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Nicotinic acid-induced flushing is mediated by activation of epidermal Langerhans cells[a]

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Running title: Langerhans cells mediate nicotinic acid-induced flushing

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Abbreviations: DP, PGD_2 receptor, DT, diphtheria toxin; DTR, diphtheria toxin receptor; LD, laser-Doppler; LDF, laser-Doppler flow; HDL, high density lipoprotein; LDL, low density lipoprotein; PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; mPGES-1 and mPGES-2, type 1 and type 2 PGE_2 synthases; NA, nicotinic acid; EP2, type-2 PGE_2 receptor; EP4, type-4 PGE_2 receptor.

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Abstract

The anti-dyslipidemic drug nicotinic acid (niacin) has been used for decades. One of the major problems of the therapeutical use of nicotinic acid is a strong cutaneous vasodilation called flushing which develops in almost every patient taking nicotinic acid. Nicotinic acid-induced flushing has been shown to be mediated by the nicotinic acid receptor GPR109A and to involve the formation of vasodilatory prostanoids. However, the cellular mechanisms underlying this acute effect are unknown. Here we show that epidermal Langerhans cells are essential for the cutaneous flushing response induced by nicotinic acid. Langerhans cells respond with an increase in $[Ca^{2+}]_i$ to nicotinic acid and express prostanoid synthases required for the formation of the vasodilatory prostanoids prostaglandin E_2 and prostaglandin D_2. Depletion of epidermal Langerhans cells but not of macrophages or dendritic cells abrogates nicotinic acid induced flushing. These data unexpectedly identify epidermal Langerhans cells as essential mediators of nicotinic acid induced flushing and may help to generate new strategies to suppress the unwanted effects of nicotinic acid. In addition, our results suggest that Langerhans cells besides their immunological roles are also involved in the local regulation of dermal blood flow.

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Introduction

Nicotinic acid (niacin) is the oldest lipid modifying drug and induces a unique spectrum of changes in lipid and lipoprotein levels (Carlson, 2005). Besides its ability to decrease triglyceride and LDL cholesterol levels, it has the strongest HDL cholesterol elevating activity among the currently available lipid modifying drugs. Since a low HDL cholesterol level is an independent risk factor for cardiovascular diseases, the HDL cholesterol elevating effect of nicotinic acid has recently led to a renewed interest in this drug (Carlson, 2006; Kontush and Chapman, 2006; Offermanns, 2006), and there is good evidence indicating a beneficial effect of nicotinic acid alone or in combination with statins (Brown et al., 2001; Coronary Drug Project Research Group, 1975; Taylor et al., 2004).

The major problem of oral nicotinic acid treatment is the occurrence of a strong flushing phenomenon associated with cutaneous vasodilation which occurs in virtually all patients and severly influences patients' compliance. It has recently been shown that the nicotinic acid receptor GPR109A (HM74A in humans, PUMA-G in mice) mediates not only the acute metabolic effects of nicotinic acid but also the flushing response (Benyo et al., 2005; Tunaru et al., 2003). However, the cells and molecular mechanisms mediating nicotinic acid-induced flushing are unclear.

The flushing response of nicotinic acid can be inhibited by pre-treatment with cyclooxygenase inhibitors (Andersson et al., 1977; Eklund et al., 1979; Kaijser et al., 1979; Svedmyr et al., 1977), and the levels of several vasodilatory prostanoids like prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2) and their metabolites are elevated after administration of nicotinic acid (Eklund et al., 1979; Morrow et al., 1989; Nozaki et al., 1987; Stern et al., 1991). These observations have led to the hypothesis that prostanoids are involved in nicotinic acid-induced flushing. Pharmacological and genetic evidence has recently been provided that PGD_2 acting

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through the DP receptor as well as PGE_2 acting via EP_2 and EP_4 mediate the nicotinic acid-induced flushing response (Benyo et al., 2005; Cheng et al., 2006).

The cells required for nicotinic acid-induced and prostanoid-mediated flushing, however, remain elusive. The ability of topically applied nicotinic acid to induce locally restricted hyperemia (Morrow et al., 1992; Wilkin et al., 1985) and the fact that the nicotinic acid receptor GPR109A is expressed in macrophages and other immune cells (Schaub et al., 2001) suggests that immune cells of the skin are involved in the flushing response. This is supported by data demonstrating that MHC class II-positive skin cells express GPR109A, and that the lack of nicotinic acid-induced flushing response in GPR109A-deficient mice can be restored by the transplantation of wild type bone marrow (Benyo et al., 2005). We therefore systematically studied the potential involvement of various cell types in the cutaneous effects of nicotinic acid. By conditional ablation of defined subpopulations of immune cells as well as by the visualization of the local effects of nicotinic acid we show in the present study that epidermal Langerhans cells respond to nicotinic acid and mediate the flushing response.

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Materials and methods

Mice. CD11b-DTR and CD11c-DTR mice were puchased (Jackson Lab, Bar Harbor, Maine, USA), and Langerin-DTR transgenic mice have been described previously (Bennett et al., 2005). GPR109A deficient mice were kindly provided by Klaus Pfeffer (Universität Düsseldorf, Germany). Mice were housed in temperature-controlled facilities on a 12 h light-dark cycle with *ad libitum* food and water access. All experimental procedures were performed in accordance with institutional guidelines of the University of Heidelberg, Germany.

Measurement of flush. Cutaneous blood flow was determined as described (9). Shortly, anaesthetized mice were placed on their left side on a controlled heating pad and blood flow in the ear was determined with an LD probe (No. 407, Perimed AB, Stockholm, Sweden). Original LDF recordings were averaged for one second intervals, and the baseline LDF was determined prior to injection of the tested compound. All data are presented as mean ± SEM, n indicates the number of experimental animals. Statistical analysis of differences between flushing responses before and after DT treatment was performed by Student's paired t-test, $p<0.05$ was considered significant. Nicotinic acid was prepared in 5% (2-hydroxypropyl)-β-cylodextrin (Sigma) and the pH of the solutions was adjusted to 6.9-7.1 with 1 M NaOH. Prostaglandin D_2 (Cayman Chemical Co.) was dissolved in DMSO and diluted 20 times with saline.

DT treatment. After control experiments the CD11b-DTR, CD11c-DTR and Langerin-DTR transgenic animals were treated with 25, 4 and 16 ng/g diphtheria toxin (Sigma), respectively, as described (Bennett et al., 2005; Duffield et al., 2005; Jung et al., 2002). DT was injected i.p. 24 and 48 hours before re-testing of the flushing responses. Wild-type littermate animals were treated with the same protocol and

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served as controls. Between two experiments the animals were allowed to recover for at least one week.

Isolation of epidermal sheets, immunohistochemistry. Mouse ears were dissected, split into ventral and dorsal sheets and incubated for 50 min in RPMI and 10 mM EDTA. The epidermis was removed from the dermis using fine forceps and was fixed on ice for 1 hour in 4 % PFA/PBS. After permeabilization with 0.2% Triton/PBS for 10 min at 25 ºC and 2 washes with PBS for 10 min, the epidermal sheets were incubated with 10 % goat serum for 30 min. Polyclonal rabbit anti-PGE synthase 1, polyclonal rabbit anti-PGE synthase 2 and monoclonal mouse PGD_2 synthase (all from Cayman, Ann Arbor, USA) were each mixed with PE-labeled anti MHC II - IA/IE (BD PharMingen) in 10% goat serum/PBS and incubated with epidermal sheets overnight at 4ºC on a rotator. Secondary FITC labeled anti-rabbit and anti-mouse antibodies were applied together with DAPI in 1.5% horse serum for 2 hours on a rotator. After washing epidermal sheets were mounted and analyzed by fluorescence microscopy.

Fluorescence activated cell sorting

Splenocytes were isolated by collagenase digestion and passing through a 40 µm nylon mesh. Red blood cells were removed using Lympholyte-M (Cedarlane Laboratories). Splenocytes were stained with FITC-anti-CD11c and PE-labeled anti-F4/80 (BD PharMingen). Peritoneal macrophages were obtained by peritoneal lavage and stained with PE-labeled anti-F4/80 (BD PharMingen). All flow cytometric analyses were performed using the FACSCalibur and Cell Quest software (BD Biosciences).

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Measurement of intracellular [Ca^{2+}].

Epidermal sheets of Langerin-GFP-DTR or GPR109A-deficient animals were incubated in RPMI with 6 µM Fura-2 AM / pluronic acid (Molecuar Probes) and in the case of Puma-G KO together with FITC-labeled anti MHC II - IA/IE (BD PharMingen) for 45 min. The epithelial sheets were placed dermal side up in a perfusion chamber and immobilized with thin silk threads tied to a steel ring. Epithelial sheets were superfused with 900 µl RPMI at a flow rate of 3.5 ml/min and stimulation was achieved by pipetting 100 µl of a 10x concentrated solution into the chamber. Excitation light was provided by a monochromator (TILL Photonics, Germany), and the fluorescence emission was captured by a cooled CCD camera (Image QE; TILL Photonics, Germany) on an upright microscope (Olympus BX51WI). The calcium concentration was visualized in Langerhans cells that were identified by GFP or FITC fluorescence, respectively. Ratio images were collected at intervals every 500 ms. Imaging was controlled and analyzed with Tillvision Software 4.0 (TILL Photonics, Germany).

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Results

As previously reported, nicotinic acid-induced cutaneous vasodilation can be observed and quantified in the mouse ear using laser-Doppler (LD) flowmetry (Benyo et al., 2005; Cheng et al., 2006). Intraperitoneal injection of nicotinic acid results within a few minutes in a biphasic increase in the blood flow which lasts for about 40 minutes. Based on the fact that transplantation of wild type bone marrow into GPR109A-deficient mice can restore the ability of nicotinic acid to induce flushing (Benyo et al., 2005), we tested the potential involvement of various cutaneous immune cells in the nicotinic acid-induced flushing response. Since nicotinic acid does not induce an increase in histamine levels (Morrow et al., 1989) and since mast cell-deficient mice still respond with flushing to nicotinic acid (Benyo et al., 2005), mast cells are obviously not required for the flushing phenomenon. Besides mast cells, dermal macrophages and dermal dendritic cells are the major immune cells present in the dermis. To examine their potential involvement in nicotinic acid-induced flushing we conditionally depleted macrophages and dendritic cells using recently developed transgenic mouse lines in which either the CD11c promoter element drives the expression of the human diphtheria toxin (DT) receptor in dendritic cells (Jung et al., 2002) or in which diphtheria toxin receptor (DTR) expression is restricted to macrophages by using the CD11b promoter element (Duffield et al., 2005). Murine cells are insensitive to DT because their DTR homologue does not bind DT. In both, CD11b-DTR and CD11c-DTR mice we were able to show that the systemic administration of DT led to depletion of macrophages and dendritic cells, respectively (Fig. 1a,b and f,g). When DT-treated CD11b-DTR and CD11c-DTR mice were injected with nicotinic acid, a normal biphasic flushing response could be observed which was indistinguishable from the flushing response induced by nicotinic acid before DT treatment (Fig. 1c-e and h-j). This clearly indicates that neither

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macrophages nor dendritic cells in the dermis or elsewhere in the body are involved in the nicotinic acid-induced flushing response.

Another major immune cell type present in the skin are epidermal Langerhans cells which densely populate the epidermal layer of the skin. By using a mouse line expressing DTR under the control of the Langerhans cell-specific *langerin* promoter (Bennett et al., 2005), we were able to study the potential role of Langerhans cells in nicotinic acid-induced flushing. DT treatment of Langerin-DTR mice resulted in ablation of the Langerhans cell population from the epidermis of mice, while dermal dendritic cells were not affected (Bennett et al., 2005) (Fig. 2a and b). Interestingly, depletion of Langerhans cells was accompanied by abrogation of nicotinic acid-induced flushing, while Langerin-DTR animals before DT treatment showed normal distribution of epidermal Langerhans cells as well as normal flushing response to nicotinic acid (Fig. 2c-e). Wild type mice pretreated with DT under the same conditions as Langerin-DTR transgenic mice exhibited a normal flushing response (data not shown). Treatment of Langerin-DTR animals with DT did not principally affect the ability of these animals to respond with cutaneous vasodilation to other stimuli as shown by the normal flushing response to the intraperitoneal injection of PGD_2 (Fig. 2f-h). Thus, epidermal Langerhans cells are specifically required for the nicotinic acid-induced cutaneous vasodilation and are positioned upstream of PGD_2 release.

To test whether Langerhans cells functionally respond to nicotinic acid, we prepared epidermal sheets from ears of Langerin-DTR mice which express EGFP fused to DTR (Bennett et al., 2005), and loaded epidermal cells with the Ca^{2+} indicator Fura-2/AM. At the same time, Langerhans cells were visualized by fluorescence microscopy (Fig. 3a). Exposure of Fura-2-loaded epidermal sheets to 100 μM nicotinic acid resulted in a transient increase in $[Ca^{2+}]_i$ of Langerhans cells

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(Fig. 3b and c). Fura-2 loaded Langerhans cells in epidermal sheets from GPR109A-deficient mice, which were identified by staining with and anti-MHCII antibody, did not respond with any increase in $[Ca^{2+}]_i$ to the application of nicotinic acid while still responding to the Ca^{2+} ionophore ionomycin (Fig. 3d).

Since PGD_2 and PGE_2 have been shown to be principal mediators of the nicotinic acid-induced flushing response, we tested whether epidermal Langerhans cells express prostaglandin D_2 and prostaglandin E_2 synthases. Staining of epidermal sheets from mouse ears with specific antibodies directed against murine PGD_2 synthase as well as type 1 and type 2 PGE_2 synthases (mPGES-1 and mPGES-2) together with an antibody against mouse MHCII showed the presence of both PGD_2 synthase and type 2 PGE synthase whereas we were unable to detect type 1 PGE_2 synthase (mPGES-1) (Fig. 4). The mPGES-2 enzyme appeared to be exclusively expressed in epidermal Langerhans cells, whereas prostaglandin D_2 synthase was also detected in other cells of the epidermis (Fig. 4).

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Discussion

The nicotinic acid-induced flushing response is a phenomenon which was first described shortly after the discovery of nicotinic acid as a vitamin (Goldsmith and Cordill, 1943; Spies et al., 1938). The ability of nicotinic acid to induce a strong cutaneous vasodilation has always been an obstacle in the clinical use of nicotinic acid as a lipid modifying drug. Despite the pharmacological importance of this phenomenon, the underlying mechanism is still not clear. Based on the recent observation that bone marrow-derived immune cells in the skin mediate the nicotinic acid-induced flushing response, we systematically studied the involvement of individual cell populations like dermal macrophages, dermal dendritic cells and epidermal Langerhans cells which are all present at relatively high numbers in human and mouse skin (Dupasquier et al., 2004; Lenz et al., 1993). Our data clearly indicate that the nicotinic acid-induced flushing response is mediated by epidermal Langerhans cells while dermal dendritic cells and macrophages are not required. Depletion of Langerhans cells alone is able to prevent nicotinic acid-induced flushing.

Previous studies have shown that PGD_2 and PGE_2 are critically involved in the nicotinic acid-induced flushing response (Benyo et al., 2005; Cheng et al., 2006). In order to test whether Langerhans cells are principally able to synthesize both prostanoids we tested them for the expression of PGD_2 and PGE_2 synthases. We found both PGD_2 synthase as well as the constitutive type 2 PGE_2 synthase (mPGES-2) to be expressed in epidermal Langerhans cells. The inducible type 1 PGE synthase form (mPGES-1) (Kudo and Murakami, 2005) could, however, not be detected. This is consistent with earlier findings which showed that Langerhans cells are a major source of prostanoids in the epidermis, and that their main prostanoid product is PGD_2 while they are also able to synthesize PGE_2 (Rosenbach et al., 1990; Ruzicka and Aubock, 1987).

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Our finding that Langerhans cells respond to nicotinic acid with a transient increase in the cytoplasmic Ca^{2+} concentration suggest that activation of G_i through the nicotinic acid receptor results in a Gβγ-mediated phospholipase C activation, a classical response of immune cells to the activation of G_i-coupled receptors (Exton, 1996; Rhee, 2001). The transient increase in the cytoplasmic Ca^{2+} concentration is a major trigger of the activation of phospholipase A_2 and subsequent formation of arachidonic acid. Arachidonic acid is then further metabolized by the ubiquitously expressed type 1 cyclooxygenase and both PGD_2 and PGE_2 synthases which are present in Langerhans cells. The release of PGD_2 and PGE_2 from nicotinic acid-activated Langerhans cells then results in vasodilation in the dermal papillae of the upper dermis layer which are just adjacent to the epidermis-dermis junction close to the localization of Langerhans cells.

The nicotinic acid-induced flushing response is subject to a tolerance phenomenon which can be observed within days after repeated administration of nicotinic acid (Olsson, 1994). Tolerance is unlikely to develop on the basis of receptor desensitization as the metabolic effects are stable even after long-term administration of nicotinic acid (Olsson, 1994). It is more likely that the downstream mechanisms specific for the nicotinic acid-induced flushing response are involved. We could rule out the possibility that tolerance to nicotinic acid-induced flushing is due to any translocation of Langerhans cells, as repeated administration of nicotinic acid did not change the number of Langerhans cells per epidermal area although the vasodilatory effect had seized (data not shown). It is therefore more likely that the nicotinic acid-induced prostanoid formation in Langerhans cells undergoes tachyphylaxis. This would be consistent with the observation that the development of tolerance to nicotinic acid-induced flushing is accompanied by a reduced formation of prostanoids (Stern et al., 1991). It has been described in the past that patients with atopic

dermatitis show a severely reduced flushing response to topical application of nicotinic acid esters (English et al., 1987; Uehara and Ofuji, 1977). Thus, activation of Langerhans cells under certain pathological conditions like atopic dermatitis (Leung et al., 2004) may lead to a resistance of Langerhans cells to nicotinic acid-induced prostanoid formation.

The observed link between the activation of epidermal Langerhans cells and the regulation of blood vessel diameter in the upper dermis raises the question whether this mechanism in addition to its pharmacological significance has any physiological or pathophysiological role. There are numerous transient and chronic forms of skin alterations which go along with erythema. It will be interesting to study the role of Langerhans cells not only in immunological responses of the skin but also in the local regulation of dermal blood flow.

Our data clearly show that epidermal Langerhans cells play a central role in the nicotinic acid-induced flushing response by mediating the formation of vasodilatory prostanoids in response to activation by nicotinic acid. This study provides new insight into the mechanism underlying the clinical problem of nicotinic acid-induced flushing and may provide the basis for new approaches aiming at the reduction of this unwanted side effect. In addition, our data point to an interesting new function of epidermal Langerhans cells in the regulation of the local blood flow in the dermis, whose potential physiological and pathophysiological role remains to be further characterized.

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Figure legends

Fig. 1. Effect of macrophage and dendritic cell depletion on nicotinic acid-induced flushing. CD11b-DTR (A-E) and CD11c-DTR transgenic mice (F-J) were analyzed before or after i.p. injection of DT. A, B, F, G, verification of macrophage and dendritic cell depletion. Cells harvested by peritoneal lavage (A and B) or splenocytes (F and G) were analyzed by FACS. C-E and H-J, original laser-Doppler flow recordings and quantitative analysis of the percentage of LDF increase in response to i.p. administration of 200 µg/g nicotinic acid in untreated or DT-treated CD11b-DTR (C-E) and CD11c-DTR mice (H-J).

Fig. 2. Nicotinic acid-induced flushing requires the presence of epidermal Langerhans cells. Langerin-DTR transgenic mice were analyzed before or after i.p. injection of DT. A, B, verification of Langerhans cell depletion. Shown is an overlay of the light microscopical image and the fluorescence image of epidermal sheets prepared from untreated (A) and DT-treated (B) Langerin-DTR mice which were stained with PE-labeled anti-MHCII antibodies. C-E, original laser-Doppler flow recordings and quantitative analysis of the percentage of LDF increase in rersponse to i.p. administration of nicotinic acid in untreated or DT-treated Langerin-DTR mice. F-H, original laser-Doppler flow recordings and quantitative analysis of the percentage of LDF increase after i.p. administration of 2 µg/g PGD_2 in untreated or DT-treated Langerin-DTR mice.

Fig. 3. Epidermal Langerhans cells respond to nicotinic acid. Langerhans cells were identified in epidermal sheets of untreated Langerin-DTR mice by EGFP fluorescence (A), and sheets were loaded with Fura-2 and exposed to 100 µM nicotinic acid (B and C). D, representative traces demonstrating the effect of nicotinic acid (100 µM) and

ionomycin (Iono, 1 μM) on $[Ca^{2+}]_i$ ion MHCII positive cells of epidermal sheets prepared from GPR109A-deficient mice. Values on the y-axis indicate the measured 340/380-nm fluorescence ratio as an indicator of the free intracellular $[Ca^{2+}]$ concentration

Fig. 4. Expression of prostanoid synthases in epidermal Langerhans cells. Epidermal sheets from wild type mice were stained with antibodies against MHCII and microsomal prostaglandin E synthase-1 (A, mPGES-1) or microsomal prostaglandin E synthase-2 (B, mPGES-2) or PGD_2 synthase (C, PGD_2-S). The length of the bar in the upper row of each panel corresponds to 50 μm, whereas bar length in the lower row corresponds to 5 μm.

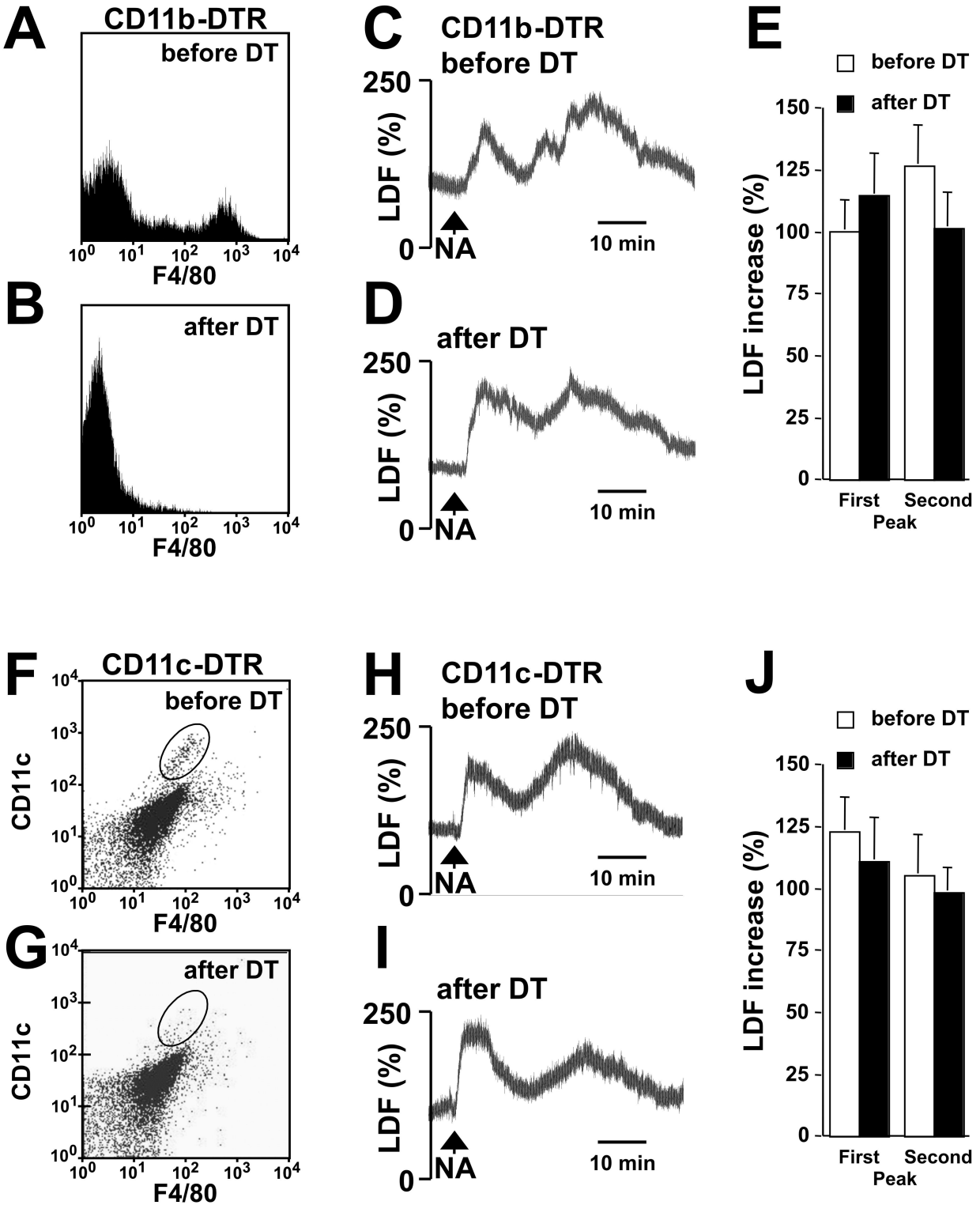
A
CD11b-DTR
before DT
F4/80
B
after DT
F4/80
C
CD11b-DTR
before DT
LDF (%)
250
0
NA
10 min
D
after DT
LDF (%)
250
0
NA
10 min
E
before DT
after DT
LDF increase (%)
150
125
100
75
50
25
0
First
Second
Peak
F
CD11c-DTR
before DT
CD11c
F4/80
G
after DT
CD11c
F4/80
H
CD11c-DTR
before DT
LDF (%)
250
0
NA
10 min
I
after DT
LDF (%)
250
0
NA
10 min
J
before DT
after DT
LDF increase (%)
150
125
100
75
50
25
0
First
Second
Peak

Fig. 1

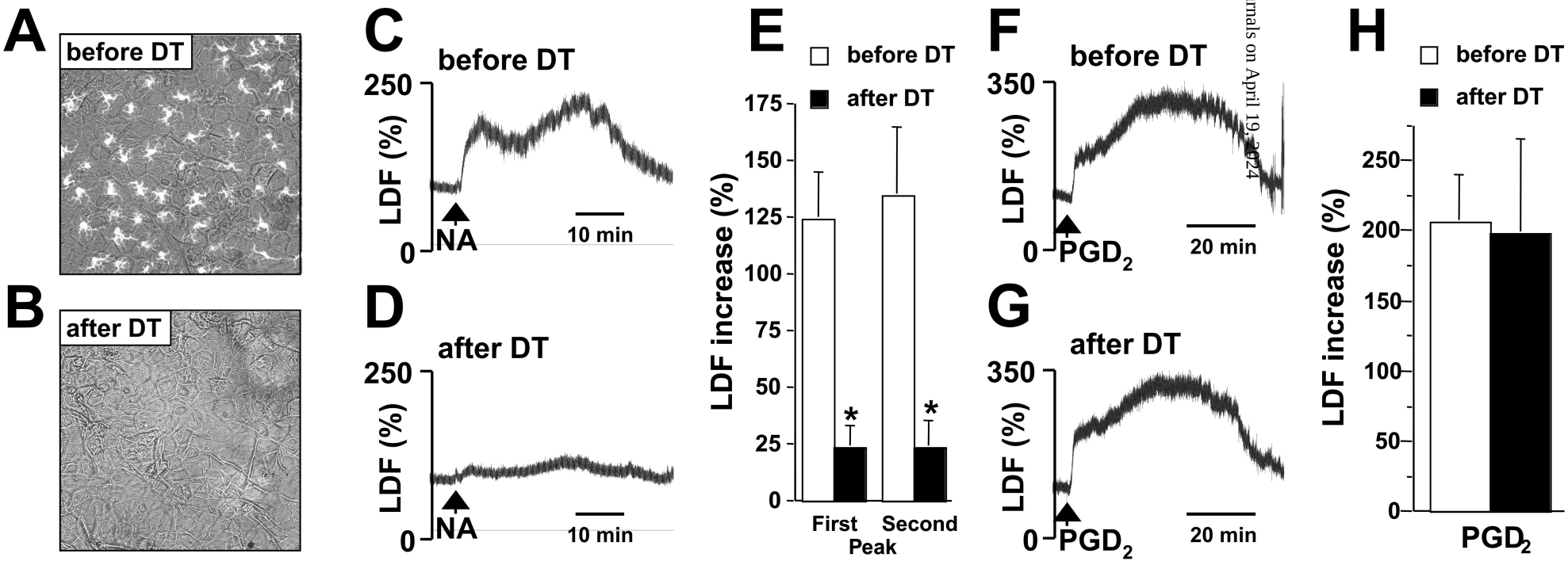

Fig. 2

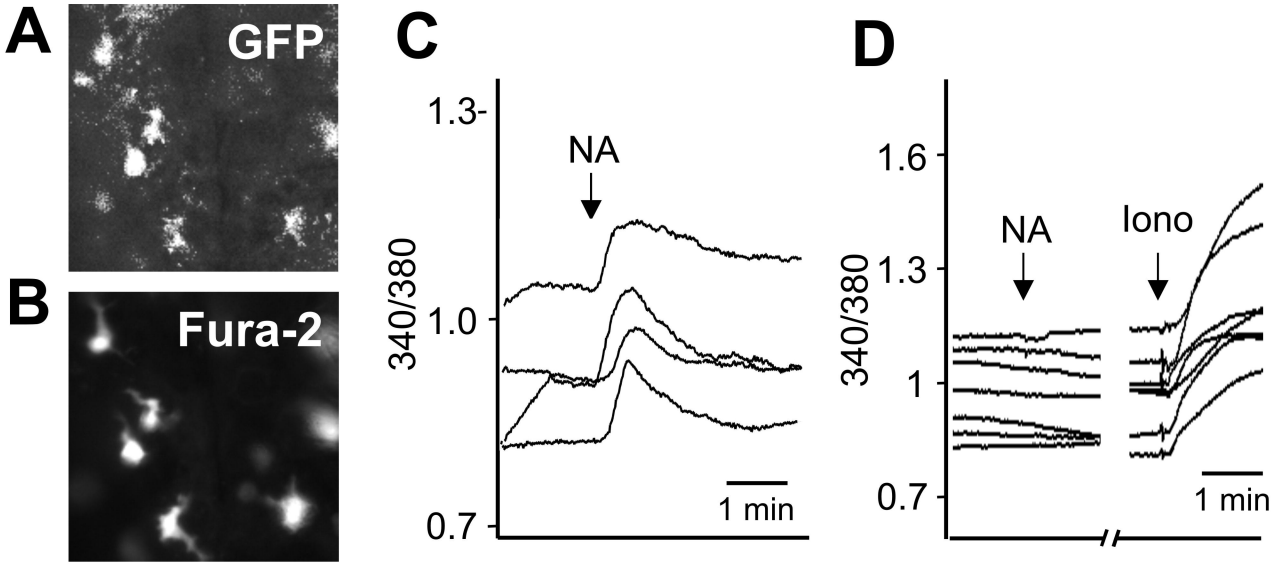
A
GFP
B
Fura-2
C
1.3-
NA
340/380
1.0
0.7
1 min
D
1.6
NA
Iono
340/380
1.3
1
0.7
1 min

Fig. 3

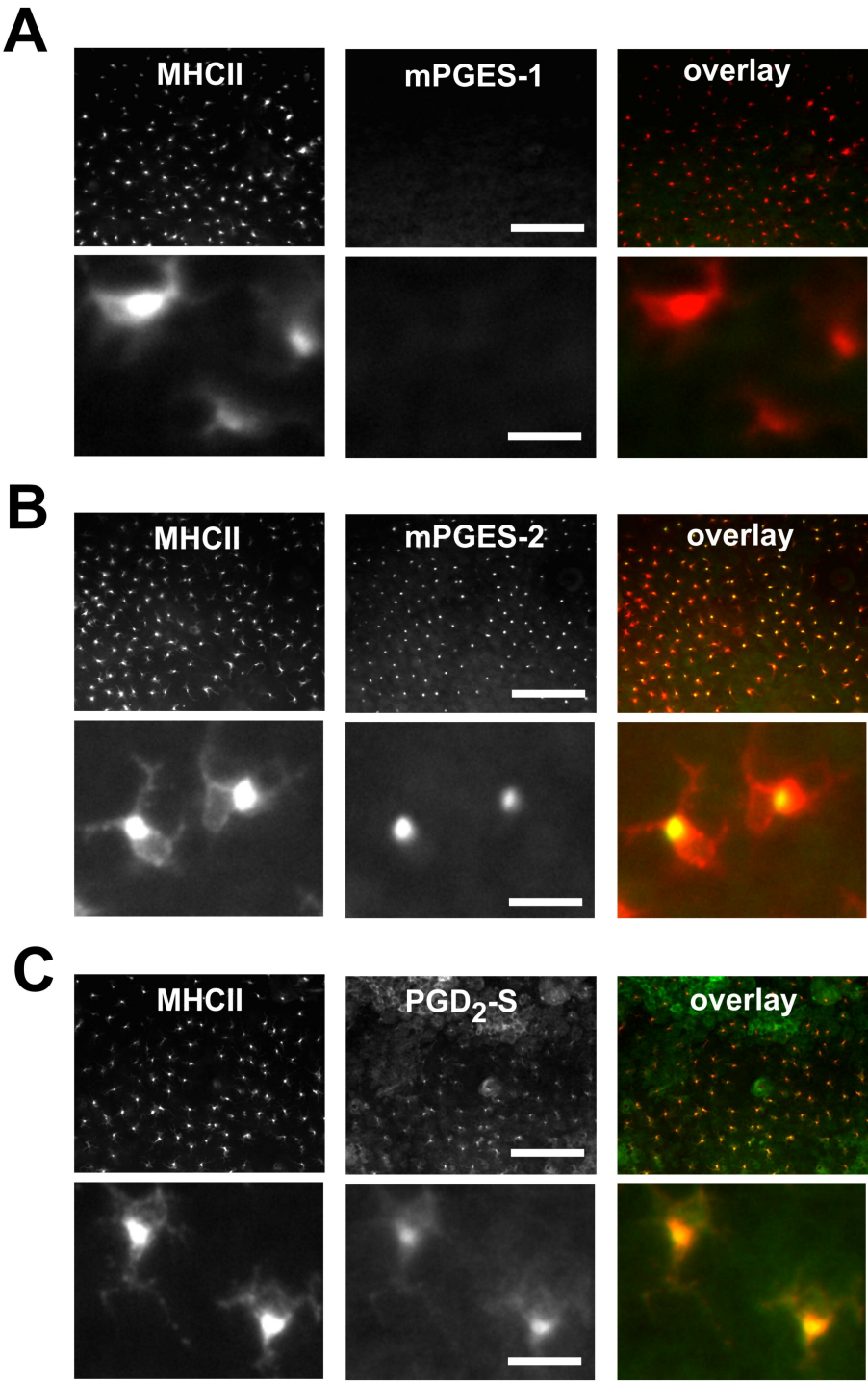
A
MHCII
mPGES-1
overlay
B
MHCII
mPGES-2
overlay
C
MHCII
PGD2-S
overlay

Fig. 4