyperalgesia, at 1, 3, 5 and 24 hours after carrageenan/saline injection, followed by paw thickness measurement, using a precision caliper (Mitutoyo, Japan). Mice underwent also to a Catwalk analysis at the same time points. Mice were sacrificed by an intraperitoneal (ip) overdose of thiopental, and paws were removed for histological evaluation.

Hargreaves' plantar test

Thermal hyper/hypoalgesia was assessed by Hargreaves' plantar apparatus (Plantar test, Ugo Basile, Italy)[19].The test was performed at 1, 3, 5 and 24 hours post 2% carrageenan injection. Animals were accustomed to the apparatus for 1 hour for 2 days preceding the test. On the test day, animals were individually placed in a clear acrylic box on a glass platform and a removable infrared generator (radiant heat 137mW/cm^2/s) was placed underneath the animal's hind paw. The apparatus automatically detects the withdrawal of the paw. Latency of each paw withdrawal was recorded and mean values of left and right paw used as reaction index for the individual animal. A cut-off of 25 seconds was used to avoid tissue damage in case of absence of response.

Automated Von Frey test

Tactile hyper/hypoalgesia was assessed by a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). The test was performed immediately after the Hargreaves's test. Animals were accustomed to the apparatus for 1 hour, for 2 days preceding the test day. On the test day, were individually placed in a clear acrylic box with a grid floor. A blunted probe was placed under the plantar surface of one of the hind paw and automatically exerted a constantly increasing force to the plantar surface (from 0 up to 5 grams over 20 s). Force applied (g) at the retraction reflex was automatically recorded. Each hind paw was tested 3 times and mean values used as individual parameter for group statistic.

CatWalk

Detailed analysis of gait was performed on walking mice using the CatWalk™ (Noldus Information Technology) method[20,21]. Briefly, light from a fluorescent tube is sent through a glass plate. Light rays are completely reflected internally. As soon as mouse's paw is in contact with the glass surface, light is reflected downwards. It results in a sharp image of a bright paw print. The whole run is recorded via a camera placed under the glass plate.

In the present study, parameters related to single paws were analyzed:

• *Intensity* (expressed in arbitrary units in the range 0-255): this parameter describes the mean pressure exerted by one individual paw during the floor contact, during the whole crossing of the walkway. The intensity parameter is highly correlated with the Von Frey thresholds [22].

• *Duty cycle* (expressed in %): the duty cycle represents stance duration as a percentage of step cycle duration. It is calculated according to the formula: stand duration/[stand + swing phases duration]×100, where the stand phase is indicated as the time of contact (in seconds) of one paw with the glass plate in a single step cycle and the swing phase is indicated as seconds of non-contact with the plate during a step cycle. The duty cycle parameter is highly correlated with the Von Frey thresholds[22].

• *Maximum contact area* (expressed in mm2): the maximal contact area describes the paw area contacted at the moment of maximal paw-floor contact, during stand phase.

• *Print area* (expressed in mm2): this parameter describes the surface area of the complete paw print during the stance phase.

LPS-induced inflammation

PTPH1-WT and KO female mice (n=3, 2 months old) received an ip injection of 1mg/kg of LPS (Escherichia coli 0127:B8, batch 032K4099, L3880, Sigma) and randomized

groups of mice were sacrificed by an ip overdose of thiopental at 30, 60 and 180 minutes after LPS injection.

Cytokine beads assay (CBA)

At sacrifice whole blood was collected from the heart of the animals and plasma was obtained by centrifugation. 25 µl of plasma were used to quantify the amount of cytokines by a inflammation CBA kit for mouse (BD Bioscience).

RTPCR on PBMC

From whole blood, red blood cells were lysed with BD PharM Lyse (BD Biosciences/BD Pharmingen) and RNA was extracted using TriZol (Invitrogen). Taqman analysis was performed on several cytokines genes: ccl2 (#Mm00441242_m1, Applied Biosystems), IL1b (#Mm01336189_m1, Applied Biosystems), IL12a (#Mm00434169_m1, Applied Biosystems), IL6 (#Mm00446190_m1, Applied Biosystems), TNF (#Mm00443258_m1, Applied Biosystems). The PCR analysis was carried out also on PTPH1-related genes: adam17 (#Mm00456428_m1, Applied Biosystems), ghr (#Mm00439093_m1, Applied Biosystems), IGF1 (#Mm00439560_m1, Applied Biosystems), IGF1R (#Mm01318459_m1, Applied Biosystems). 200ng of total RNA were used to perform the RT-PCR reaction (SuperScript II RT kit, Invitrogen). The qPCR experiment was carried out using the Taqman Universal PCR master mix (Applied Biosystems) and the gene assays mentioned above.

Statistical analysis

Statistical comparisons were performed by Two-way Anova followed by T-test and Bonferroni's post-hoc analysis ($p<0.05$) at each time points. Results are expressed as mean±SEM.

RESULTS

Carrageenan (CARR)-induced inflammation

No complication after injection of 2% carrageenan in the right paw of the mice was observed. All the animals stayed alive until the end of the experiment.

Paw thickness- A significantly higher paw thickness has been measured by a precision caliper in the CARR-treated paws, compared to the controlateral vehicle treated one. This difference is statistically significant in both WT and KO groups and is detectable already after 1 hour from carrageenan injection. The edema is still present 24 hours post- carrageenan injection (PTPH1-WT: P_{1h}=0.0028; P_{3h}=0.0022; P_{1h}=0.0049; P_{1h}=0.0006) (PTPH1-KO: P_{1h}=0.0001; P_{3h}=0.0002; P_{1h}=0.0002; P_{1h}=0.0003) (Fig.1). No statistical differences in paw thickness have been detected in PTPH1-WT versus PTPH1-KO animals.

Hargreaves's test- A significantly decreased response at the Hargreaves'test is detected in the CARR-treated paws compared to the controlateral vehicle treated one. This decrease in withdrawal time is statistically significant in both WT and KO groups, it was detectable already at 1 hour after carrageenan injection through 24 h maintaining the same intensity (PTPH1-WT: P_{1h}=0.00004; P_{3h}=0.0122; P_{1h}=0.0016; P_{1h}=0.0039) (PTPH1-KO: P_{1h}=0.0001; P_{3h}=0.00001; P_{1h}=0.0005; P_{1h}=0.0005) (Fig.2). No statistical differences in withdrawal time have been detected in PTPH1-WT versus PTPH1-KO animals.

Von Frey test- A significantly decreased response at the Von Frey test is detected in the CARR-treated paws compared to the controlateral vehicle treated one. This decrease in withdrawal force is statistically significant in both WT and KO groups, it is detectable already at 1 hour after carrageenan injection through 24 h maintaining the same intensity till 24 hours post-injection (PTPH1-WT: P_{1h}=0.017; P_{3h}=0.0002; P_{1h}=0.001621; P_{1h}=0.002458) (PTPH1-KO: P_{1h}=0.004; P_{3h}=0.00977; P_{1h}=0.001272; P_{1h}=0.007833) (Fig.3). No statistical differences in withdrawal force have been detected in PTPH1-WT versus PTPH1-KO animals.

CatWalk test

Intensity- The carrageenan injection provokes a decrease of the intensity parameter in PTPH1-WT group 1 hour post-injection ($P_{WT\ 1h}$<0.05). No significant difference is observed at this early time point in the PTPH1-KO group between CARR-treated and vehicle-treated paws. No differences have been detected between WT and KO mice. No differences in intensity due either to treatment or to genotype are detectable at 3 hours post CARR-injection. A significant decrease in intensity is detectable in the CARR-

treated paws compared to vehicle-treated one in both WT and KO mice at 5 and 24 hours after carrageenan injection (PTPH1-WT: P_{5h}, P_{24h} <0.05) (PTPH1-KO: P_{5h}, P_{24h} <0.05). No differences in the intensity at 5 and 24 hours post-injection are genotype-related (Fig.4).

Duty cycle- In the WT group, a slight significant decrease in duty cycle is detectable in the CARR-treated paws compared to the vehicle-treated ones, already 1 hour after CARR-injection ($P_{WT\ 1h}$<0.05). At this time point, no significant differences have been disclosed either in the KO mice group or between WT and KO mice. No differences in duty cycle due either to treatment or to genotype are detectable at 3 hours post CARR-injection. At 5 hours post CARR-injection, PTPH1-KO CARR-treated paws display a significant decreased duty cycle compared to the controlateral vehicle-treated one ($P_{KO\ 5h}$<0.01), but not compared to the WT CARR-treated. This difference is maintained in KO mice, CARR*vs*vehicle, also at 24 hours post-injection ($P_{KO\ 24h}$<0.05), and it is detectable also in WT mice group (CARR*vs*vehicle $P_{WT\ 24h}$<0.05) (Fig.5).

Maximum contact area (max area)- In WT group, no differences in maximum contact area are detectable in CARR*vs*vehicle treated paws. KO CARR-treated paws display a significant decrease in max area compared to the controlateral vehicle-treated paws only at 5 hours post-injection ($P_{KO\ 5h}$<0.05). No other differences due to treatment or to genotype are detectable (Fig. 6).

Print area- No differences in print area due to either treatment or genotype are detectable at 1 and 3 hours post CARR-injection. At 5 and 24 hours post-injection KO CARR-treated paws show a significant decreased print area compared to controlateral vehicle-treated paws ($P_{KO\ 5h}$<0.05; $P_{KO\ 24h}$<0.05). No differences have been detected in WT CARR-treated *vs* vehicle treated paws at 5 hours post-injection, but a non-significant decreased print area was present in WT CARR-treated *vs* vehicle paws at 24 hours time point ($P_{WT\ 24h}$=0.0642) (Fig. 7).

LPS-induced inflammation

No complication after ip injection of 1mg/kg LPS on PTPH1-WT and KO female mice was observed. All the animals stayed alive until the end of the experiment.

Cytokine Beads Assay- The mouse inflammation CBA kit allows the analysis of 6 cytokines: TNFα, MCP-1, IL-6, IL-10, IFN-γ, IL-12p70. IFN-γ and IL-12p70 values has been recorded below detection limit and were excluded from the final analysis.

TNF overall release is increased in LPS-treated mice compared to vehicle-treated ones 30 minutes post LPS injection (P_{2way}=0.0199), but Bonferroni's post hoc test reveals no significant differences in PTPH1-WT and KO group either due to treatment or to genotype (Fig. 8).

MCP-1 levels in plasma do not change at 30 minutes post LPs injection in PTPH1-WT and KO mice.

IL6 overall release is significantly higher in the plasma of LPS-treated compared to vehicle-treated mice 30 minutes post LPS injection (P_{2way}=0.011), and Bonferroni's post hoc test shows a significant treatment-related increase in PTPH1-WT mice group LPS*vs*vehicle treated (P_{WT}<0.05). No treatment-related differences have been recorded in PTPH1-KO mice group. No genotype-related differences in IL6 release have been detected in LPS*vs*vehicle treated animals.

IL10 levels in plasma do not change at 30 minutes post LPs injection in PTPH1-WT and KO mice.

At 60 minutes post LPS/vehicle injection TNFα, MCP-1, IL-6 and IL-10 release is highly significant increased in the plasma of LPS-treated compared to vehicle-treated mice (Fig. 9). No genotype-related differences is detected in TNFα, IL-6 and IL-10 plasma levels, but Bonferroni's post hoc analysis reveals a significantly higher MCP-1 plasma level of PTPH1-WT *vs* KO LPS-treated mice (P_{WtvsKO}=0.014).

At 180 minutes post LPS/vehicle injection, TNFα, MCP-1, IL-6 and IL-10 overall release is highly significant increased in the plasma of LPS-treated compared to vehicle-treated mice, furthermore MCP-1 and IL-6 levels in LPS-treated mice is above detection limit (Fig. 10). Bonferroni's post hoc analysis shows that TNFα and IL-10 levels are significantly higher in PTPH1-KO LPS*vs*vehicle treated mice (P_{TNF} =0.0032; $P_{IL\text{-}10}$ =0.0004), but not in the PTPH1-WT mice group (Fig. 11).

RT-PCR on cytokine-related genes- RT-PCR on PBMC analysis has been carried out on the genes encoding for the cytokines already analyzed by CBA and also for other cytokine genes of interest.

TNF gene expression levels are slightly increased in LPS-treated mice compared to vehicle-treated ones 30 minutes post treatment (mpt) (Fig. 12a), and this trend becomes significant for PTPH1-KO mice group at 60 mpt ($P_{KO\ 60mpt}<0.01$), when KO LPS-treated mice display a 10 fold TNF expression increase compared to KO vehicle-treated age-matched animals (Fig 12b). At 180 mpt, both WT and KO LPS-treated mice show a significantly higher TNF gene expression level compared to their respective vehicle-treated animals, displaying respectively a 5.5 (PTPH1-WT LPS *vs* vehicle) and 6.4 (PTPH1-KO LPS *vs* vehicle) fold increase (Fig. 12c). No genotype-related differences in TNF expression have been recorded.

Ccl2 is MCP-1 encoding gene. Ccl2 gene expression is not altered at 30 mpt (data not shown) and an overall trend in increasing ccl2 level is detectable in LPS-treated compared to vehicle treated mice at 60 mpt (Fig. 13a). At 180 mpt post hoc analysis reveals a significant 15-fold increased ccl2 gene expression in PTPH1-WT LPS-treated mice compared to vehicle-treated ones ($P<0.05$). On the other hand a trend in increased ccl2 expression (12x) is detectable in PTPH1-KO LPS *vs* vehicle –treated mice ($P=0.09$) (Fig. 13b). No genotype-related differences in ccl2 expression have been recorded.

IL12A gene encodes for IL12p35 subunit of IL12p70 cytokine. At 30 mpt no differences in IL12A was recorded in PTPH1-WT and KO treated and untreated mice (Fig 14a). At 60 mpt no changes in IL12A expression level was detected in PTPH1-WT PBMC, but a trend in decrease of its expression was recorded in PTPH1-KO mice (Fig. 14b). Indeed a genotype-related difference ($P<0.05$) is present in WT *vs* KO LPS-treated mice. At 180 mpt an overall increase of IL12A has been detected in LPS treated *vs* vehicle-treated mice. This increase is statistically significant for PTPH1-WT mice group and not for KO mice group (Fig. 14c). No genotype-related differences in IL12A expression at 180 mpt have been recorded.

IL1b gene expression levels in PBMC are not significantly altered at 30 and 60 mpt in LPS-treated *vs* vehicle-treated PTPH1-WT and KO mice (data not shown). At 180 mpt, PTPH1-WT LPS-treated mice display a slightly higher (not significant) IL1b

expression, while a slight decrease is detectable in PTPH1-KO LPS *vs* vehicle-treated mice. Thus a genotype-related IL1b gene expression decrease ($P<0.05$) is detectable in WT *vs* KO LPS-treated mice (Fig. 14d).

IL-10 and IL-6 gene expression level is not significantly altered in PBMC extracted from LPS-treated vs vehicle-treated PTPH1-WT and KO mice (data not shown).

RT-PCR on PTPH1-related genes- RT-PCR has been carried out on those genes known to be involved in PTPH1 signaling cascade or to interact with PTPH1 as GHR, IGF1, IGF1R and ADAM17.

GHR, IGF1 and IGF1R expression level is not significantly altered in PBMC extracted from LPS-treated vs vehicle-treated PTPH1-WT and KO mice at any time points (data not shown). Furthermore no differences in ADAM17 gene expression level has been recorded in PBMC from WT and KO LPS treated and untreated mice (Fig 15a-c).

DISCUSSION

PTPH1 role in the inflammatory response is controversial. PTPH1 was identified as the only PTP able of interacting physically with TCRζ and dephosphorylating TCRζ ITAMs in vitro[16]. On the other hand a recent PTPH1-KO *ex vivo* study demonstrated that no alteration is occurring in stimulated cells concerning the TCR-induced signal transduction, cytokine production, and proliferation [17]. We therefore decided to better understand PTPH1 role in the immune response by in vivo studies, in particular using a CARR-induced and a LPS-induced inflammatory model.

Carrageenan-induced inflammation was originally described by Winter et al. (1962), is acute and highly reproducible {WINTER, 1962 6 /id}. Edema, measured by paw thickness, is the first sign of the induced inflammation and a sensible increased CARR-induced edema is detectable already 1 hour post treatment in both WT and KO animals. Indeed PTPH1 does not have an effect of the edema induction and as well as on tactile and thermal hyperalgesia, measured at the Hargreaves' and Von Frey's tests. CatWalk analysis reveals no altered major signs between genotypes, but displays CARR-

induced modifications of intensity, maximum area, duty cycle and print area parameters. However PTPH1-KO mice seem to be susceptible to pain (indicated as duty cycle) already at 5 hours post carrageenan injection (pci), while this sensitization is visible only at 24 hours pci in WT mice. This hypothesis is corroborated by the course of print area parameter in PTPH1-KO mice. In KO CARR-treated mice the print area is significantly lower than the matched vehicle-treated ones at 5 and 24 hours pci, while no differences are detectable in WT mice. Furthermore a trend of a genotype-related difference is visible at 24 hours pci between WT and KO CARR-treated mice. The finer sensitivity of the Catwalk test compared to the Von Frey's test allowed us to detect these minute differences in the pain response of CARR-treated PTPH1-KO mice compared to their matched vehicle-treated paws and to WT animals.

A deeper evaluation at histological level is ongoing to better understand PTPH1 role in this process that could involve T-cell recruitment.

The LPS-induced inflammatory model is a well defined model of septic shock. LPS leads to a massive TNFα release in the plasma that reaches the maximum 1-hour post injection. Our CBA analysis supports this mechanism of action and does not reveal any significant genotype-related differences. Anyway at 180 mpt PTPH1-KO LPS-treated TNFα and IL10 plasma levels are still significantly higher compared to the vehicle-treated controls, while this difference is present but not significant in WT mice group. These data support the concept that in PTPH1 lacking mice an inflammatory event is still massively ongoing, while it is decaying in WT mice. This hypothesis is strengthened and widened by the gene expression analysis on PBMC that showed a significantly 10-fold increase in TNFα expression already 60 mpt in KO LPS-treated mice compared to vehicle-treated controls. No differences have been detected at 60 mpt in WT mice, thus indicating an earlier inflammatory response. A genotype-related alteration of IL12A and IL1b gene expression at 60 mpt strengthens the hypothesis of a strong involvement of PTPH1 in the control of cytokine expression. No major differences have been detected in the expression of PTPH1-related genes in PBMC under LPS challenge.

In summary the present work demonstrates PTPH1 involvement in the inflammatory response in two different mouse models, leading to increase pain sensitivity

and to an augmented cytokine expression in the PBMC and cytokine release in the plasma.

Figures

Fig.1: Paw thickness measurement in PTPH1-WT and KO. CARR-treated paws display increased paw thickness (edema) compared to controlateral vehicle treated ones starting 1 hour post-injection upwards. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05; **:p<0.01; ***:p<0.001.

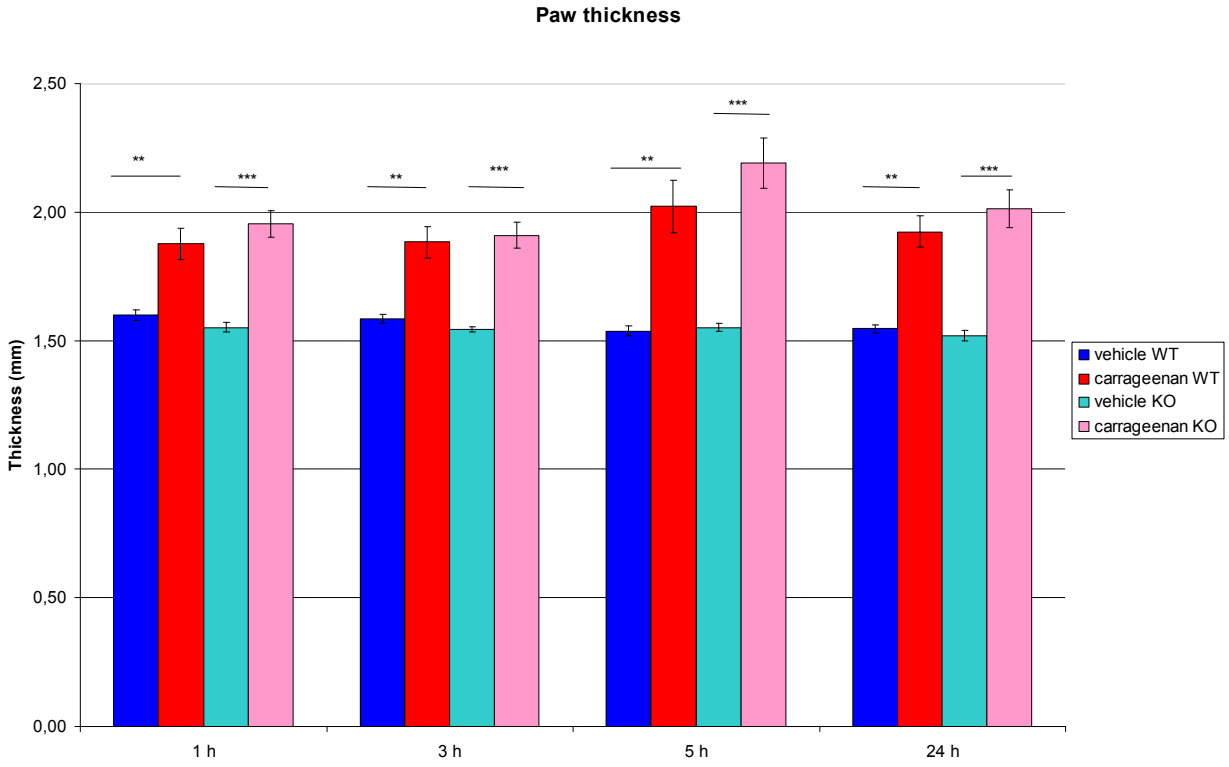


Fig.2: Hargreaves'test on PTPH1-WT and KO mice. CARR-treated paws show decreased withdrawal time compared to controlateral vehicle-treated ones in both WT and KO groups starting from 1 hour post-injection upwards. No differences in Hargreaves's response due to genotype. In bold the delta between CARR and vehicle-treated paws indicate the percentage of hyperalgesic effect. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05; **:p<0.01; ***:p<0.001.

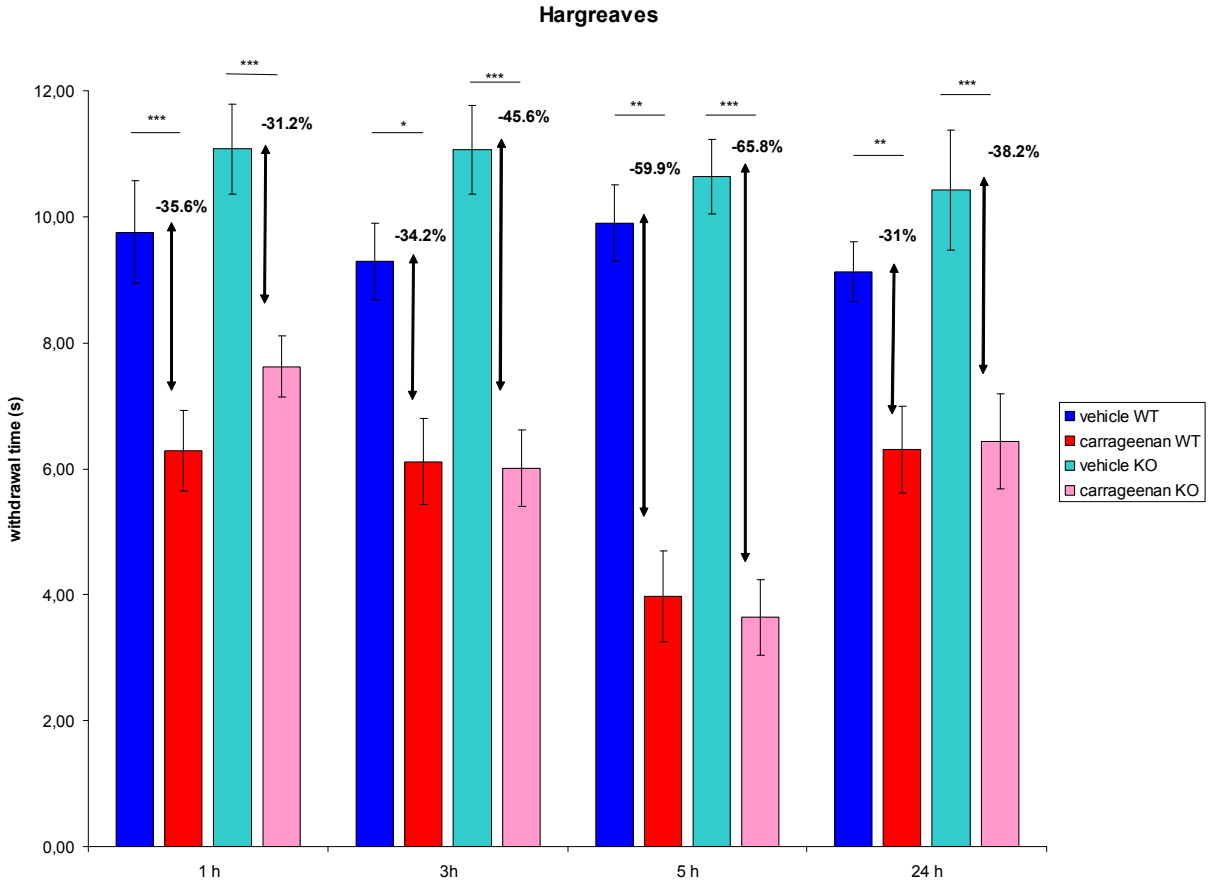


Fig.3: Automated Von Frey test on PTPH1-WT and KO mice. CARR-treated paws show decreased withdrawal force compared to controlateral vehicle-treated ones in both WT and KO groups starting from 1 hour post-injection upwards. No differences in Von Frey response due to genotype. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05; **:p<0.01; ***:p<0.001.

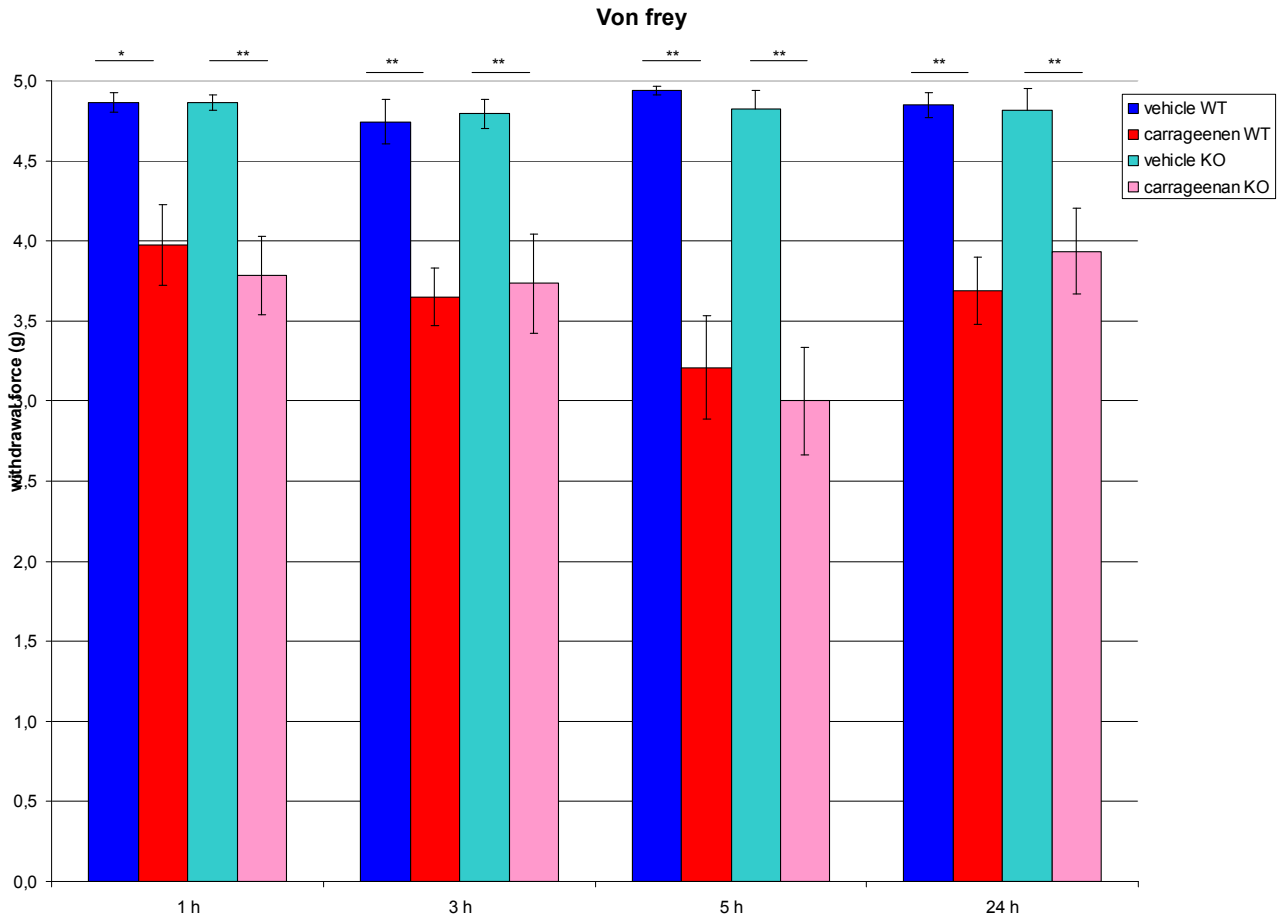


Fig.4: CatWalk test: Intensity parameter on PTPH1-WT and KO mice. Lower intensity detected in WT CARR-treated paws compared to vehicle-treated ones at 1, 5 and 24 h post-injection. A decreased intensity is recorded in KO CARR-treated paws compared to vehicle-treated ones at 5 and 24 h post-injection. No differences in intensity is genotype-related. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05.

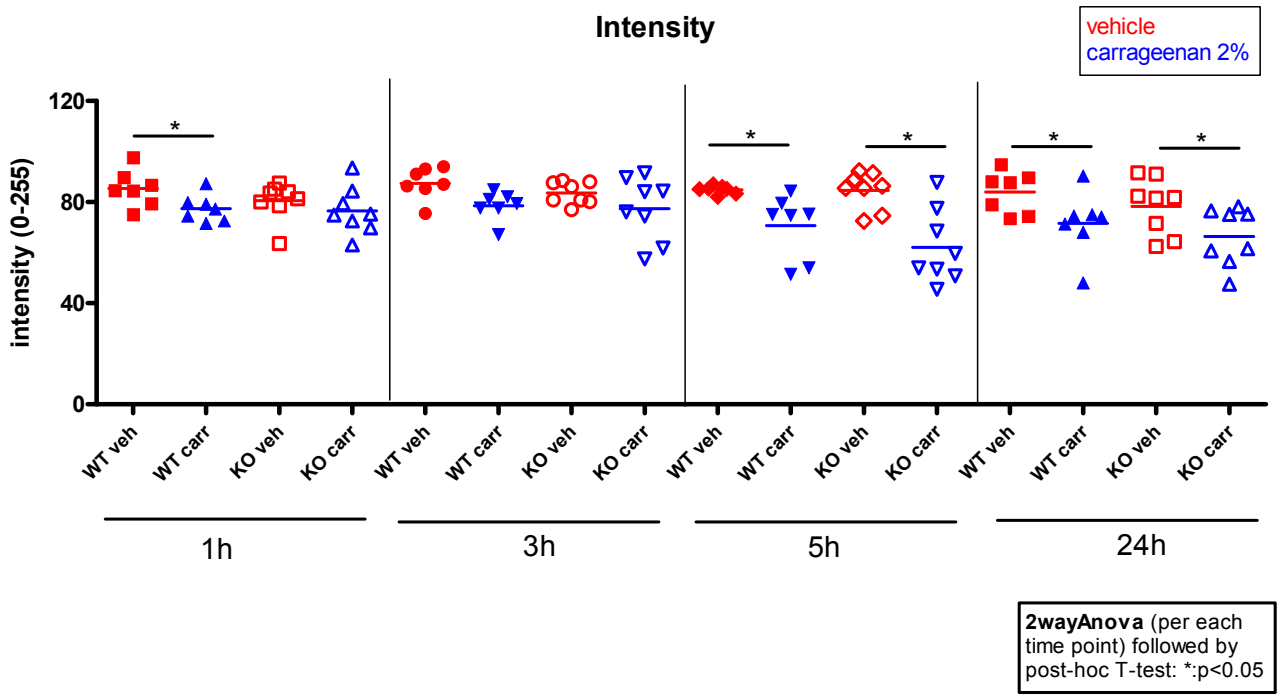


Fig. 5: CatWalk test: Duty cycle parameter on PTPH1-WT and KO mice. A decreased percentage of duty cycle is recorded in WT CARR*vs*vehicle- treated paws at 1 and 24 hours post-injection. Lower percentage of percentage of duty cycle is detectable at 5 and 24 hours post carrageenan injection. No differences in duty cycle is genotype-related. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05; **:p<0.01.

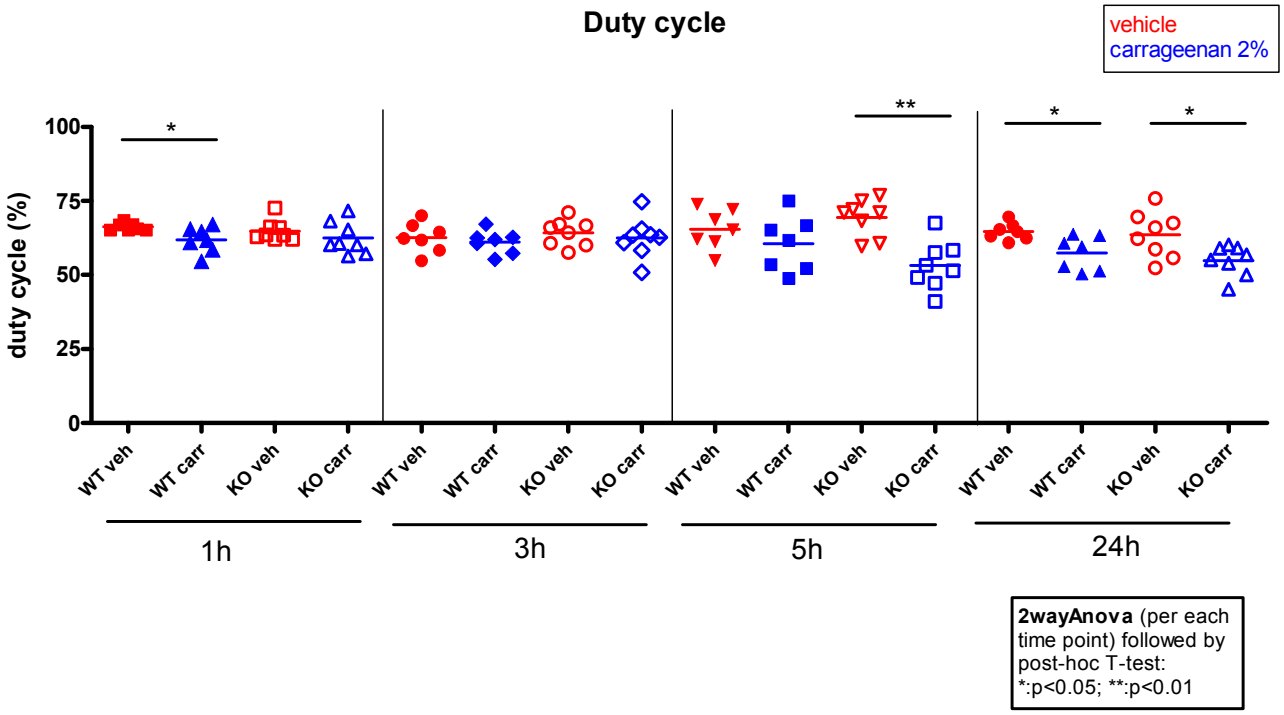


Fig. 6: CatWalk test: Maximum contact area parameter on PTPH1-WT and KO mice. No gross differences have been detected in WT and KO mice groups. A trend in decreased max area is recorded in CARR*vs*vehicle-treated paws, that is significant in KO mice at 5 hours post carrageenan injection. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05.

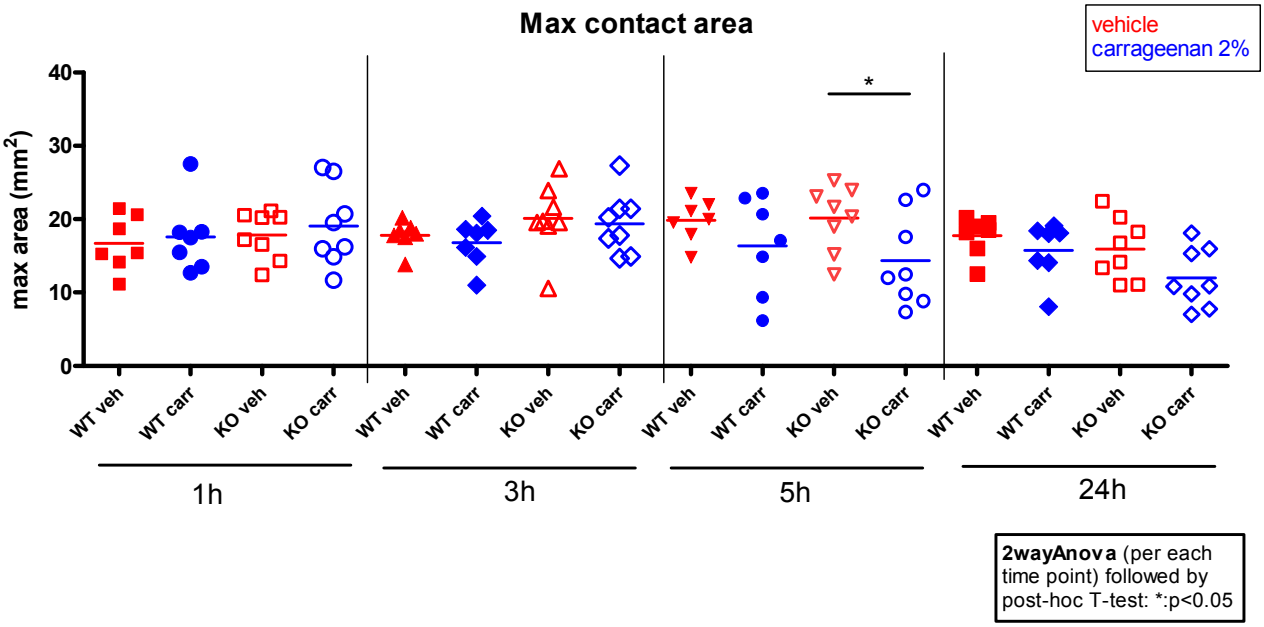


Fig. 7: CatWalk test: Print area parameter on PTPH1-WT and KO mice. Significant lower print area is recorded in KO CARR*vs*vehicle-treated paws at 5 and 24 hours post carrageenan injection. No differences in WT CARR*vs*vehicle-treated paws are detected at any time points but a trend in decreased print area is visible at 24 hours post injection. No significant differences are genotype-related. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05.

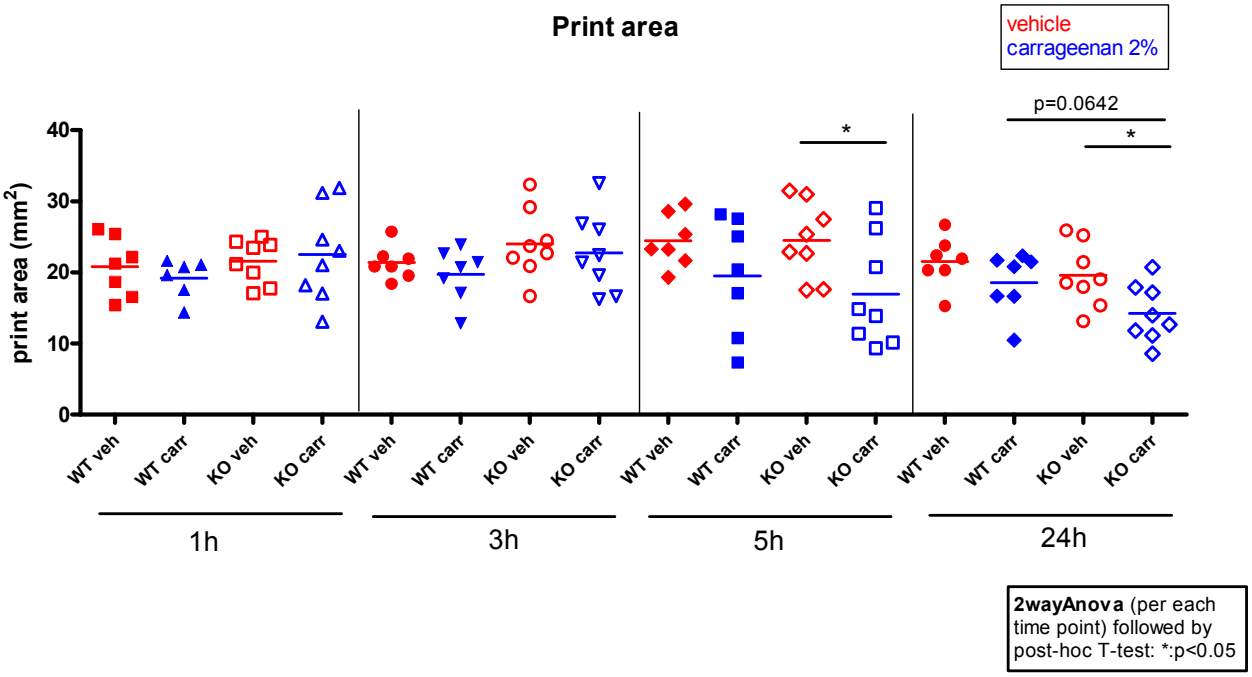


Fig. 8: CBA-30 minutes post treatment. Pink line indicates the detection limit. Data were analyzed by 2way Anova followed by Bonferroni's post-hoc test; *:p<0.05.

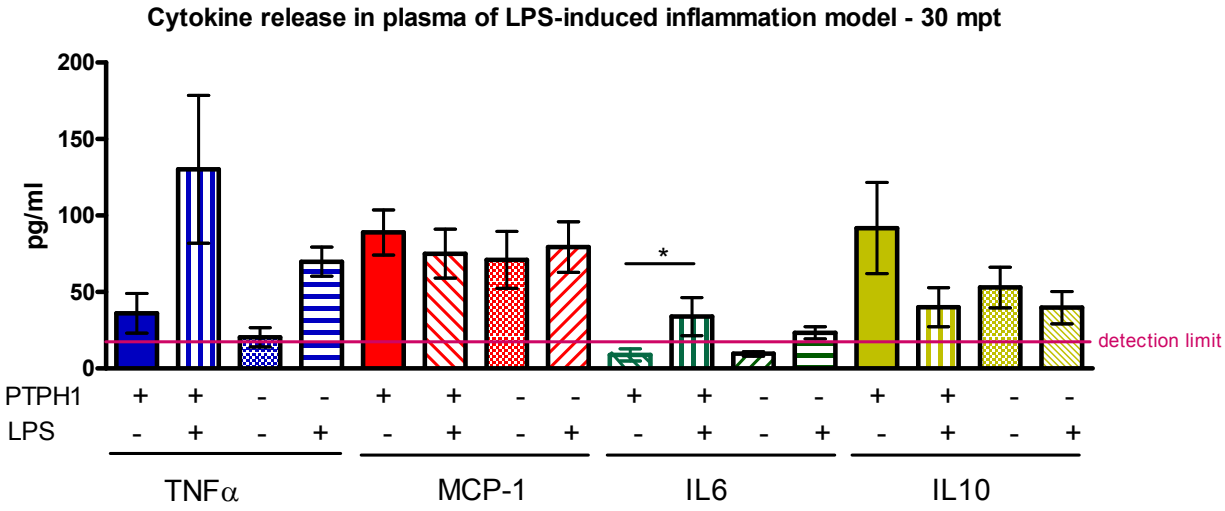


Fig. 9: CBA-60 minutes post treatment. Data were analyzed by 2way Anova followed by Bonferroni's post-hoc test; **:p<0.01; ***:p<0.001

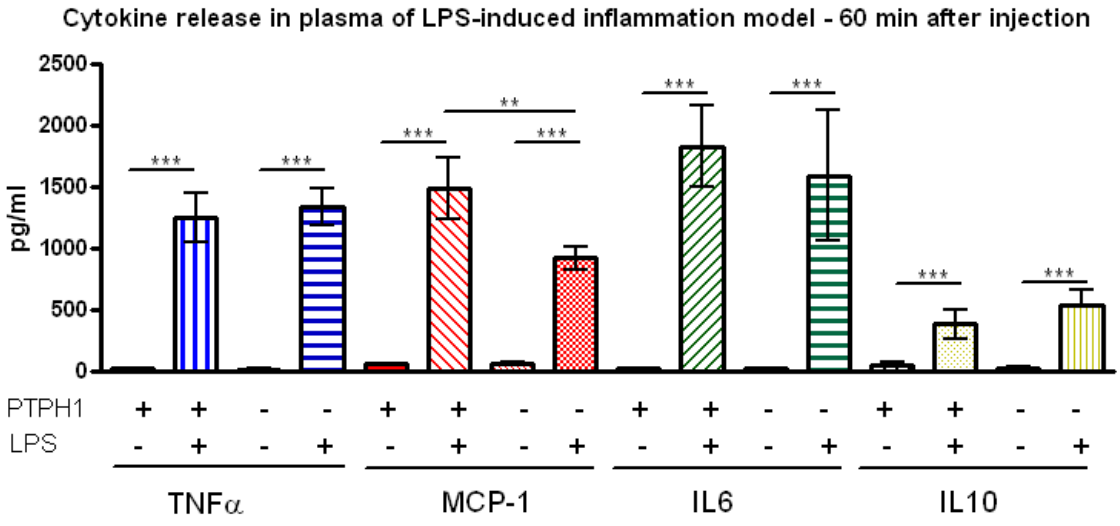


Fig. 10: CBA-180 minutes post treatment. Pink line indicates the detection limit. Data were analyzed by 2way Anova followed by Bonferroni's post-hoc test; **:p<0.01; ***:p<0.001.

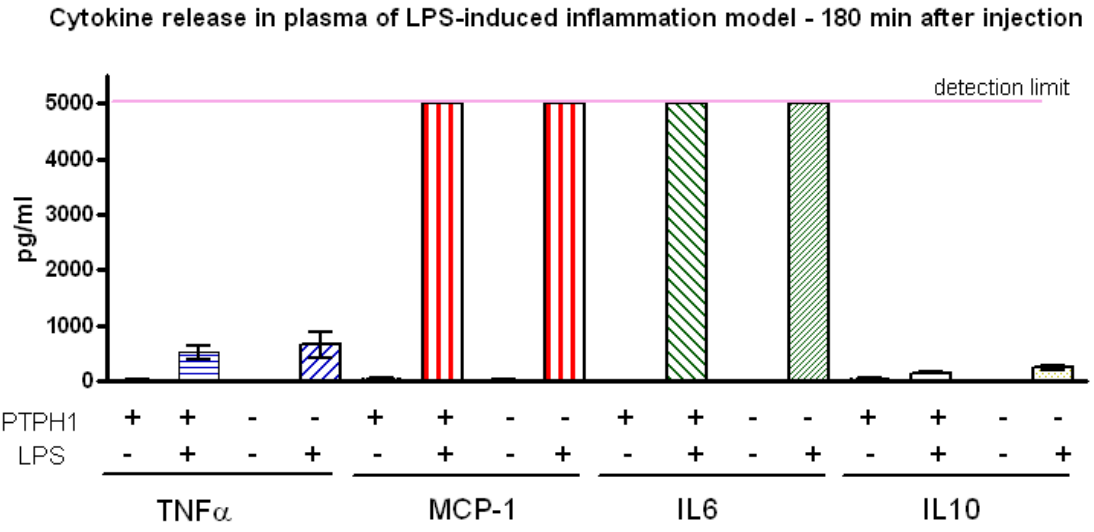


Fig. 11: Detail of CBA-180 minutes post treatment: TNF and IL10. Data were analyzed by 2way Anova followed by Bonferroni's post-hoc test; *:p<0.05; ***:p<0.001.

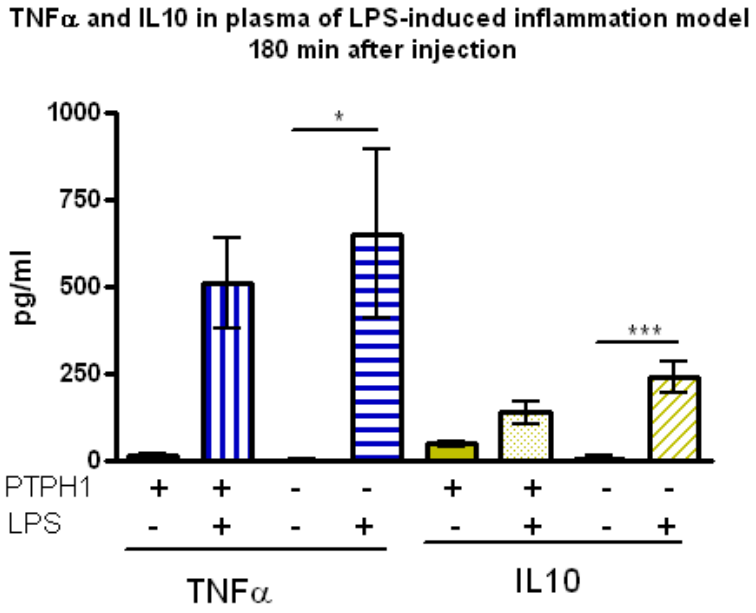


Fig. 12: RT-PCR analysis for TNF gene in PBMC. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05; **:p<0.01.

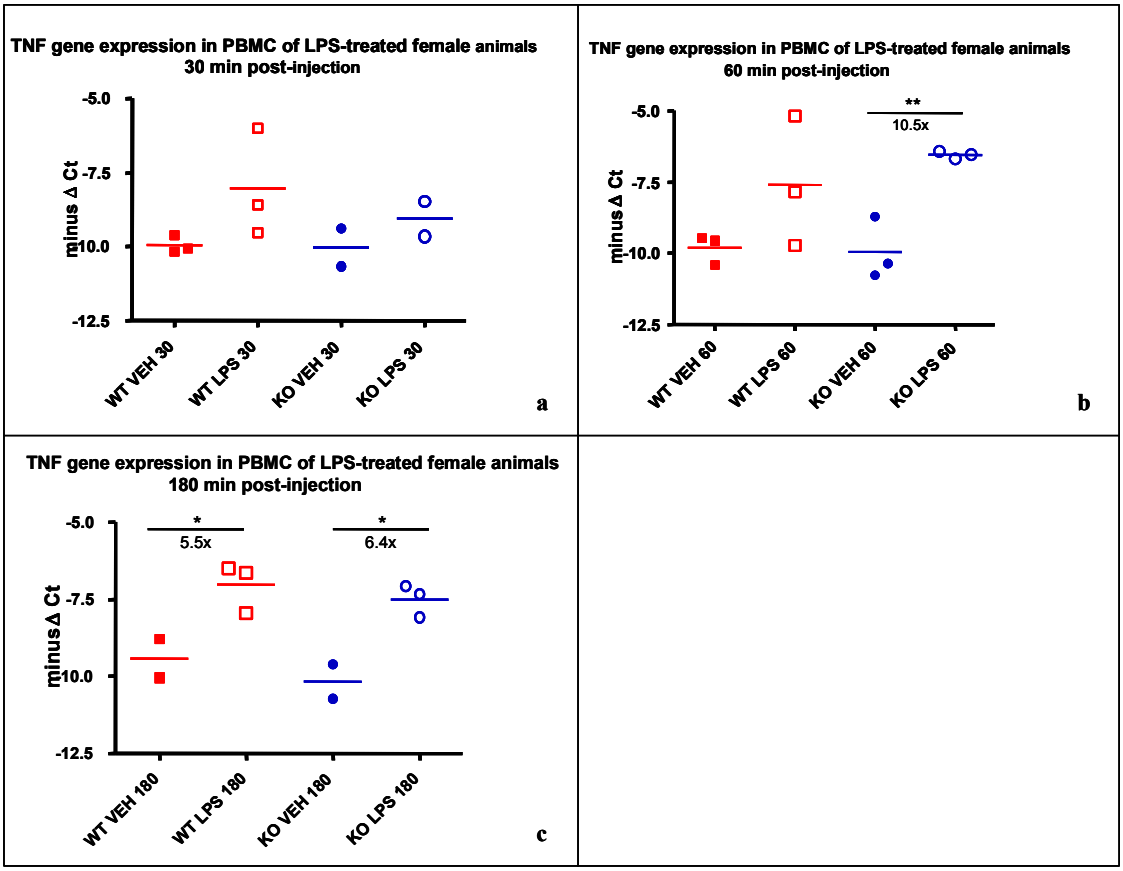


Fig. 13: RT-PCR analysis for ccl2 gene in PBMC. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05.

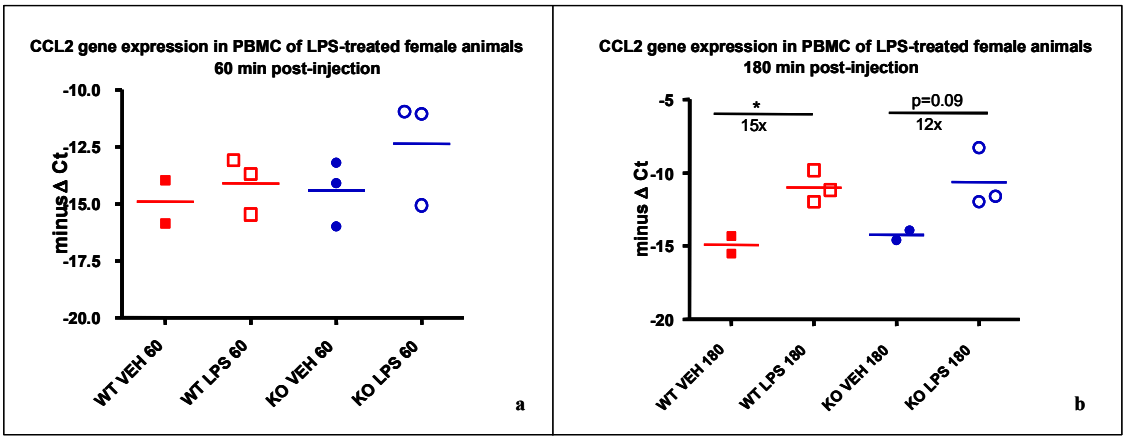


Fig. 14: RT-PCR analysis for IL12A and IL1b genes in PBMC. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05.

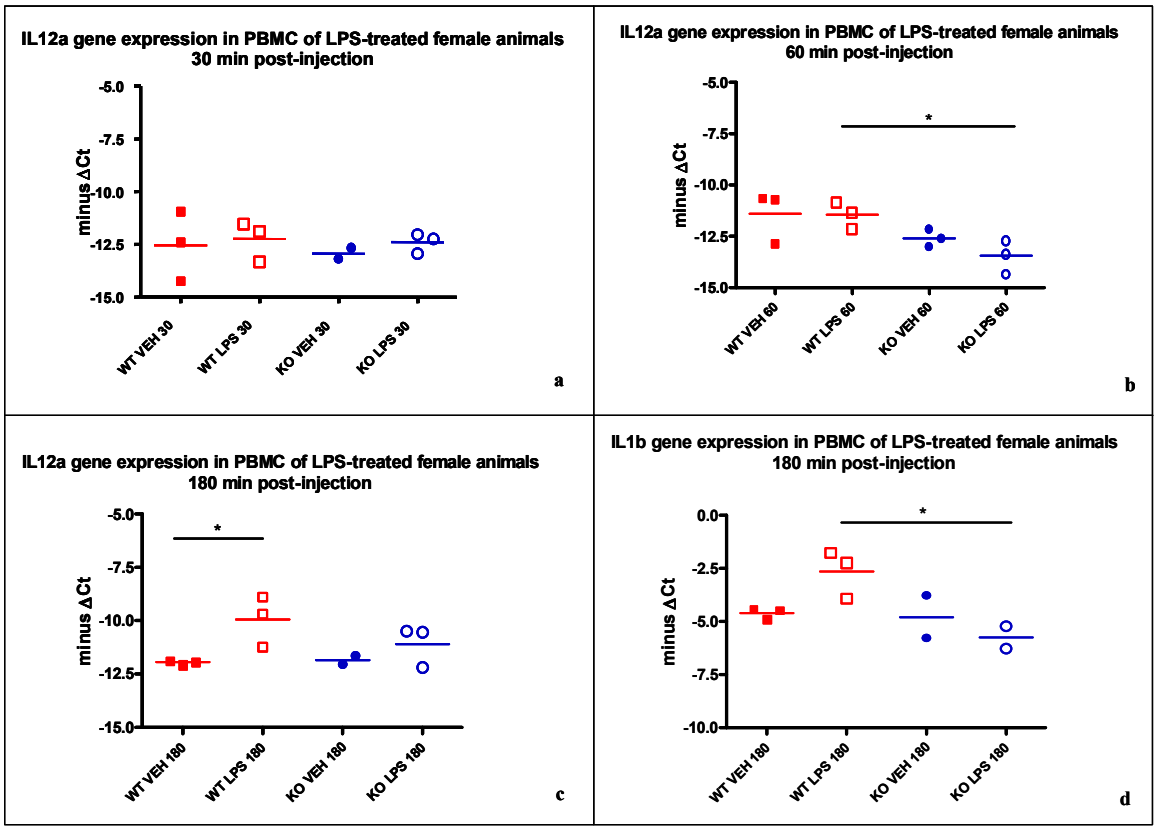
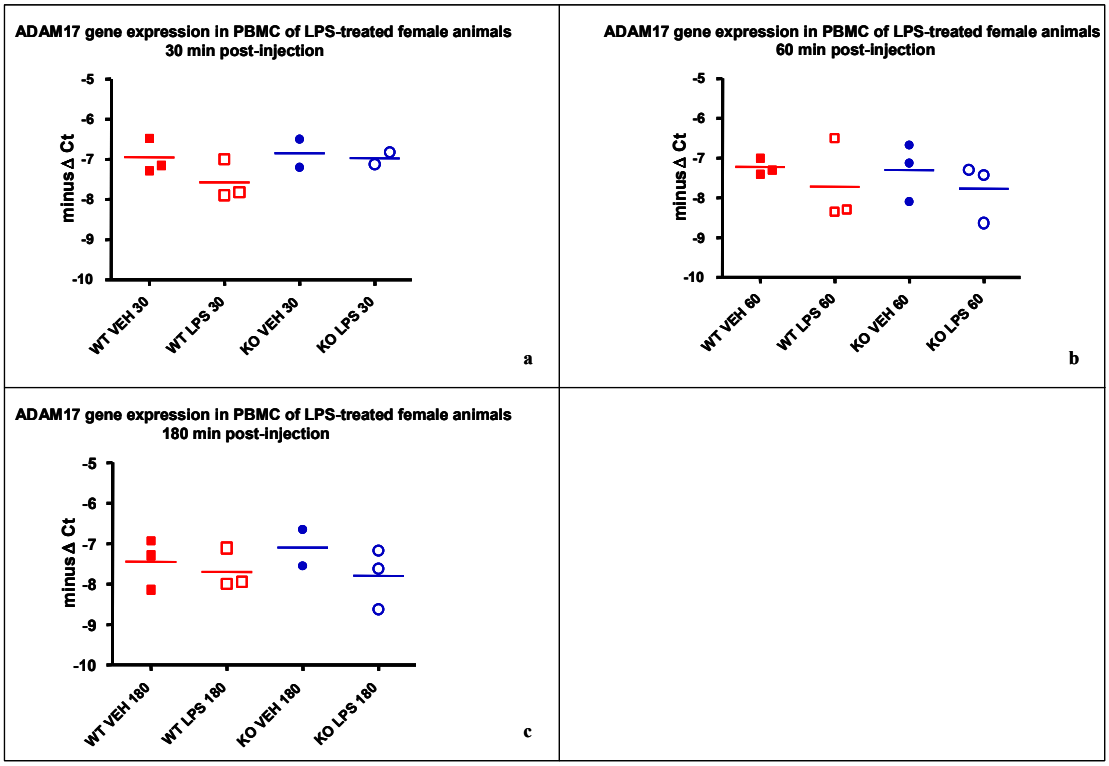


Fig. 15: RT-PCR analysis for ADAM17 gene in PBMC. Data were analyzed by 2way Anova followed by post-hoc T-test; *:p<0.05.



Reference List

1. Stoker, A. W.: **Protein tyrosine phosphatases and signalling**. *J.Endocrinol.* 2005, **185**: 19-33.

2. Andersen, J. N., Jansen, P. G., Echwald, S. M., Mortensen, O. H., Fukada, T., Del Vecchio, R., Tonks, N. K., and Moller, N. P. H.: **A genomic perspective on protein tyrosine phosphatases: gene structure, pseudogenes, and genetic disease linkage**. *FASEB J.* 2004, **18**: 8-30.

3. Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J., and Mustelin, T.: **Protein tyrosine phosphatases in the human genome**. *Cell* 2004, **117**: 699-711.

4. Zhang, S. H., Eckberg, W. R., Yang, Q., Samatar, A. A., and Tonks, N. K.: **Biochemical characterization of a human band 4.1-related protein-tyrosine phosphatase, PTPH1**. *J.Biol.Chem.* 1995, **270**: 20067-20072.

5. Zhang, S. H., Kobayashi, R., Graves, P. R., Piwnica-Worms, H., and Tonks, N. K.: **Serine phosphorylation-dependent association of the band 4.1-related protein-tyrosine phosphatase PTPH1 with 14-3-3beta protein**. *J.Biol.Chem.* 1997, **272**: 27281-27287.

6. Takeuchi, K., Kawashima, A., Nagafuchi, A., and Tsukita, S.: **Structural diversity of band 4.1 superfamily members**. *J.Cell Sci.* 1994, **107 (Pt 7)**: 1921-1928.

7. Zhang, S. H., Eckberg, W. R., Yang, Q., Samatar, A. A., and Tonks, N. K.: **Biochemical characterization of a human band 4.1-related protein-tyrosine phosphatase, PTPH1**. *J.Biol.Chem.* 1995, **270**: 20067-20072.

8. Sozio, M. S., Mathis, M. A., Young, J. A., Walchli, S., Pitcher, L. A., Wrage, P. C., Bartok, B., Campbell, A., Watts, J. D., Aebersold, R., Van Huijsduijnen, R. H., and van Oers, N. S.: **PTPH1 is a predominant protein-tyrosine**

phosphatase capable of interacting with and dephosphorylating the T cell receptor zeta subunit. *J.Biol.Chem.* 2004, **279**: 7760-7769.

9. Zhang, S. H., Liu, J., Kobayashi, R., and Tonks, N. K.: **Identification of the cell cycle regulator VCP (p97/CDC48) as a substrate of the band 4.1-related protein-tyrosine phosphatase PTPH1**. *J.Biol.Chem.* 1999, **274**: 17806-17812.

10. Lavoie, C., Chevet, E., Roy, L., Tonks, N. K., Fazel, A., Posner, B. I., Paiement, J., and Bergeron, J. J.: **Tyrosine phosphorylation of p97 regulates transitional endoplasmic reticulum assembly in vitro**. *Proc.Natl.Acad.Sci.U.S.A* 2000, **97**: 13637-13642.

11. Jespersen, T., Gavillet, B., van Bemmelen, M. X., Cordonier, S., Thomas, M. A., Staub, O., and Abriel, H.: **Cardiac sodium channel Na(v)1.5 interacts with and is regulated by the protein tyrosine phosphatase PTPH1**. *Biochem.Biophys.Res.Commun.* 2006, **348**: 1455-1462.

12. Zheng, Y., Schlondorff, J., and Blobel, C. P.: **Evidence for regulation of the tumor necrosis factor alpha-convertase (TACE) by protein-tyrosine phosphatase PTPH1**. *J.Biol.Chem.* 2002, **277**: 42463-42470.

13. Patrignani, C., Magnone, M. C., Tavano, P., Ardizzone, M., Muzio, V., Greco, B., and Zaratin, P. F.: **Knockout mice reveal a role for protein tyrosine phosphatase H1 in cognition**. *Behav.Brain Funct.* 2008, **4**: 36-

14. Gjorloff-Wingren, A., Saxena, M., Han, S., Wang, X., Alonso, A., Renedo, M., Oh, P., Williams, S., Schnitzer, J., and Mustelin, T.: **Subcellular localization of intracellular protein tyrosine phosphatases in T cells**. *Eur.J.Immunol.* 2000, **30**: 2412-2421.

15. Han, S., Williams, S., and Mustelin, T.: **Cytoskeletal protein tyrosine phosphatase PTPH1 reduces T cell antigen receptor signaling**. *Eur.J.Immunol.* 2000, **30**: 1318-1325.

16. Sozio, M. S., Mathis, M. A., Young, J. A., Walchli, S., Pitcher, L. A., Wrage, P. C., Bartok, B., Campbell, A., Watts, J. D., Aebersold, R., Van Huijsduijnen, R. H., and van Oers, N. S.: **PTPH1 is a predominant protein-tyrosine phosphatase capable of interacting with and dephosphorylating the T cell receptor zeta subunit**. *J.Biol.Chem.* 2004, **279**: 7760-7769.

17. Bauler, T. J., Hughes, E. D., Arimura, Y., Mustelin, T., Saunders, T. L., and King, P. D.: **Normal TCR signal transduction in mice that lack catalytically active PTPN3 protein tyrosine phosphatase**. *J.Immunol.* 2007, **178**: 3680-3687.

18. Pilecka, I., Patrignani, C., Pescini, R., Curchod, M. L., Perrin, D., Xue, Y., Yasenchak, J., Clark, A., Magnone, M. C., Zaratin, P., Valenzuela, D., Rommel, C., and Hooft van, Huijsduijnen R.: **Protein-tyrosine phosphatase H1 controls growth hormone receptor signaling and systemic growth**. *J.Biol.Chem.* 2007, **282**: 35405-35415.

19. Hargreaves, K., Dubner, R., Brown, F., Flores, C., and Joris, J.: **A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia**. *Pain* 1988, **32**: 77-88.

20. Moller, K. A., Berge, O. G., and Hamers, F. P.: **Using the CatWalk method to assess weight-bearing and pain behaviour in walking rats with ankle joint monoarthritis induced by carrageenan: Effects of morphine and rofecoxib**. *J.Neurosci.Methods* 2008, **174**: 1-9.

21. Gabriel, A. F., Marcus, M. A., Honig, W. M., Walenkamp, G. H., and Joosten, E. A.: **The CatWalk method: a detailed analysis of behavioral changes after acute inflammatory pain in the rat**. *J.Neurosci.Methods* 2007, **163**: 9-16.

22. Vrinten, D. H. and Hamers, F. F.: **'CatWalk' automated quantitative gait analysis as a novel method to assess mechanical allodynia in the rat; a comparison with von Frey testing**. *Pain* 2003, **102**: 203-209.

SEZIONE 2:

CORSI FREQUENTATI (per ciascun anno del corso)

1st year PhD

- Summer course: "Cell Culture and Cellular Model Systems", organized by EMIL: European Network of Excellence, Milan 18-22/07/2005;

2nd year PhD

- *"Inflammatory Mechanisms in Neurodegenerative Disease"*, organized by the MS Centre ErasMS and Erasmus Postgraduate School for Molecular Medicine, held in Rotterdam, 30-31 March 2006;
- *"English course"*, organized by RBM and by the M.I.T. Center of Turin.

3rd year PhD

- Baltic Summer School 2007-"Analysis of models for multiple sclerosis. Analysis of cells in the central nervous system", organized by Shohreh Issazadeh-Navikas, Lund 17-21/09/07.

4th year PhD

- 6th Summer School of Neuroscience: "Drug discovery in Nueroscience", Catania 19-25 July 2008, organized by Filippo Drago, University of Catania.

SEMINARI FREQUENTATI (during 3rd year PhD)

- "Measurement parameters of animals chronic pain" held by Angelo Ceci.
- "Role of serotonin in controlling L-DOPA induced dyskinesia" held by Mahmoud Iravani.
- "Discovery of a putative biomarker of anti-IL17 activity *in vivo*" held by Mara Fortunato.
- "In vivo neurochemistry: microdialysis from accademia to drug discovery" held by Gael Hedou.
- "In vivo monitoring of curative MMP-12 inhibition suppresses proinflammatory macrophage activation in relapsing rat EAE" held by Klaus Petry.
- "Modulating dendritic cells: from immunogenic to tolerogenic responses" held by Clara Ballerini.

CONGRESSI FREQUENTATI (elenco completo: denominazione congresso, sede, data)

1st year PhD

- "Le Biotecnlogie per progettazione, sviluppo e produzione dei farmaci", organized by Charles River Lab., Milan 20-21/05/2005;
- "20th Biennial Meeting of the International Society For Neurochemistry", organized by European Society for Neurochemistry, Innsbruck 21-26/08/2005;

2nd year PhD

- *"Neurodegenerative Diseases: Molecular Mechanisms in a Functional Genomics Framework",* organized by the Max Delbruck Center for Molecular Medicine, held in Berlin, 6-9 September 2006.

3rd year PhD

- Baltic Summer School 2007 – "Inflammation: A Key to Common Complex Diseases" Organized by the Faculty Members of the Baltic Summer School, coordinator Prof. Rikard Holmdahl and course-leader Dr. Bo Nilson, Lund 2-13/09/07.

4th year PhD

- Bracco Imaging Workshop – "From basic research to clinical translation: the challenge of molecular imaging" Organized by Bracco Imaging S.p.A., Colleretto Giacosa 26/06/08.

COMUNICAZIONI A CONGRESSI (elenco completo: autori, titolo, denominazione congresso, sede, data)

A) poster1: C. Patrignani[1,2], P. Tavano[1], A. Graziani[2] , R. Hooft [3], C. Rommel[3] and M.C. Magnone[1]
[1] LCG-RBM/ Serono Research, Turin, Italy; [2] Università del Piemonte Orientale, Novara, Italy Serono Pharmaceutical Research Institute, Geneva, Switzerland: **"PTPH1 in central nervous system: possible involvement in neurological functions"** presented at the conference *"Neurodegenerative Diseases: Molecular Mechanisms in a Functional Genomics Framework",* organized by the Max Delbruck Center for Molecular Medicine, Berlin, 6-9 September 2006.

A) poster2: C. Patrignani[1,2],S. Carboni[1], V. Muzio[1], B. Greco[1], P. Zaratin[1]
[1] :RBM/Merck Serono International S.A. (an affiliate of Merck KGaA, Darmstadt , Germany,) Turin, Italy; [2] :Università del Piemonte Orientale, Novara, Italy: **"TACE expression in late stage mouse chronic experimental autoimmune encephalomyelitis"** presented at the Baltic Summer School 2007 – "Inflammation: A Key to Common Complex Diseases" Organized by the Faculty Members of the Baltic Summer School, Lund 2-13/09/07.

ARTICOLI SCIENTIFICI PUBBLICATI NEL CORSO DEL DOTTORATO

1) JBC 2007 Nov 30;282(48):35405-15: **Control of Growth Hormone Receptor Signaling by Protein Tyrosine Phosphatase H1 (PTP-H1/PTPN3)** by Iwona Pilecka[1,3], Claudia Patrignani[2], Rosanna Pescini[1], Marie-Laure Curchod[1], Dominique Perrin[1], NN[5] , Ann Clark[6], Maria Chiara Magnone[1,4], Paola Zaratin[2], Christian Rommel[1] and Rob Hooft van Huijsduijnen[1,7]
[1] Merck Serono International S.A., 9, chemin des Mines, 1211 Geneva, Switzerland
[2] Merck Serono Istituto di Ricerche Biomediche "Antoine Marxer" (RBM); Via Ribes, 1 Colleretto Giacosa(TO)10010, Italy
[3] Present address: International Institute of Molecular and Cell Biology, Ks. Trojdena 4, 02-109 Warsaw, Poland
[4] Present address: Novartis Pharma AG Lichtstrasse 35 Basel CH-4002 Switzerland

[5] Regeneron Pharmaceuticals, Inc. 777 Old Saw Mill River Road, Tarrytown, NY 10591-6707, USA
[6] Serono, Inc. One Technology Place. Rockland, Massachusetts 02370, USA

2) Behavioral and Brain Funtions 2008 Aug 12;4:36: **Knockout mice reveal a role for protein tyrosine phosphatase H1 in cognition** by Claudia Patrignani[1, 2]*, Maria Chiara Magnone[1], Patrizia Tavano[1], Michele Ardizzone[1], Valeria Muzio[1], Béatrice Gréco[1] and Paola F. Zaratin[1]

[1]:MerckSerono Ivrea, Colleretto G. (TO) 10010, Italy

[2]:University of Eastern Piedmont, Novara, Italy