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CD154 (CD40L): a novel aid to document nonimmediate hypersensitivity to amoxicillin or amoxicillin clavulanic acid

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The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

To the editor,

Diagnosis of nonimmediate drug hypersensitivity reactions (NIDHRs) to β-lactams starts with a history and revision of the patient's records complemented with intradermal tests (IDT) with delayed readings and/or patch tests [1, 2]. However, history is not always straightforward and skin test responses do not always predict the clinical outcome of subsequent exposure [3]. Consequently, some patients might require controlled drug challenges (DC) to have their diagnosis established. Unfortunately, DCs can elicit severe reactions and are contraindicated in patients who experienced potentially life-threatening SCARs. In such patients, a reliable and safe in vitro substitute would be more than welcome.

In vitro tests for NIDHRs have focused on quantifying activated drug-specific T lymphocytes. The main advantages of flow cytometric assays over traditional lymphocyte transformation tests (LTTs) are speed and the fact that they do not require a radioisotope. Beeler et al [4], demonstrated up-regulation of CD69 on T cells to be a promising substitute for classical LTT to diagnose NIDHRs to β-lactams such as amoxicillin (AX). However, high concentrations of clavulanic acid (CL) induced CD69 expression by $CD4^{+ve}$-T lymphocytes in control individuals.

CD154 is a member of the TNF-superfamily and is expressed by $CD8^{+ve}$-cytotoxic T cells, which can also be involved in NIDHRs [5], e.g. a maculopapular exanthema to β-lactams. With respect to the application of a CD154-based lymphocyte activation test (LAT) in NIDHRs, we located a single series of 14 patients [6]. This study provided encouraging data about the potential of a CD154-LAT in NIDHRs, but no cases with NIDHR to aminopenicillins were enrolled. Because aminopenicillins constitute a predominant cause of NIDHRs, we sought to explore the potential of the CD154-LAT in documenting AX or AX/CL hypersensitivity.

As shown in table E1 in the repository file, 15 patients with a NIDHR to AX or AX/CL were selected. Ten patients had their diagnosis confirmed by delayed readings of intradermal skin tests (IDT). The remainder 5, required a DC with AX or AX/CL because of negative IDT. In 2/5 patients requiring a DC, repeated IDT after their positive DC was negative. The majority of the patients (8/15) presented with an undefined delayed skin eruption. In these patients AX/CL was chosen for diagnostic work-up as this is by far the most common used B-lactam in our region [7]. The median interval between the index reaction and IDT or DC was 2.8 years and 1.3 years between initial diagnostic work-up and the CD154-LAT. Ten tolerant individuals (6 female; median age 47 years) with negative delayed readings of IDT and an uneventful DC with AX or AX/CL served as a control group. The local ethics committee approved this study (B300201524055) and patients provided an informed consent in accordance with the Declaration of Helsinki.

Skin testing with AX or AX/CL were performed according to the ENDA guidelines [8] (table E2 in this article's Online Repository). Delayed reading of IDT was judged positive when an induration surrounded by erythema exceeded 5 mm and occurred within 48-96 hours. Drug challenges are described elsewhere [3].

For our CD154-LAT experiments, peripheral blood mononuclear cells were collected after a density gradient isolation with Histopaque (Merck, St. Louis, Missouri, USA). Each tube contains 1.106 cells in AIM-V medium (Thermo Fisher Scientific (TFS), Merelbeke, Belgium) with autologous plasma. The experiment includes the AIM-V negative control and a positive control (Staphylococcus aureus enterotoxin B, Merck, St. Louis, Missouri, USA). After preincubation of 2 hours at 37°C 5%CO_2 and saturated humidity with anti-human CD28 (TFS, final concentration 5 µg/mL), a protein transport inhibitor containing Brefeldin A (BD

Biosciences, Erembodegem, Belgium; end concentration 10 µg/mL) was added followed by AX/CL (ratio 7/1, Sandoz, Vilvoorde, Belgium) in three concentrations (875/125, 437.5/62.5 and 175/25 µg/mL). To evaluate the effect of CL, in 5 patients experiments with AX (1000, 500 and 200 µg/mL) were done. Subsequently, incubation at 37°C 5%CO_2 and saturated humidity for an additional 16 hours was performed. After centrifugation, the cell pellet was resuspended in phosphate buffered saline (PBS pH = 7.4) containing 0.5% bovine serum albumin (BSA). Then, cells were stained with anti-CD3-PercP (BD Biosciences) during 20 minutes at 4°C. After lysis and fixation with lysing solution (BD Biosciences) during 20 minutes at room temperature, cells were centrifuged and permeabilized with 0.3% saponin in PBS (Merck, St. Louis, Mis-souri, USA). Cells were stained with anti-CD154-PE (BD Biosciences) for 30 minutes on 4°C in the dark. Cells were washed in 0.3% saponin and resuspended in PBS for analysis using FacsCanto II (BD Biosciences) flow cytometer. At least 350,000 living (LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (ThermoFisher Scientific)) CD3 positive cells were gated out to determine intracellular CD154 upregulation. Figure E1 shows a representative plot.

GraphPad Prism version 8.00 software was used for data analysis (GraphPad Software, La Jolla California USA, www.graphpad.com). Percentages of CD154 upregulation after stimulation with AX or AX/CL were calculated by subtracting the % of CD154 upregulation after stimulation with AIM-V medium (negative control) and costimulation with CD28.

As shown in figure 1, all patients had a CD154-based LAT with three concentrations of AX/CL. From the dose-finding experiments, 437.5/62.5 µg/mL appeared the most discriminative concentration between patients and tolerant individuals. For this optimal stimulation concentration, 7/15 patients and 4/10 tolerant individuals showed a median upregulation in

the $CD3^{+ve-}$cells of CD154 of 0.63% (range 0.14–1.62; 95%CI 0.28-1.62) and 0.13% (range 0.06–0.19; 95%CI 0.06-0.19) respectively. In 6/7 patients (3 with a positive IDT and 3 with a positive DC) who showed upregulation of CD154, upregulation exceeded the maximal value of 0.19% of our tolerant individuals.

To examine whether CL triggers nonspecific CD154 upregulation by T-lymphocytes, comparative analyses between AX/CL 437.5/62.5 µg/mL and AX 500 µg/mL were done in 5 tolerant individuals. As shown in figure 2, CL did not induce nonspecific CD154 upregulation.

To ascertain that our results are not due to inter-assay variations, in 15 individuals experiments with AX/CL 437.5/62.5 µg/mL were done in duplicate. A median variation of CD154 upregulation of 0.05% (range 0.01 – 0.12) was recorded (data not shown).

Here we provide the first evidence on the potential of CD154 as an alternative readout for drug-specific T-lymphocyte activation in NIDHR to AX or AX/CL. Our findings suggest that the CD154-LAT is a safe and quick diagnostic for mild cutaneous NIDHRs to AX or AX/CL. It provides a result within 24 hours without need for additional markers to identify $CD4^{+ve}$- or $CD8^{+ve}$-T-lymphocytes. Although CD154 upregulation was also observed in some AX/CL tolerant individuals, the median upregulation was numerically lower than in patients with AX or AX/CL hypersensitivity responsive in the LAT. Using an optimal concentration, 7/15 patients with an established NIDHR, demonstrated upregulation of CD154. In all, except in one of these 7 patients, upregulation exceeded the maximum upregulation of our tolerant control individuals. It cannot be excluded the interval between initial diagnostic work-up and lymphocyte activation experiments to be in disadvantage of the latter. Most importantly, the CD154-LAT was positive in 3/5 patients who required DC for diagnosis . Our proposal would be to offer a CD154-LAT before DC to patients with a history compatible with a NIDHR who

demonstrate negative or uncertain delayed readings in skin testing. However, larger studies are needed to confirm this proposal. As it has been shown that the performance of different T-lymphocyte proliferation assays can be influenced by the clinical phenotype (for review: [9, 10]), for the time being, the exact positioning of the CD154-LAT in SCARs remains elusive and unpredictable.

A potential issue of this study is that the CD154-LAT experiments in the patients with positive DC were performed after their delayed reaction. Questions remain whether upregulation of CD154 would also have been present before the DC. However, in 2/5 patients, of whom 1 patient showed upregulation of CD154, skin testing was repeated after the positive drug challenge and remained negative.

In conclusion, the CD154-based LAT is an attractive instrument to document mild cutaneous NIDHR to AX or AX/CL, especially in cases who demonstrate uncertain skin tests and thus require additional challenges. Additional larger studies are required to confirm our observations, to calculate predictive values and to determine whether the technique can enter mainstream applications, especially in severe cases in whom a challenge is precluded.

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We thank Dr. Oliver Hausmann and Dr. Daniel Yerly for careful revision of the manuscript and their contribution in the interpretation of the data and discussion. DGE is a Senior Clinical Researcher of the Research Foundation Flanders/Fonds Wetenschappelijk Onderzoek (FWO: 1800614N). ALVG is a fellow of the Research Foundation Flanders/Fonds Wetenschappelijk Onderzoek (FWO: 1113617N). VS is a Senior Clinical Researcher of the Research Foundation

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Figure legends

Figure 1. Dose finding experiments with amoxicillin clavulanic acid

Dose finding experiments with amoxicillin clavulanic acid in three concentrations, i.e. 175/25 µg/mL, 437.5/62.5 µg/mL and 875/125 µg/mL were performed in 15 patients with a diagnosis of a NIDHR to amoxicillin or amoxicillin clavulanic acid documented by delayed readings of intradermal tests (red), or, if negative a controlled drug challenge (green) and 10 uneventfully challenged individuals (black). CD154 upregulation in $CD3^{+ve}$ T cells is presented on the y-axis. *IDT = intradermal test; DC = drug challenge; + = positive; Δ = percentages of CD154 upregulation were calculated by subtracting the % of CD154 upregulation after stimulation with buffer (negative control).*

Figure 2. Comparative analyses with amoxicillin clavulanic acid and amoxicillin

In 5 individuals tolerant to amoxicillin clavulanic acid comparative analyses with amoxicillin clavulanic acid (437.5/62.5 µg/mL) and amoxicillin (500 µg/mL) were done. CD154 upregulation in $CD3^{+ve}$ T cells is presented on the y-axis. 3 patients did not show any upregulation and their curves fall together with x-axis. *Δ = percentages of CD154 upregulation were calculated by subtracting the % of CD154 upregulation after stimulation with buffer (negative control).*

Repository file figure/table legends

Figure E1. CD154 upregulation plots in a patient with positive reading of the intradermal test with amoxicillin clavulanic acid

Selection of $CD3^{+ve}$ T cells. Stimulation with *S. Aureus* as a positive control triggers upregulation of CD154. Amoxicillin clavulanic acid (concentration 437.5/62.5 µg/mL) also leads to some upregulation of CD154 compared to stimulation with buffer (negative control).

Table E1. Clinical data and allergy test results of our 15 patients with a nonimmediate amoxicillin (clavulanic acid) hypersensitivity reaction

Patient	Sex	Age (y)	Culprit drug	Delay work-up (y)	Symptoms	Diagnostic work-up				Delay experiments (y)
						IDT AX	IDT AX/CL	Positive DC	IDT after DC	
1	F	64	Undefined penicillin	Un	Un	NP	+	NP	NP	2.9
2	F	55	AX/CL	0.7	Undefined rash	NP	+	NP	NP	0.2
3	F	27	Undefined penicillin	Un	Un	NP	+	NP	NP	5.8
4	F	51	Undefined penicillin	Un	Un	NP	+	NP	NP	0.6
5	M	42	AX/CL	Un	Undefined rash	NP	+	NP	NP	4.7
6	F	37	AX	10.0	Undefined rash	+	NP	NP	NP	2.0
7	F	33	Undefined penicillin	Un	Un	NP	+	NP	NP	0.5
8	F	64	AX/CL	4.8	Undefined rash	NP	+	NP	NP	4.2
9	F	26	AX/CL	Un	Undefined rash	NP	+	NP	NP	0.3
10	F	44	Undefined penicillin	0.3	MPE	NP	+	NP	NP	0.2
11	F	62	AX/CL	8	Undefined rash	NP	-	AX/CL	NP	2.9

12	M	25	AX	1.9	MPE	-	NP	AX	-	1.3
13	F	42	Undefined penicillin	5	Undefined rash	NP	-	AX/CL	-	1.0
14	M	70	AX/CL	3.1	Undefined rash	NP	-	AX/CL	NP	2.9
15	F	37	AX	Un	MPE	-	NP	AX	NP	1.4

F = female; M = male; AX = amoxicillin; AX/CL = amoxicillin clavulanic acid; Un = unknown; IDT = intradermal test; DC = drug challenge; MPE = maculopapular exanthema; NP = not performed

Table E2. Protocol for skin testing

Amoxicillin or amoxicillin clavulanic acid

	Concentration
Skin prick test	0.2 mg/ml
	2 mg/ml
	20 mg/ml
Intradermal test	0.2 mg/ml
	2 mg/ml
	20 mg/ml

Histamine 10 mg/ml (ALKAbello, Hørsholm, Denmark) and isotonic sodium chloride were used as positive and negative controls respectively.

Skin prick tests were read after 15 minutes and considered positive when the wheal of the wheal and flare reaction exceeded 3 mm. Patients with negative SPT had additional IDT. IDT responses were considered positive when the wheal of the wheal and flare reaction equalled or exceeded 5 mm.

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5	M	42	AX/CL	Un	Undefined rash	NP	+	NP	NP	4.7
6	F	37	AX	10.0	Undefined rash	+	NP	NP	NP	2.0
7	F	33	Undefined penicillin	Un	Un	NP	+	NP	NP	0.5
8	F	64	AX/CL	4.8	Undefined rash	NP	+	NP	NP	4.2
9	F	26	AX/CL	Un	Undefined rash	NP	+	NP	NP	0.3
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12	M	25	AX	1.9	MPE	-	NP	AX	-	1.3
13	F	42	Undefined penicillin	5	Undefined rash	NP	-	AX/CL	-	1.0
14	M	70	AX/CL	3.1	Undefined rash	NP	-	AX/CL	NP	2.9
15	F	37	AX	Un	MPE	-	NP	AX	NP	1.4

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