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**Cellular recognition of drug antigenic determinants by
allergic patients. Inclusion of new parameters to
improve cellular *in vitro* tests.**

Tesis doctoral
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2. Fernandez-Santamaria R, Bogas G, Montañez MI, Ariza A, Salas M, Cespedes JA, Labella M, Paris JL, Perez-Sanchez N, Perez-Inestrosa E, Vida Y, Fernandez TD, Mayorga C, Torres MJ. **Synthetic antigenic determinants of clavulanic acid induce dendritic cell maturation and specific T cell proliferation in patients with immediate hypersensitivity reactions.** Allergy. 2022 Oct;77(10):3070-3083. doi: 10.1111/all.15383.

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ABBREVIATIONS AND ACRONYMS

- ◆ **AA:** 4-AminoAntipyrine
- ◆ **AAA:** 4-AcetylAmino-Antipyrine
- ◆ **AD:** Antigenic Determinant
- ◆ **ADR:** Adverse Drug Reaction
- ◆ **AGEP:** Acute Generalised Exanthematous Pustulosis
- ◆ **AMP:** Ampicillin
- ◆ **ANA:** Anaphylaxis
- ◆ **ANG:** Angioedema
- ◆ **APC:** Antigen Presenting Cell
- ◆ **AS:** Anaphylactic Shock
- ◆ **ASPS:** Algorithm of the Spanish Pharmacovigilance System
- ◆ **AX:** Amoxicillin
- ◆ **BDCA:** Blood Dendritic Cell Antigens
- ◆ **BL:** Betalactam
- ◆ **BM:** Bone Marrow
- ◆ **BP:** BenzylPenicillin
- ◆ **Breg:** B Regulatory cell
- ◆ **CAST:** Cellular Allergen Stimulation Test
- ◆ **CCR7:** C-C chemokine receptor type 7
- ◆ **cDC:** Conventional Dendritic Cell
- ◆ **CDP:** Common Dendritic Cell progenitor
- ◆ **CFSE:** CarboxyFluorescein Succinimidyl Ester
- ◆ **CLA:** Cutaneous Lymphocyte-associated Antigen
- ◆ **C-LTT:** Conventional Lymphocyte Transformation Test
- ◆ **cLT:** Cysteinyl Leukotrienes
- ◆ **CLV:** Clavulanic acid
- ◆ **cMop:** Common Monocyte Progenitor
- ◆ **Cox-1:** Cyclooxygenase-1
- ◆ **CTL:** Cytotoxic T Lymphocyte
- ◆ **DC:** Dendritic Cell

ABBREVIATIONS AND ACRONYMS

- ◆ **dDC-LTT:** Drug-Dendritic Cell Lymphocyte Transformation Test
- ◆ **DHR:** Drug Hypersensitivity Reaction
- ◆ **DIAT:** DIAminoThiazine
- ◆ **DRESS:** Drug Reaction with Eosinophilia and Systemic Symptoms
- ◆ **EAACI:** European Academy of Allergy and Clinical Immunology
- ◆ **ELISA:** Enzyme-Linked ImmunoSorbent Assay
- ◆ **ENDA:** European Network on Drug Allergy
- ◆ **ESCD:** European Society of Contact Dermatitis
- ◆ **EPX:** Carbamazepine-10,11-EPoXide
- ◆ **FAA:** 4-FormylAmino-Antipyrine
- ◆ **FBS:** Fetal Bovine Serum
- ◆ **FCεRI:** High-affinity receptor
- ◆ **FL:** Fetal Liver
- ◆ **GM-CSF:** Granulocyte-Macrophage Colony-Stimulating Factor
- ◆ **HLA:** Human Leukocyte Antigen
- ◆ **HRUM:** Hospital Regional Universitario de Málaga
- ◆ **HRT:** Histamine Release Test
- ◆ **HSA:** Human Serum Albumin
- ◆ **HSC:** Hematopoietic Stem Cell
- ◆ **ICM:** Iodinated Contrast Media
- ◆ **ICON:** International Consensus on Drug Allergy
- ◆ **IDHR:** Immediate Drug Hypersensitivity Reaction
- ◆ **IgA:** Immunoglobulin A
- ◆ **IgD:** Immunoglobulin D
- ◆ **IgE:** Immunoglobulin E
- ◆ **IgG:** Immunoglobulin G
- ◆ **IgM:** Immunoglobulin M
- ◆ **IFN-γ:** Interferon gamma
- ◆ **IRF:** Interferon Regulatory Factor
- ◆ **L-ASA:** L-Aspirin
- ◆ **LCS:** Langerhans cells

- ◆ **LN:** Lymph Node
- ◆ **LPS:** Lipopolysaccharide
- ◆ **LTT:** Lymphocyte Transformation Test
- ◆ **MAA:** MethylAmino-Antipyrine
- ◆ **mAb:** Monoclonal antibody
- ◆ **mDC:** Myeloid Dendritic Cell
- ◆ **MDH:** Multiple Drug Hypersensitivity
- ◆ **MDM:** Minor Determinant Mixture
- ◆ **moDC:** Monocyte Derived Dendritic Cell
- ◆ **MPE:** Maculopapular Exanthema
- ◆ **MRGPRX2:** Mas-Related G-Protein Coupled Receptor Member X2
- ◆ **NIDHR:** Non-immediate Drug Hypersensitivity Reaction
- ◆ **NK:** Natural Killer
- ◆ **NMBA:** Neuromuscular Blocking Agent
- ◆ **NSAID:** Nonsteroidal Anti-Inflammatory Drug
- ◆ **PAMP:** Pathogen-Associated Molecular Pattern
- ◆ **PBMC:** Peripheral Blood Mononuclear Cell
- ◆ **pDCs:** Plasmacytoid Dendritic Cells
- ◆ **PGE2:** ProstaGlandin E2
- ◆ **PHO:** PHOlcodine
- ◆ **PLL:** Poly-L-Lysine
- ◆ **PPL:** Penicilloyl-PolyLysine
- ◆ **PPV:** Positive Predictive Value
- ◆ **PT:** Patch Test
- ◆ **PV:** Penicillin V
- ◆ **QAM:** Quaternary Ammonium Morphine
- ◆ **RIA:** RadiImmunoAssay
- ◆ **ROC:** Receiver Operating Characteristic
- ◆ **SJS/TEN:** Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis
- ◆ **sLT:** Sulfide Leukotrienes
- ◆ **SMX:** Sulfametoxazol

ABBREVIATIONS AND ACRONYMS

- ◆ **SPT:** Skin Prick Test
- ◆ **ST:** Skin Test
- ◆ **TAP:** Transporter Associated with Antigen Processing
- ◆ **TCR:** T Cell Receptor
- ◆ **TLR:** Toll-Like Receptor
- ◆ **TNF- α :** Tumor Necrosis Factor α
- ◆ **Treg:** T Regulatory cell
- ◆ **Trm:** Tissue Resident Memory T cell
- ◆ **URT:** Urticaria
- ◆ **US:** United States of America
- ◆ **WHO:** World Health Organization
- ◆ **WTM:** Wortmannin
- ◆ **YS:** Yolk Sac

RESUMEN

Las reacciones de hipersensibilidad a fármacos (DHRs) son reacciones adversas a fármacos (ADRs), no relacionadas con la dosis o la farmacocinética del fármaco, produciéndose solamente en ciertas personas expuestas al mismo. La incidencia de estas reacciones ha aumentado en las últimas décadas, probablemente debido al aumento del consumo de fármacos, principalmente en el mundo occidental, a los cambios en nuestros hábitos alimentarios y de higiene, que pueden alterar nuestro microbioma, así como nuestra mayor exposición a contaminación y aeroalérgenos.

Aunque la clasificación de DHRs es importante, no existe un consenso sobre la mejor manera de clasificarlas, principalmente debido a la gran diversidad y complejidad de los fármacos implicados, así como al gran número de manifestaciones clínicas y mecanismos inmunológicos existentes.

Una clasificación útil de DHRs es aquella que divide a las reacciones en función del tiempo transcurrido entre la administración o ingesta del fármaco y la aparición de síntomas. Las reacciones inmediatas (IDHRs) son aquellas en que los síntomas se producen en menos de una hora desde la administración del fármaco. En cambio, los síntomas en reacciones no inmediatas (NIDHRs) aparecen después de 1 hora tras la administración del fármaco, pudiendo aparecer tras varios días o incluso semanas. No obstante, existe cierta controversia en las reacciones cuyos síntomas aparecen entre 1 y 6 horas tras la exposición del fármaco, aunque algunos autores las clasifican como aceleradas. Por lo tanto, aunque esta clasificación es muy útil, principalmente para los procedimientos de diagnóstico clínico, esta clasificación también es controvertida ya que no todas las reacciones encajan de forma perfecta. No obstante, para los objetivos de la presente tesis, se utilizará esta clasificación, diferenciando entre IDHRs y NIDHRs.

En cuanto a las IDHRs, las reacciones pueden clasificarse en inmunológicamente mediadas (alérgicas) o aquellas sin mecanismo inmunológico implicado (no alérgicas). Las reacciones no alérgicas están causadas por la interacción directa del fármaco con receptores de mastocitos o basófilos, o mediante la inhibición de enzimas, como es el caso de la ciclooxygenasa-1 (COX-1), causando la reducción de prostaglandinas, y la consiguiente acumulación de leucotrienos, provocando la activación y desgranulación de basófilos y mastocitos. De acuerdo con esta clasificación, esta tesis se centrará solamente en reacciones alérgicas.

Otra manera de clasificar las DHRs es mediante el mecanismo inmunológico concreto implicado en cada reacción. Combinando esta clasificación y la anterior que divide las reacciones en función del intervalo de tiempo entre la administración del fármaco y la aparición de síntomas, las IDHRs se encuentran mayoritariamente mediadas por inmunoglobulina E específica (sIgE), mientras que las NIDHRs están normalmente mediadas por linfocitos. Por lo tanto, para el propósito de esta tesis, y para facilitar la comprensión del lector, se realizará la asociación entre IDHRs y mecanismo IgE mediado y entre NIDHRs y mecanismo mediado por linfocitos.

Las DHRs también pueden clasificarse en función de cómo los fármacos interaccionan con el sistema inmune. Esta interacción puede ser explicada por 3 diferentes modelos. El primer modelo descrito fue el del hapteno, principalmente evaluado para antibióticos betalactámicos (BLs). Este modelo se basa en el hecho de que los fármacos son moléculas demasiado pequeñas para poder ser reconocido por el sistema inmune. Para ello, los fármacos necesitan unirse a proteínas, para formar el conjugado fármaco-proteína que puede interaccionar con el sistema inmune. El segundo modelo de interacción descrito es aquel que se basa en la interacción farmacológica, principalmente descrito para bloqueadores neuromusculares. Este modelo sugiere que los fármacos pueden interaccionar directamente con los complejos mayores de histocompatibilidad de las células presentadoras de antígenos, o los receptores de linfocitos T, provocando la activación del sistema inmune. El último modelo descrito para la interacción entre el fármaco y el sistema inmune es el modelo de alteración del péptido, principalmente descrito para Abacavir. Este modelo se basa en que el fármaco puede modificar de forma específica ciertos péptidos de los complejos mayores de histocompatibilidad, permitiendo su reconocimiento por linfocitos T. En esta tesis, para las IDHRs solo se evalúan reacciones a BLs, por lo que se puede asumir que el modelo de interacción del fármaco con el sistema inmune corresponde al modelo del hapteno. No obstante, debido a que para las NIDHRs se evalúan diferentes fármacos y manifestaciones clínicas, no se puede excluir la participación de otros modelos de interacción.

Teóricamente, todos los fármacos tienen la capacidad de provocar DHRs, aunque ciertos fármacos como los BLs se encuentran fuertemente implicados en este tipo de reacciones, tanto en adultos como en niños. No obstante, otros fármacos como los

antiinflamatorios no esteroideos, bloqueadores musculares, fluoroquinolonas, inhibidores de la bomba de protones, heparinas, o contrastes yodados son fármacos frecuentemente implicados en reacciones de hipersensibilidad.

Aunque la prevalencia de estas reacciones parece haber aumentado en las últimas décadas, establecer la incidencia concreta en la población general es complicado, principalmente debido al sobrediagnóstico e infradiagnóstico. El sobrediagnóstico es debido a que sujetos son clasificados como alérgicos, pero realmente no lo son, pudiendo tolerar el fármaco. El infradiagnóstico, aunque menos frecuente, consiste en clasificar al paciente como no alérgico, cuando realmente lo es, por lo que tiene implicaciones muy importantes para la salud del paciente, especialmente en aquellos con reacciones graves.

El sobrediagnóstico de DHRs es un grave problema de salud pública, ya que solamente entre el 10% y 30% de los pacientes clasificados como alérgicos a BLs pueden ser confirmados mediante diferentes métodos diagnósticos disponibles, mientras que el resto, entre 70% y 90% de paciente clasificados como alérgicos, realmente toleran los fármacos. Este sobrediagnóstico tiene importantes implicaciones ya que la alergia a fármacos implica la necesidad de utilizar medicamentos de segunda línea, normalmente asociados con mayor número de efectos secundarios, siendo menos efectivos y con mayores costes. Además, ser clasificado como alérgico se relaciona con la necesidad de utilizar mayor número de fármacos, con mayor riesgo de ingresos hospitalarios e infecciones bacterianas.

El diagnóstico de DHRs es un proceso complejo, basados en la combinación de tests *in vivo* e *in vitro*. La primera fase del proceso diagnóstico es la evaluación de la historia clínica del paciente, aunque no siempre es fiable, siendo difícil en ocasiones determinar la entidad clínica y el fármaco inductor. Esto es incluso más complicado en NIDHRs, debido a la variabilidad de manifestaciones clínicas, mecanismos inmunes y células implicadas.

La existencia de infecciones víricas cuyos síntomas se asemejan a los de las reacciones de hipersensibilidad hacen difícil determinar si la causa real de la reacción es un fármaco. Además, el prolongado intervalo de tiempo entre la exposición al fármaco y la aparición de síntomas hace difícil identificar el medicamento concreto causante de la reacción, especialmente en aquellos pacientes que utilizan más fármacos durante

dicho periodo. Todo ello hace que sea necesario la realización de diferentes métodos para poder realizar un diagnóstico correcto.

Las pruebas cutáneas son las pruebas más comúnmente utilizadas para el diagnóstico de DHRs, ya sean inmediatas o no inmediatas. No obstante, su sensibilidad no es óptima, o no están disponibles para todo tipo de fármacos. Además, existe la posibilidad de provocar una reacción sistémica como consecuencia de su realización, por lo que deben realizar con precaución, especialmente en pacientes con reacciones graves.

Debido a la limitada sensibilidad de las pruebas cutáneas, la realización de la prueba de exposición controlada es necesaria en muchos casos para poder determinar o comprobar la tolerancia a un fármaco concreto, tanto en IDHRs como en NIDHRs. Sin embargo, es una prueba larga, costosa, y no está exenta de riesgo para los pacientes, por lo que sólo debe realizarse en centros especializados con monitorización constante por profesionales entrenados. Es por ello por lo que no se recomienda realizar esta prueba en aquellos pacientes con reacciones más graves.

Para reducir la necesidad de la realización las pruebas de exposición controlada, los ensayos *in vitro* pueden representar alternativas útiles y seguras para ser incluidas en los procedimientos de diagnóstico. En el caso de IDHRs, el biomarcador más frecuentemente utilizado es la cuantificación de IgE en sueros de pacientes mediante inmunoensayos. Sin embargo, el inmunoensayo más validado y disponible comercialmente es el inmunoensayo fluoroenzimático (FEIA) ImmunoCAP®, para el que el número de fármacos disponibles es muy escaso, y su sensibilidad y especificidad es reducida y limitada, como ha sido demostrado para BLs.

Otro ensayo *in vitro* para evaluar IDHRs es el test de activación de basófilos. Esta prueba se basa en la estimulación de basófilos de pacientes alérgicos con el fármaco implicado, ya que los basófilos, junto con los mastocitos, son las células efectoras en estas reacciones. La técnica consiste en evaluar la desgranulación de los basófilos en respuesta a un fármaco midiendo la expresión de ciertos marcadores en su superficie mediante citometría de flujo. Aunque se ha demostrado su utilidad, el test no tiene una sensibilidad óptima a la hora de evaluar fármacos, aunque la posibilidad de utilizar medicamentos o incluso sus metabolitos directamente, sin necesidad de conjugarlos

previamente con portadores o proteínas, hace que el test de activación de basófilos sea una técnica versátil, útil, y con una gran potencialidad.

En NIDHRs, la prueba *in vitro* más utilizada es la prueba de transformación linfocitaria (LTT). Este test analiza la proliferación de linfocitos en respuesta a un fármaco específico. Sin embargo, su sensibilidad es variable dependiendo de los estudios al evaluar diferentes fármacos y entidades clínicas. Además, presenta varios problemas, como el uso de radiactividad, la dificultad de la técnica, la falta de estandarización, o el largo tiempo que transcurre entre la recogida de la muestra y la obtención de los resultados. Todo ello ha hecho que el LTT sea considerado más una herramienta de investigación que una prueba con potencial diagnóstico. Aunque este test se ha utilizado principalmente para evaluar NIDHRs, también puede utilizarse para evaluar IDHRs.

Las células dendríticas (DCs) son las células presentadoras de antígenos (APCs) más potentes del organismo, desempeñando un papel importante en el inicio de la respuesta de tolerancia o hipersensibilidad, debido a su capacidad de comunicación entre el sistema inmunitario innato y adaptativo. Las DCs son capaces de encontrar, captar y procesar antígenos tanto en sangre como en tejidos periféricos. Tras reconocer y captar antígenos, las células dendríticas procesan el antígeno intracelularmente. Este procesamiento está acompañado por un complejo proceso de maduración, en el que se aumenta la expresión de moléculas de migración, responsables de la migración a los ganglios linfáticos. Durante este proceso, las DCs reducen su capacidad de captación y procesamiento de antígenos, mientras que expresan mayores niveles de marcadores de maduración y activación, tales como moléculas coestimuladoras.

En los ganglios linfáticos, las DCs presentan los fármacos a los linfocitos T vírgenes a través de los complejos mayores de histocompatibilidad de clase I y II, junto con moléculas coestimuladoras y la liberación de citoquinas. Si este proceso se produce, los linfocitos T vírgenes se diferencian principalmente a células CD4⁺Th1 en NIDHRs, y a células CD4⁺Th2 en IDHRs.

Por lo tanto, el estudio de la expresión de marcadores de activación y maduración en DCs puede ser una herramienta útil para evaluar la sensibilización a diferentes fármacos tanto en IDHRs como en NIDHRs. Además, diferentes estudios han demostrado que la inclusión de DCs previamente estimuladas por fármacos, en el LTT

puede aumentar la sensibilidad del test, ya que estas células tienen una gran capacidad para activar diferentes poblaciones de linfocitos.

No obstante, esta aproximación ha sido descrita principalmente en NIDHRs, y utilizando DCs derivadas de monocitos (moDCs), ya que pueden ser transformadas fácilmente y en gran número a partir de monocitos circulantes de la sangre. Sin embargo, otras poblaciones como las células dendríticas mieloides (mDCs), podrían tener una mayor capacidad para ser activadas por fármacos y desencadenar la activación de linfocitos de una forma más eficiente que las moDCs.

Otro factor importante que puede afectar a la sensibilidad del LTT es la propia naturaleza del fármaco. Se ha demostrado que algunos fármacos se degradan rápidamente en solución, generando diferentes metabolitos. Estos metabolitos pueden tener diferentes capacidades para interactuar con proteínas, siendo necesario para poder ser reconocidos por el sistema inmunitario y, por tanto, para desencadenar una respuesta de hipersensibilidad, como ha sido descrito para BLs. Por lo tanto, se ha sugerido que, cuando el fármaco completo se incluye en el ensayo, este se degrada rápidamente, generando una mezcla heterogénea de metabolitos, siendo sólo algunos de ellos inmunogénicos. Por ello, la concentración específica del metabolito con capacidad de ser reconocido puede no ser suficiente para desencadenar la activación de DCs y la consiguiente proliferación de linfocitos.

Por ello, la identificación de estos metabolitos específicos, así como su capacidad para estimular el sistema inmunitario de pacientes con DHRs, pero no de controles sanos, podría representar una alternativa útil para mejorar la sensibilidad de los actuales ensayos celulares *in vitro*.

Por ello, el objetivo principal de esta tesis fue determinar el reconocimiento celular de los fármacos y sus determinantes antigénicos por parte del sistema inmune de pacientes alérgicos con IDHRs y NIDHRs, analizando la expresión de marcadores en las DCs, y su capacidad de activar a linfocitos autólogos. Además, se estudió la inclusión de diferentes parámetros que pudiesen mejorar la sensibilidad de los actuales ensayos celulares *in vitro*.

El primer objetivo de esta tesis fue evaluar la inclusión de parámetros que pueden ser relevantes para mejorar la sensibilidad de LTT para evaluar NIDHRs. El principal reto a la hora de evaluar estas reacciones es la gran variabilidad de

manifestaciones clínicas, mecanismos inmunológicos y células implicadas en los mismos. En este sentido, existen importantes diferencias de sensibilidad entre diferentes estudios, principalmente porque cada uno de ellos se centra en evaluar la utilidad del LTT en un número reducido de pacientes con características clínicas similares, y sólo evaluando un número reducido de fármacos, en su mayoría pertenecientes a la misma familia.

Por ello, para determinar la sensibilidad general del test como herramienta para evaluar NIDHRs, se incluyeron muestras de 37 pacientes con NIDHRs confirmadas a diferentes fármacos, con diferentes manifestaciones clínicas, desde reacciones leves o moderadas como exantema maculopapular (MPE), a reacciones más graves como pustulosis aguda generalizada (AGEP), reacciones con eosinofilia y síntomas sistémicos (DRESS), o Stevens-Johnson/necrólisis epidérmica tóxica (SJS/TEN).

Se evaluaron dos metodologías diferentes de LTT. La primera fue el LTT convencional (C-LTT), que consiste en la estimulación directa de todas las células mononucleares de sangre periférica con el fármaco. El segundo enfoque consistió en estimular las moDC con los fármacos específicos y, a continuación, cultivarlas con linfocitos autólogos, evaluando su proliferación (dDC-LTT).

En este estudio, también se evaluó la proliferación de los linfocitos midiendo la dilución de carboxyfluorescein succinimidyl ester (CFSE) mediante citometría de flujo, ya que la intensidad de CFSE se reduce a la mitad con cada ciclo de división si se produce la proliferación. El uso de la citometría de flujo para evaluar dicha proliferación tiene dos ventajas importantes. La primera es que se evita el uso de materiales radiactivos y, por tanto, es una técnica más segura, lo que permite su uso potencial en hospitales y rutina clínica. La segunda ventaja es que la citometría de flujo permite la identificación de diferentes subpoblaciones celulares de forma simultánea, lo que hace posible determinar la respuesta de proliferación de dichas poblaciones en un único ensayo. Además, podría ser extremadamente útil para evaluar la respuesta de proliferación específica de subpoblaciones celulares que, aunque son importantes en el mecanismo inmunitario, su número es muy reducido.

Los resultados indican que cuando se evalúa por citometría de flujo la proliferación de la población general de linfocitos T ($CD3^+T$) en respuesta al fármaco, la sensibilidad del C-LTT fue sólo del 29,4%. Sin embargo, la inclusión de moDCs pre-

RESUMEN

estimuladas con el fármaco, aumentó la sensibilidad hasta un 61,8%. En ambas aproximaciones, la especificidad fue de un 85%. Estas diferencias ponen de manifiesto la importancia de incluir moDCs como APCs para aumentar la sensibilidad del LTT en NIDHRs. Nuestros resultados también indican que el C-LTT tiene una sensibilidad menor en comparación con la observada en otros estudios. Esto puede explicarse por la heterogeneidad de las manifestaciones clínicas incluidas en el presente estudio, así como por la alta variabilidad de fármacos implicados, en contraste con estudios anteriores.

En relación con esto, evaluamos ambas técnicas en las tres manifestaciones clínicas más representadas en nuestra cohorte de pacientes, SJS/TEN, MPE y AGEP. La sensibilidad del dDC-LTT fue superior a la observada en el C-LTT, independientemente de la manifestación clínica implicada (87,5% frente a 62,5% en SJS/TEN, 47,4% frente a 15% en MPE, y 80% frente a 33% en AGEP), todo ello con una especificidad del 85%. Es interesante destacar que las reacciones más graves, como SJS/TEN y AGEP, tuvieron los valores de sensibilidad más altos, en comparación con reacciones leves/moderadas, como MPE. Estas observaciones tienen importantes implicaciones, ya que es precisamente el diagnóstico de las reacciones graves el que tiene una disponibilidad más limitada de pruebas *in vivo*.

Además, también demostramos que la combinación de los resultados de la respuesta proliferativa de linfocitos CD3⁺T entre C-LTT y dDC-LTT no mejora la sensibilidad general del ensayo en comparación con la observada para el dDC-LTT (61,8%) por sí sola, lo que sugiere que el rendimiento de la segunda aproximación es suficiente para lograr alcanzar la mayor sensibilidad general del LTT.

Una vez demostrado que con el dDC-LTT se obtienen unos mejores niveles de sensibilidad en comparación con el C-LTT, el segundo objetivo de la tesis fue la evaluación de la respuesta proliferativa de poblaciones específicas de linfocitos mediante dDC-LTT. Los resultados indican que la mayor respuesta proliferativa se detectó en linfocitos CD4⁺Th1, sugiriendo el importante papel de esta subpoblación celular en NIDHRs. Cuando combinamos la respuesta proliferativa de diferentes poblaciones de linfocitos, la sensibilidad global del LTT aumentó, sin reducir su especificidad. Del 61,8% de sensibilidad alcanzada con linfocitos CD3⁺T, la combinación de los resultados de proliferación de linfocitos CD3⁺T y CD4⁺Th1 aumentó la sensibilidad

al 73,9%, mientras que entre los linfocitos CD3⁺T y células natural killer (NK), aumentó al 82,6%. Cuando se combinaron los resultados de proliferación de linfocitos CD3⁺T, CD4⁺Th1 y NK, la sensibilidad del LTT aumentó hasta un 87% con un 85% de especificidad. Por último, aunque la inclusión de los resultados proliferativos de linfocitos CD3⁺T y CD8⁺T aumentó la sensibilidad del LTT hasta un 91%, también se observó una reducción de la especificidad (80%).

Independientemente de la entidad clínica implicada, la mayor respuesta proliferativa se observó en linfocitos CD4⁺Th1. Además, la combinación de los resultados de proliferación de linfocitos CD3⁺T y CD4⁺Th1, o de CD3⁺T y NK permitió identificar todas las reacciones de SJS/TEN con una especificidad del 85%. El valor de sensibilidad más bajo se encontró en MPE al evaluar la respuesta proliferativa de linfocitos CD3⁺T (47,4%). Tras combinar los resultados de proliferación de linfocitos CD3⁺T y CD4⁺Th1, la sensibilidad aumentó hasta el 50%, y de CD3⁺T y NK hasta un 58,3%, similar a la observada con los linfocitos CD3⁺T y CD8⁺T, pero con una especificidad menor. La combinación de los resultados de proliferación de linfocitos CD3⁺T, CD4⁺Th1 y NK aumentó la sensibilidad del LTT hasta un 68,4% sin reducir su especificidad. Por último, en AGEP, la combinación de resultados proliferativos de linfocitos CD3⁺T y CD4⁺Th1 no aumentó la sensibilidad del dDC-LTT (80%) en comparación con la proliferación de células CD3⁺T por si solas. En cambio, la combinación de resultados de proliferación de linfocitos CD3⁺T y NK permitió identificar a todos los pacientes con AGEP, de forma similar a la combinación de resultados de linfocitos CD3⁺T y CD8⁺T, pero con una especificidad menor.

Curiosamente, se observó una implicación diferente de cada subpoblación celular en función de la manifestación clínica estudiada. En cuanto a las subpoblaciones de linfocitos CD4⁺T, aquellos con un patrón de citoquinas Th1 fueron los más implicados en todas las manifestaciones clínicas (88% en SJS/TEN, 100% en MPE, 100% en AGEP). No obstante, la implicación de linfocitos CD4⁺Th2 fue diferente dependiendo de la manifestación clínica concreta, siendo de sólo el 37,5% en SJS/TEN, pero del 67% en MPE, y del 50% en AGEP.

De forma similar, se observó una contribución diferente en relación con las subpoblaciones de NK y la manifestación clínica implicada. En SJS/TEN se observó una mayor contribución de las células NK citotóxicas (87,5%) en comparación con las células

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NK inflamatorias (50%). Por el contrario, el papel de las células NK citotóxicas (28,6%) fue menor que el de las células NK inflamatorias (71,4%) en MPE. La contribución entre NK citotóxicas (66%) e inflamatorias (80%) parece estar más equilibrada en pacientes con AGEp.

En conjunto, nuestros datos sugieren que, aunque C-LTT por citometría de flujo no tiene una sensibilidad óptima cuando se evalúan NIDHRs con diferentes manifestaciones con diferentes fármacos implicados, la inclusión de moDCs pre-estimuladas aumentó significativamente la sensibilidad del LTT. Además, y gracias a la ventaja de utilizar la tecnología de citometría de flujo, el análisis de proliferación de poblaciones de linfocitos concretas es útil para aumentar la sensibilidad del LTT, con una especial relevancia para las reacciones más graves, ya que cuentan con herramientas de diagnóstico más limitadas. Por lo tanto, avances en la determinación de los mecanismos específicos implicados en cada manifestación clínica pueden tener importantes implicaciones para mejorar la sensibilidad de los test celulares *in vitro* actuales.

El tercer objetivo de este trabajo fue evaluar el reconocimiento del ácido clavulánico (CLV) y de sus determinantes antigénicos por parte de moDCs de pacientes alérgicos selectivos (AP) y de controles sanos (HC), así como determinar la capacidad de estas células para estimular a linfocitos autólogos.

Las IDHRs tras la ingesta de amoxicilina (AX)- CLV han aumentado en las últimas décadas, probablemente debido al elevado consumo de estos fármacos. Aunque el CLV tiene una capacidad antibiótica limitada, se ha convertido en un fármaco muy frecuentemente prescrito con AX debido a su elevada actividad betalactamasa.

Aunque inicialmente se pensó que el CLV no tenía capacidad inmunogénica, los estudios realizados en las últimas décadas han demostrado que el CLV puede ser el fármaco responsable de desencadenar DHRs tras la exposición a AX-CLV. Curiosamente, un sujeto tras la exposición a AX-CLV podría sensibilizarse a un fármaco u otro, siendo infrecuente la sensibilización simultánea a ambos fármacos en un mismo paciente. Por lo tanto, determinar si AX o CLV es el fármaco responsable implicado en la reacción tiene importantes implicaciones para los pacientes, ya que, si el CLV es el fármaco responsable, el paciente puede tolerar la AX, evitando la necesidad de utilizar antibióticos de segunda línea de tratamiento, normalmente asociados con mayores costes, más efectos secundarios y siendo generalmente menos efectivos.

No obstante, el diagnóstico de IDHRs a CLV es un proceso complejo, ya que las pruebas cutáneas tienen una baja sensibilidad, y la prueba de exposición controlada no puede realizarse con CLV, por lo que estas reacciones deben confirmarse mediante la tolerancia a AX tras una reacción con AX-CLV. Además, no es posible la detección de CLV-slgE, y el test de activación de basófilos no tiene una sensibilidad óptima. Por ello, la búsqueda de nuevas técnicas *in vitro* para la evaluación de IDHRs a CLV, así como la introducción de factores que puedan mejorar la sensibilidad de los ya disponibles, puede tener importantes implicaciones para el diagnóstico de este tipo de reacciones.

Una posible explicación a la reducida sensibilidad encontrada en las pruebas *in vitro* podría deberse a la elevada tasa de degradación del CLV en comparación con otros BLs como AX o penicilina. En el caso del CLV, los metabolitos derivados de su degradación pueden observarse en los primeros minutos en disolución. Además, estos metabolitos pueden ser reactivos, haciendo que la reacción sea auto catalítica. Por lo tanto, la identificación de los metabolitos generados a partir de la degradación de la CLV es un proceso complicado.

Nuestra hipótesis es que, cuando se incluye el CLV en los ensayos *in vitro*, se generan diferentes metabolitos, presentes en diferentes y reducidas concentraciones. Dado que el CLV pertenece a la familia de BLs, está bien descrito que necesita unirse covalentemente a proteínas para formar conjugados de fármacos y proteínas, que pueden ser reconocidos por el sistema inmune y, por tanto, desencadenar la reacción de hipersensibilidad. Es posible que los diferentes metabolitos generados tengan diferentes capacidades para interactuar con diferentes proteínas, siendo sólo algunos de ellos los responsables de ser reconocidos, y en consecuencia de desencadenar la reacción. Sin embargo, dada la gran heterogeneidad de metabolitos, la concentración de aquellos específicos responsables de desencadenar la reacción podría no ser suficiente para activar a las células del sistema inmune incluidas en los ensayos *in vitro*, pudiendo explicar la baja sensibilidad encontrada en los ensayos *in vitro* cuando se evalúan IDHRs a CLV.

En un estudio reciente demostramos que uno de estos determinantes antigénicos de CLV (AD-I), puede desencadenar la desgranulación de basófilos en AP al CLV, pero no en HC. Por lo tanto, su inclusión en el test de activación de basófilos aumentó su sensibilidad. No obstante, no se conoce completamente el reconocimiento

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del CLV, así como de sus posibles ADs. En consecuencia, para entender mejor el reconocimiento inmunitario de los dos determinantes antigénicos propuestos del CLV (AD-I y AD-II), se sintetizaron diferentes análogos de cada uno (Clav1-3 de AD-I, y Clav4-7 de AD-II) con diferentes grupos funcionales, otorgándolos una diferente capacidad para interactuar con proteínas. El reconocimiento de CLV y de los análogos sintéticos de cada AD se evaluó analizando la maduración y activación de las moDCs, así como su capacidad para estimular a linfocitos autólogos en un grupo de 27 pacientes con IDHRs a CLV y en 13 sujetos tolerantes.

Nuestros resultados sugieren que los análogos sintéticos tanto de AD-I como de AD-II fueron eficientemente reconocidos por las moDCs de AP selectivos a CLV en comparación con las de HC. Este reconocimiento se produjo de forma más eficiente que el reconocimiento de la forma completa de CLV, como se ha demostrado por la expresión de los marcadores CD40, CD80, CD83, y en menor medida de CD86 y CCR7.

Resulta interesante que la combinación de resultados de maduración tras la estimulación de moDCs de CLV-AP con análogos sintéticos de AD-I y AD-II, aumentó significativamente la sensibilidad de los ensayos de maduración hasta el 76% para CD40, 67% para CD80, 62% para CD86, 57% para CD83 y 48% para CCR7, con una especificidad general de más de 75%. La inclusión de los resultados del fármaco completo no aumentó el % de positividad en comparación con la inclusión de la combinación de resultados de análogos sintéticos de AD-I y AD-II sin reducir la especificidad.

Además, se observó un patrón de reconocimiento diferente, ya que los análogos sintéticos de AD-I se asocian más a la expresión de CCR7 y CD40, mientras que los análogos sintéticos de AD-II se encuentran más asociados a la expresión de CD80, CD83 y CD86. Este diferente patrón de reconocimiento entre los análogos sintéticos de ambos ADs pone de manifiesto la importancia de la inclusión de análogos de ambos ADs para aumentar la sensibilidad de los ensayos de maduración de moDCs.

Nuestros datos mostraron que moDCs de CLV-AP estimuladas con CLV y sus diferentes análogos sintéticos de ambos AD, son capaces de estimular proliferación de linfocitos CD3⁺T, CD4⁺T y principalmente CD4⁺Th2, pero no de CD8⁺T y CD4⁺Th1. Estas observaciones coinciden con el mecanismo implicado en IDHRs, caracterizado por una mayor contribución de las células CD4⁺Th2 en comparación con otras poblaciones de linfocitos.

Combinando los resultados de proliferación de linfocitos obtenidos tras la estimulación de moDCs con los análogos sintéticos de AD-I y AD-II, la sensibilidad del LTT aumentó hasta un 46% en linfocitos CD3⁺T, 50% en CD4⁺T y 65% en CD4⁺Th2. Al igual que en los ensayos de maduración de moDCs, la inclusión de CLV completo no aumentó la sensibilidad del test en ninguna de las poblaciones de linfocitos analizadas.

Además, también se observó un patrón de reconocimiento diferente entre los análogos sintéticos de ambos ADs. Aunque la mayoría de los casos positivos reconocieron ambos ADs simultáneamente, entre un 18% y 35% de los casos positivos reconocieron específicamente estructuras de uno u otro AD. Esto apoya la necesidad de incluir análogos sintéticos de ambos ADs para alcanzar los valores más altos de sensibilidad.

Al contrario de lo encontrado para NIDHRs, en IDHRs a CLV, la mejor sensibilidad de LTT se obtuvo cuando se evaluó la respuesta proliferativa de linfocitos CD4⁺Th2, sin necesidad de combinar los resultados de diferentes poblaciones celulares.

En resumen, en este estudio hemos demostrado que los análogos sintéticos de AD-I y AD-II derivados de la degradación de CLV, son eficientemente reconocidos por moDCs de AP selectivos de CLV con IDHRs. Además, estas moDCs estimuladas pueden desencadenar eficazmente la proliferación de linfocitos T, principalmente de CD4⁺Th2. La combinación de análogos sintéticos de ambos ADs de CLV aumentó significativamente la sensibilidad de los ensayos de maduración de moDCs y LTT, ambos con alta especificidad, en comparación con la sensibilidad obtenida con el fármaco completo. Estos resultados, aunque en una fase inicial, pueden representar un enfoque prometedor para la mejora del diagnóstico de IDHRs tras la ingesta de AX-CLV, con la ventaja de que estos ensayos *in vitro* no sólo pueden diferenciar entre sujetos alérgicos y tolerantes, sino también identificar específicamente si la reacción es desencadenada por un fármaco u otro.

El último objetivo de esta tesis fue evaluar la implicación de las mDCs en la evaluación de IDHRs, tanto a AX como a CLV.

Como ha sido descrito en estudios previos, y en los dos objetivos anteriores de esta tesis, el análisis de la expresión de marcadores en moDCs tras la estimulación con el fármaco responsable de la reacción es un método útil para evaluar la sensibilización a un fármaco en pacientes con IDHRs y NIDHRs. De forma similar, se ha demostrado que

la inclusión de moDCs pre-estimuladas con el fármaco en el LTT aumenta su sensibilidad, aunque principalmente se ha descrito para NIDHRs, conociéndose poco sobre su papel en IDHRs.

Aunque la inclusión de moDCs pre-estimuladas con fármacos sirve para aumentar la sensibilidad del LTT en comparación con el C-LTT, su sensibilidad sigue siendo subóptima. Una posible explicación para esto podría ser la naturaleza de las APCs incluidas en el ensayo. La mayoría de los estudios utilizan moDCs, principalmente porque pueden transformarse fácilmente *in vitro* en grandes cantidades a partir de monocitos sanguíneos circulantes en grandes cantidades. Aunque las moDC existen en la naturaleza, sólo aparecen durante los procesos inflamatorios y, tras su resolución, desaparecen. Por lo tanto, otras poblaciones celulares como las mDC, verdaderos “centinelas” en la sangre y los tejidos periféricos, con gran capacidad para orquestar la respuesta inmunitaria, permitiendo la interacción entre los sistemas inmunitarios innato y adaptativo, podrían ser más sensibles a los fármacos y ser más capaces de activar a linfocitos autólogos en los pacientes alérgicos, pero no en los sujetos tolerantes.

El desarrollo de ensayos *in vitro* con suficiente sensibilidad es esencial para el diagnóstico de IDHRs, ya que permiten discriminar entre pacientes alérgicos y sujetos tolerantes. Sin embargo, también deben tener la suficiente especificidad para identificar el fármaco responsable de desencadenar la reacción, incluso entre fármacos de la misma familia y con importantes similitudes químicas como es el caso de AX y CLV.

Por todo ello, en este estudio se evaluó el reconocimiento de AX y CLV por parte de moDCs y mDCs de 14 pacientes selectivos con IDHRs a AX, y de 14 pacientes selectivos con IDHRs a CLV. Para ello, se estudió la expresión de marcadores de migración, maduración y activación en ambas poblaciones de DCs tras la estimulación con cada fármaco en pacientes comparados con sujetos tolerantes. Además, se analizó su capacidad para activar poblaciones específicas de linfocitos, comparando los resultados con el C-LTT.

Los resultados muestran que las mDC no estimuladas expresan más CCR7 en comparación con moDC, mientras que estas últimas expresan mayores niveles de CD40, CD80 y CD86 en comparación con las mDC. Estas observaciones pueden sugerir que, en estado basal, las mDC están más preparadas para migrar a los nódulos linfáticos que las moDC, y que esta última población se encuentra más activada que las mDC. Esto podría

tener importantes implicaciones en la posterior estimulación con fármacos, ya que la elevada activación de las moDCs en condición basal podría reducir su capacidad de expresar estos marcadores como consecuencia de la estimulación con el fármaco, ya que es sabido que los fármacos desencadenan una débil respuesta de activación.

De acuerdo con esto, se observó que la estimulación de mDCs de AX-AP con el fármaco responsable, aumentó la expresión de marcadores de migración, activación y maduración en comparación con HC. Por el contrario, la estimulación con AX de moDC de los mismos pacientes sólo indujo la expresión de CCR7, CD83 y CD86, todos ellos en menor medida que en las mDC.

Del mismo modo, la estimulación de mDCs de CLV-AP con el fármaco responsable, aumentó la expresión de todos los marcadores analizados, excepto de CD40 en comparación con HC. Por otro lado, la estimulación de las moDCs de CLV-AP con IDHRs a este fármaco sólo aumentó la expresión de CD83 en comparación con HC. Estos resultados sugieren que las mDCs tienen una mayor capacidad de activación por parte de ambos fármacos que las moDCs, aunque existen diferencias en cuanto al fármaco específico implicado.

Esto también se ha confirmado tras analizar el porcentaje de casos positivos. En AX-AP, se obtuvo una mayor positividad para todos los marcadores en mDC tras la estimulación con AX (47% para CCR7, CD40 y CD83, y 60% para CD80) en comparación con los AX-moDC (31% para CD40 y CD80, 39% para CD83 y 15% para CCR7), excepto para CD86 (27% en mDC y 31% en moDC). Del mismo modo, el porcentaje de positividad fue también mayor en CLV-AP para todos los marcadores en mDC estimuladas con CLV (43% para CCR7, 50% para CD40, 21% para CD80 y 36% para CD86) en comparación con lo observado en CLV-moDC (0% para CCR7 y CD40, 8% para CD80 y 13% para CD86), excepto para CD83 (14% en mDC y 17% en moDC).

Otra característica importante de estos ensayos de maduración de DCs es su capacidad para diferenciar entre el fármaco responsable de la reacción y fármacos similares. Por este motivo, se estimularon las DCs de AX-AP con el fármaco no responsable, en este caso el CLV, y viceversa. Aunque las mDC de AX-AP estimuladas con CLV presentaron un índice de maduración más alto que las moDCs, el porcentaje global de casos positivos al fármaco no responsable fue del 0% para CCR7, CD80 y CD83, del

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18% para CD86 y del 46% para CD40, mientras que fue del 0% para CD80, del 10% para CCR7 y CD40 y del 20% para CD83 y CD86 en las CLV-moDC.

La estimulación de DCs de CLV-AP con AX, produjo resultados de maduración más bajos en mDCs que en moDCs. El porcentaje de positividad fue del 0% para todos los marcadores analizados, excepto para el CD86 (13%) en mDCs. Por el contrario, el porcentaje de casos positivos en moDCs fue mayor (5% para CCR7, 11% CD40 y CD80, 21% para CD83 y 26% para CD86).

En cuanto a la capacidad de las mDC pre-estimuladas con AX en AX-AP de estimular a linfocitos autólogos, su inclusión como APC aumentó el índice de proliferación en todas las poblaciones de linfocitos T analizada en comparación con HC, excepto en linfocitos CD8⁺T. La inclusión de moDCs estimuladas con AX procedentes de AX-AP sólo desencadenó la respuesta proliferativa de CD4⁺Th2 en comparación con HC, mientras que en C-LTT se observó una mayor proliferación en AX-AP en comparación con HC, independientemente de la población de linfocitos analizada, excepto en células CD4⁺Th1.

En CLV-AP, las mDC pre-estimuladas con CLV produjeron una respuesta proliferativa en todas las poblaciones de linfocitos analizados, excepto en CD8⁺T y CD4⁺Th1. No se obtuvo un aumento de la respuesta proliferativa en ninguna de las poblaciones de linfocitos analizados, independientemente de la inclusión de moDC pre-estimuladas con el fármaco, o de la realización de C-LTT.

Cabe destacar que el mayor porcentaje de positividad tanto en AX- como en CLV-AP se obtuvo cuando se incluyeron mDCs como APCs, mayor que la proliferación observada tras la inclusión de moDCs pre-estimuladas o la realización de C-LTT. El porcentaje de positividad de linfocitos CD4⁺Th2, la subpoblación celular más relevantemente implicada en IDHRs fue del 67% en AX-AP, pero sólo del 25% en CLV-AP cuando se incluyeron mDCs pre-estimuladas con el fármaco implicado. Por lo tanto, los datos aquí presentados sugieren que las mDCs están mucho más preparadas para reconocer fármacos y ser activadas que las moDCs en IDHRs, pudiendo requerir una menor cantidad de fármaco para ello. Como hemos demostrado anteriormente en este trabajo, debido a la alta inestabilidad del CLV, pueden formarse metabolitos derivados de su degradación, con diferentes capacidades para interactuar con proteínas, siendo sólo algunos de ellos los responsables de activar las células inmunes de pacientes

alérgicos. Por lo tanto, su concentración puede no ser suficiente para activar las moDCs pero sí para activar las mDCs.

Por último, determinamos la capacidad de las tres aproximaciones de LTT para identificar el fármaco responsable de la reacción, evaluando la respuesta proliferativa de linfocitos CD4⁺Th2 de AX- o CLV-AP estimuladas con el fármaco no responsable, CLV o AX respectivamente. El porcentaje de casos positivos cuando se utilizaron mDCs pre-estimuladas con CLV como APCs en AX-AP fue del 0%, mientras que fue del 11% en moDCs estimuladas con CLV. El porcentaje de casos positivos cuando se incluyeron como mDCs pre-estimuladas con AX y moDCs de CLV-AP fue del 0% en ambos casos. Sin embargo, el porcentaje de positividad en C-LTT estimulados con el fármaco no responsable fue mucho más elevado en comparación con las otras dos aproximaciones (39% en los C-LTT procedentes de AP-AX estimulados con CLV y 21% en LTT procedentes de AP-CLV estimulados con AX).

En conjunto, estos datos demuestran la importancia de incluir APCs profesionales para aumentar la sensibilidad de los test celulares *in vitro*. En particular, hemos demostrado que las mDC reconocen eficazmente AX y CLV de forma específica y, por lo tanto, el análisis de los marcadores de activación y maduración podría utilizarse para evaluar la sensibilización en pacientes con IDHRs tras la exposición a AX-CLV. La sensibilidad del test de maduración encontrada con mDCs fue mayor que la obtenida con moDCs. Además, la inclusión de mDCs pre-estimuladas aumentó la sensibilidad del LTT en comparación con la inclusión de moDCs pre-estimuladas o C-LTT, especialmente cuando se evaluó la respuesta proliferativa de CD4⁺Th2, aunque existen diferencias en cuanto al fármaco implicado en la reacción. No obstante, aunque estos datos nos dan una visión más completa sobre el mecanismo específico implicado en el reconocimiento de AX y CLV por el sistema inmunitario de pacientes alérgicos, son necesarios más estudios, ya que podría tener importantes implicaciones para un futuro diagnóstico preciso de IDHRs a BLs.

SUMMARY

Drug hypersensitivity reactions (DHRs) are unpredictable adverse drug reactions (ADRs) unrelated to the dose or pharmacokinetics of the drug, which only occur in some of the subjects exposed to the drug. The incidence of DHRs has increased in recent decades, probably due to increased drug consumption, especially in the western world, changes in hygiene and dietary habits, which may alter our microbiome, or environmental exposure to pollution or aeroallergens.

Although the classification of DHRs is important, there is not clear consensus regarding the best way to classify DHRs, mainly because of the complex chemical structure and diversity of the drugs, as well as the great number of clinical entities and immune mechanisms involved.

A useful classification divides DHRs according to the time elapse between the intake of the drug and onset of symptoms. Based on this classification, DHRs can be categorised into immediate hypersensitivity reactions (IDHRs) and non-immediate hypersensitivity reactions (NIDHRs). In IDHRs, the time between drug intake and the onset of symptoms occurs in less than one hour, whereas symptoms in NIDHRs appear later than 1 hour or even days or weeks after drug intake. Nevertheless, the time interval between 1 and 6 hours is controversial, and some authors refer them as accelerated reactions. Therefore, although useful, especially for clinical routine, the overlap between IDHRs and NIDHRs made this classification controversial for some reactions. Nevertheless, for the purpose of this thesis, this classification will be used to differentiate between IDHRs and NIDHRs.

Regarding IDHRs, reactions can be classified into allergic and nonallergic reactions. The formers are mediated by a known immunological mechanism, while the latter ones are not mediated by specific immunologic mechanisms. These reactions are usually caused by direct drug off-target interaction with receptors on basophils and mast cells, or by inhibition of enzymes such as the cyclooxygenase 1 (COX-1), leading to the reduction of prostaglandins and the accumulation of leukotrienes, resulting in degranulation of basophils and mast cells. According to this classification, this thesis will focus only on DHRs mediated by immunological mechanisms, leaving aside the nonallergic reactions.

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Another useful classification categorises DHRs depending on the immune mechanism involved. Combining the above classification with the immune mechanism involved, IDHRs are mainly mediated by specific immunoglobulin E (IgE), while NIDHRs are usually mediated by T cells. Therefore, such association between IDHRs and IgE, and between NIDHRs and T cells will be made in the present thesis, to facilitate the reader's comprehension.

DHRs can also be divided according to the interaction of the drug with the immune system. This interaction could be explained by the hapten model, mainly described for betalactams (BLs), where drugs, due to their small size, need to covalently bind to proteins to form drug-protein conjugates that can be recognised by the immune system. Another interaction model is the p-i model, mainly described by neuromuscular blocking agents (NMBAs). It is based on the fact that a drug can interact directly with major histocompatibility complexes (MHCs) on antigen presenting cells (APCs),¹ or T cell receptor (TCRs) on T cells, triggering the activation of the immune system. The last model proposed is the altered peptide ones, described mainly for abacavir. This interaction is based on the fact that once the drug is present, it can modify specific MHC peptides, triggering their recognition by T cells and causing the DHR. In this thesis, as we will only focus on the recognition of BLs in IDHRs, it is assumed that the haptenic model occurs, whereas in NIDHRs, as we evaluate different drugs and clinical entities, we cannot exclude different drug-immune system interaction models.

In theory, all drugs can be responsible for triggering DHRs, although BLs are the drugs most frequently implicated in DHRs in both children and adults. However, other drugs such nonsteroidal antiinflammatory drugs (NSAIDs), NMBAs, fluoroquinolones (FQs), proton pump inhibitors, heparins, and iodinated contrast media (ICM) are drugs frequently involved in DHRs.

Although the prevalence of DHR has increased in recent decades, the actual incidence of these reaction in the population is difficult to address, mainly due to two problematic issues, the over- and under-diagnosis. The over-diagnosis occurs because some patients were labelled as "allergic", when they are not allergic and can therefore, tolerate the drug. The under-diagnosis, although less frequent, has important implications for the health of patients, because a patient is labelled as "non-allergic"

when he or she reacts to the drug, thus compromising the health of the patient, especially relevant for patients with severe DHRs.

The over-diagnosis of DHR is a major health problem, as it is estimated that only between 10%-30% of patients labelled as “BL allergic” can be confirmed by the different tests available, while the rest (70%-90%) can tolerate the drugs. The implication of this is relevant, because labelling as “allergic” implies the need for second-line treatments, generally associated with less effectiveness, more side effects, and higher costs. In addition, DHRs are associated with the need to use more drugs, with more hospital and ambulatory admissions and bacterial infections.

The diagnosis of DHRs is a complex process, mainly based on the combination of *in vivo* and *in vitro* tests. The first clinical approach is a thorough study of the history of the patient. Nevertheless, this is not always reliable, and it is sometimes difficult to determine the clinical or immunological mechanism involved, as well as the specific drug causing the reaction. This is even more complicated in NIDHRs, due to the great variability of clinical manifestations, immunological mechanisms, and cells involved. Moreover, viral infections whose symptoms are similar to those observed during the allergic reactions make it difficult to determine whether the reaction is triggered by a drug or not. In addition, the long-time interval between the intake of the drug and the onset of symptoms hampers determining the specific drug causing the reaction, especially in patients who use several drugs before the onset of symptoms. Altogether, this indicates that the performance of different tests is necessary for a correct diagnosis.

Skin test (ST), mainly skin prick test (SPT) and intradermal test (IDT) for IDHRs, and patch test (PT) and IDT for NIDHRs are the most frequent tests used to assess a DHRs. Nevertheless, their sensitivity is not optimal and for some drugs they are not available. Moreover, there is a possibility of triggering a systemic reaction, so they should be performed with caution, especially in patients with severe DHRs.

Because of the limited sensitivity of STs, in order to determine the tolerance or not to a concrete drug, the performance of drug provocation test (DPT) is needed in most cases. Because of this, DPT is considered the gold standard for both IDHRs and NIDHRs. Nevertheless, it should only be performed in specialised centres with trained professionals, is time-consuming, costly, and not risk-exempt for patients. In fact, it is not recommended for patients with severe reactions.

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To reduce the need for DPT, *in vitro* assays may represent useful and safe alternatives to be included in diagnostic procedures as complementary tools. In the case of IDHRs, as the most frequent biomarker is sIgE, the quantification of drug-sIgE in serum from patients with IDHRs by immunoassays is the most widely used and validated technique in clinical routine. Nevertheless, the most validated and commercially available immunoassay for the detection of sIgE is the fluoroenzyme immunoassay (FEIA) ImmunoCAP®, that needs drug conjugation to a carrier, and for which the number of available drugs is limited.

Another *in vitro* assay to evaluate IDHRs is the basophil activation test (BAT). This assay is based on the stimulation of basophils from allergic patients, since basophils together with mast cells are the effector cells in these reactions. The technique consists of assessing basophil degranulation in response to a drug by measuring the expression of certain markers on their surface by flow cytometry. Although it has been demonstrated to be useful, BAT does not have an optimal sensitivity when evaluating drugs, although the possibility of using drugs or even their metabolites directly, without the need to prior conjugation with carriers or proteins makes BAT a versatile and useful technique with a wide range of potential uses.

In NIDHR, the most used *in vitro* test was the lymphocyte transformation test (LTT). This test measures the lymphocyte proliferation in response to a specific drug. However, its sensitivity is variable between studies when evaluating different drugs and clinical entities. Moreover, it presents several problematic issues such as the use of radioactivity, the difficulty of the technique, the lack of standardisation, or the long time between sample collection and results obtaining. All of this has led to LTT being considered more a research tool rather than a potential diagnostic test. Although LTT has been mainly used to assess NIDHRs, it can also be used to evaluate IDHRs.

Dendritic cells (DCs) are the most potent antigen presenting cells (APCs) in the body, with an important role in initiating a DHR, or a tolerance response, due to their ability to communicate between the innate and adaptive immune system. DCs are highly capable of finding, capturing, and processing antigens from blood and peripheral tissues. DCs then undergo a complex maturation process, in which they increase the expression of migration molecules, such as CCR7, which is responsible for migrating to lymph nodes (LNs). Moreover, these DCs reduce their antigen uptake and processing capacity, while

expressing higher levels of maturation and activation markers, such as costimulatory molecules. DCs in LNs present the drugs to naïve T cells via major histocompatibility complexes (MHC) of class I and II, along with costimulatory molecules and cytokine release. If this occurs, T naïve cells differentiate mainly to CD4⁺Th1 cells in NIDHRs, and to CD4⁺Th2 cells in IDHRs.

Therefore, measuring the expression of activation and maturation markers on DCs can be a useful tool to evaluate sensitisation to different drugs in both NIDHRs and IDHRs. Furthermore, it has been reported that the inclusion of drug stimulated DCs, such as APCs in LTT, may increase the sensitivity of LTT, because these pre-primed DCs have a great capacity to activate different lymphocyte populations.

Nevertheless, this has been mainly described for NIDHRs, and mainly using monocyte derived DCs (moDCs), because they can be easily transformed from circulant monocytes from blood, although other DC populations, such as myeloid DCs (mDCs), may have an even better capacity to be activated by drugs and to trigger the activation of lymphocytes in a more efficient way than moDCs.

Another important factor that can affect the sensitivity of *in vitro* test is the nature of the drug included in the assay. Some drugs have shown to degrade rapidly in solution, generating different drug metabolites. These metabolites may have different capacities to interact with proteins, necessary to be recognised by the immune system and thus to trigger a DHR, following the hapten model of interaction, as it has been described for BLs. Therefore, it has been suggested that, when the native drug is included in the *in vitro* assay, it is highly degraded, generating a heterogeneous mixture of metabolites, with only some of them being immunogenic. Thus, their specific concentration may not be sufficient to trigger DCs activation and the subsequent T cells proliferation. The identification of these specific metabolites, as well as their ability to stimulate the immune system from patients with DHRs, but not from healthy controls, could represent a useful approach to improve the sensitivity of current cellular *in vitro* tests.

Therefore, the main purpose of this thesis is to determine the cellular recognition of drugs and their antigenic determinants by the immune system from allergic patients with NIDHRs and IDHRs, analysing the expression of markers on DCs and their capacity to stimulate autologous lymphocytes. In addition, we studied the inclusion of different

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parameters that may improve the sensitivity of current *in vitro* cellular test to assess the sensitisation to the culprit drugs.

The first objective of the thesis was to evaluate the inclusion of some parameters that may be relevant to be included in LTT to evaluate NIDHRs. The main problem when evaluating NIDHRs is the great variability of clinical manifestations, immunological mechanisms, and cells involved. In this regard, there are important differences in sensitivity between studies, mainly because each study focused on assessing the usefulness of LTT in a small number of patients with similar clinical characteristics, and only evaluating a small number of different drugs, mostly belonging to the same family.

Because of this, to evaluate the overall sensitivity of LTT as general tool to evaluate NIDHRs, we included in the study 37 patients with confirmed NIDHRs to different drugs, and with different clinical entities, ranging from mild/moderate reactions such as maculopapular exanthemas (MPE) to more severe reactions such as acute generalised pustulosis (AGEP), drug reaction with eosinophilia and systemic symptoms (DRESS), or Stevens-Johnson/toxic epidermal necrolysis (SJS/TEN).

Here, we evaluated two different LTT methodologies. The first was the conventional LTT (C-LTT), consisting of the direct stimulation of all peripheral blood mononuclear cells (PBMCs) with the drug. The second approach consists of stimulating moDCs with the specific drugs, and then, culture them with autologous lymphocytes and assess their proliferative response (dDC-LTT), as previous studies have shown that the inclusion of moDCs as APCs increased the sensitivity of LTT.

In this study, we also evaluated the proliferation of lymphocytes by measuring the dilution of carboxyfluorescein succinimidyl ester (CFSE) by flow cytometry, as the intensity of CFSE is reduced to the half with each division cycle if proliferation occurs. The use of flow cytometry to assess lymphocyte proliferation has two important advantages. The first is that flow cytometry avoids the use of radioactive materials, and is therefore safer, allowing its potential use in the hospitals and clinical routines. The second advantage is that flow cytometry allows the identification of different cell subpopulations simultaneously, making it possible to determine the proliferation response of specific cell populations with a single assay. Moreover, it could be extremely useful to evaluate the specific proliferation response of cell subpopulations that, although important in the immune mechanism, are present at low rates.

Results indicated that when the proliferation of the general T cell population (CD3⁺T cells) in response to the culprit drug was assessed by flow cytometry, the sensitivity of C-LTT was only of 29.4%. Nevertheless, the inclusion of pre-primed moDCs increased the sensitivity to 61.8%, and in both techniques the specificity observed was of 85%. These differences highlight the importance of including moDCs as APCs to increase LTT sensitivity in NIDHRs. Our results also indicated that C-LTT had a lower sensitivity compared to that observed in other studies. This may be explained by the heterogeneity of clinical manifestations included in the present study, as well as the high variability of drugs tested, in contrast to previous studies.

Related to this, we evaluated both LTT approaches in three more represented clinical entities in our cohort, SJS/TEN, MPE, and AGEP. The sensitivity of dDC-LTT was superior to that of C-LTT, regardless of the clinical entity involved (87.5% vs. 62.5% in SJS/TEN, 47.4% vs. 15% in MPE, and 80% vs. 33% in AGEP), all with a specificity of 85%. Interestingly, the most severe reactions, such as SJS/TEN and AGEP had the highest sensitivity values, compared to mild/moderate reactions, such as MPE. These findings have important implications, as they may provide better diagnosis for severe reactions, which currently have limited *in vivo* tests available.

We also showed that combining the results of CD3⁺T cell proliferative response between C-LTT and dDC-LTT did not improve the sensitivity of LTT compared to the performance of dDC-LTT (61.8%) alone, suggesting that the performance of the second LTT approach is sufficient to achieve the highest overall sensitivity of LTT.

Once we have demonstrated that dDC-LTT has better sensitivity values compared to C-LTT, the second objective of the thesis was the evaluation of the proliferative response of specific lymphocyte populations using this LTT approach. We observed that the highest proliferative response was detected in CD4⁺Th1 cells, supporting the important role of this cell subpopulation in NIDHRs elicitation. When we combined the proliferative response of different cell populations, we were able to increase the overall LTT sensitivity, without reducing its specificity. From the 61.8% of sensitivity achieved with CD3⁺T cells, the combination of proliferative results of CD3⁺T and CD4⁺Th1 cells increased to 73.9%, while between CD3⁺T and natural killer (NK) cells, increased to 82.6%. When the proliferative results of CD3⁺T, CD4⁺Th1 and NK cells were combined, the sensitivity of LTT increased to 87% with 85% of specificity. Finally,

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although the inclusion of CD3⁺T and CD8⁺T cell proliferative results reported the highest sensitivity value (91%), a reduction in specificity (80%) was also observed.

By clinical entity, the highest proliferative response was observed for CD4⁺Th1 cells, independently of the clinical entity involved. Moreover, the combination of proliferative results of CD3⁺T and CD4⁺Th1 cells, or CD3⁺T and NK cells allowed the identification of all the SJS/TEN reactions with a specificity of 85%. The lowest sensitivity value was found in MPE when assessing the proliferative response of CD3⁺T cells (47.4%). After combining the proliferative results from CD3⁺ and CD4⁺Th1 cells, the sensitivity in MPE increased to 50%, and from CD3⁺T and NK the sensitivity increased to 58.3%, similar to that observed with CD3⁺T and CD8⁺T cells, but with a lower specificity. The combination of CD3⁺T, CD4⁺Th1 and NK cells increased the sensitivity of dDC-LTT to 68.4% without reducing its specificity. Finally, in AGEP, the combination of proliferative results of CD3⁺T and CD4⁺Th1 cells did not increase the sensitivity of LTT (80%) compared to CD3⁺T cells proliferation. In contrast, the combination of proliferative results of CD3⁺T and NK cells allowed the identification of all patients with AGEP, similar to the combination of CD3⁺T and CD8⁺T cells, but with lower specificity.

Of interest, a differential involvement of specific cell subpopulations was observed depending on the clinical entity studied. Regarding the subpopulations of CD4⁺T cells, although the % of positive proliferative results was higher in CD4⁺Th1 cells independently the clinical entity involved (88% in SJS/TEN, 100% in MPE, 100% in AGEP), the involvement of CD4⁺Th2 cells was different, being of only 37.5% in SJS/TEN, but of 67% in MPE, and 50% in AGEP.

Similarly, a different contribution was observed in relation to the NK subpopulations and the clinical entity involved. In SJS/TEN a higher contribution of cytotoxic NK cells (87.5%) was observed compared to inflammatory NK cells (50%). In contrast, the role of cytotoxic NK cells (28.6%) was lower than of inflammatory NK cells (71.4%) in MPE. The contribution between cytotoxic (66%) and inflammatory (80%) NK cells seemed to be more balanced in AGEP patients.

Taken together, our data suggest that although C-LTT by flow cytometry does not have an optimal sensitivity when assessing different clinical entities and different drugs, the inclusion of pre-primed moDCs significantly increased the sensitivity of LTT. In addition, and thanks to the advantage of using flow cytometry technology, the analysis

of proliferative results in specific cell populations, with an important role, albeit at low rates, is useful to increase the sensitivity of LTT, with important relevance for the most severe reactions, with more limited diagnostic tools. Therefore, further advances in determining the specific mechanisms involved in each clinical entity could represent useful approaches with important implications for LTT sensitivity.

The third objective of the thesis was to evaluate the recognition of clavulanic acid (CLV), and its synthetic antigenic determinants by moDCs from selective allergic patients (AP) and healthy controls (HC), as well as to determine the ability of these cells to stimulate autologous lymphocytes.

IDHRs after the intake of amoxicillin (AX)- CLV have increased in recent decades, probably due to the high consumption pattern of these drugs. Although CLV has limited antibiotic capacity, due to its high betalacatamase activity it has become highly prescribed alongside AX.

Although CLV was initially thought not to be immunogenic, studies in recent decades have reported that CLV can be the drug responsible for triggering IDHRs following exposure to AX-CLV. Interestingly, a subject after the intake of AX-CLV could be sensitised to one or the other drug, with the simultaneously sensitisation to both drugs in a patient being infrequent. Therefore, determining whether, AX or CLV is the responsible drug involved in the IDHR has important implications for patients with IDHRs, since if CLV is the responsible drug involved, the patient can tolerate AX, avoiding the need to use second-line antibiotics, usually associated with higher costs, more side effects and generally less effective.

Nevertheless, the diagnosis of CLV IDHRs is a complex process, because ST reported low sensitivity, and DPT cannot be performed with CLV itself, so IDHRs to CLV must be confirmed by tolerance to AX after a reaction with AX-CLV. Additionally, the detection of CLV-sIgE cannot be performed, and BAT reported low sensitivity. Because of this, the search of new *in vitro* alternatives for evaluating IDHRs to CLV, as well as introducing factors to those available, can help to improve their sensitivity, extremely important for assessing IDHRs to CLV.

A possible explanation for the reduced sensitivity found in *in vitro* tests could be explained by the high degradation rate of CLV compared to other BLs such as AX or benzylpenicillin (BP), where CLV degradative metabolites can be observed within the

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first minutes in solution. Furthermore, these metabolites can be also reactive, making the reaction self-catalytic. Therefore, the identification of the metabolites generated from CLV degradation is a difficult process.

We hypothesise that, when native CLV is included in *in vitro* tests, different metabolites are generated, at low and different concentrations. Because CLV belongs to the family of BLs, it is well described that CLV needs to covalently bind to proteins to form drug-protein carrier conjugates, which can be recognised by the immune system, and thus trigger the IDHR. It is possible that the different degradative metabolites generated have different capacities to interact with different proteins, being only some of them responsible for being recognised, and consequently triggering the IDHR. However, due to the heterogeneity, the concentration of the specific metabolites responsible for the reaction might not be sufficient for immune activation, and it may explain the low sensitivity found in *in vitro* assays when assessing IDHRs to CLV.

In a recent study we have demonstrated that an antigenic determinant of CLV (AD-I), can trigger the degranulation of basophils in CLV AP, but not in HC. Therefore, its inclusion in BAT increased its sensitivity. Nevertheless, the full picture of CLV recognition and its potential ADs is not fully understood. Consequently, to better understand the immune recognition of the two proposed antigenic determinants of CLV (AD-I and AD-II), we synthesised different analogues of each (Clav1-3 of AD-I, and Clav4-7 of AD-II) with different functional groups, endowing them with a different capacity to interact with proteins. The recognition of CLV and its synthetic AD analogues was assessed by analysing the maturation and activation of moDCs, as well as their ability to stimulate autologous lymphocytes in a selected group of 27 patients with IDHRs to CLV and in 13 tolerant subjects.

Our results showed that synthetic analogues from both AD-I and AD-II were efficiently recognised by moDCs from CLV-selective AP compared to those from HC. This recognition occurred more efficiently than the recognition of native CLV, as it has been demonstrated by the expression of the markers CD40, CD80, CD83, and to a lesser extent of CD86 and CCR7.

Interestingly, the combination of maturation results after stimulation of moDCs from CLV AP with synthetic analogues from AD-I and AD-II, significantly increased the sensitivity of moDCs maturation assays to 76% for CD40, 67% for CD80, 62% for CD86,

57% for CD83, and 48% for CCR7, with an overall specificity of over 75%. Notably, the inclusion of native CLV results did not increase the % of positivity compared to the inclusion of the combination of results of synthetic analogues from AD-I and AD-II without reducing their specificity.

In addition, a different recognition pattern was observed, with synthetic analogues from AD-I being more associated with the expression of CCR7 and CD40, whereas synthetic analogues from AD-II were more associated with the expression of CD80, CD83, and CD86. This different recognition pattern between synthetic analogues from both ADs highlights the importance of including all of them to increase the sensitivity of moDCs maturation assays.

Our data demonstrated that moDCs from CLV AP stimulated with CLV and its different synthetic analogues from both ADs, triggered the proliferation of CD3⁺T, CD4⁺T and mainly CD4⁺Th2 cells, but not of CD8⁺T cells and CD4⁺Th1 cells. This is consistent with the pathomechanism implicated in IDHRs, characterised by a greater contribution of CD4⁺Th2 cells compared to other T cell populations.

Combining the proliferative results of synthetic analogues from AD-I and AD-II, the sensitivity of LTT increased to 46% in CD3⁺T cells, 50% in CD4⁺T cells, and 65% in CD4⁺Th2 cells. As in the moDCs maturation assays, the inclusion of native CLV did not increase the sensitivity of LTT in any of the cell populations analysed.

Moreover, a different recognition pattern was also observed between the synthetic analogues from both ADs. Although most positive cases recognised both ADs simultaneously, between 18% and 35% of positive cases specifically recognised one or the other AD, again supporting the idea of the need to include synthetic analogues from both ADs to achieve the highest sensitivity values.

Contrary to what was found for NIDHRs, in IDHRs to CLV, the best LTT sensitivity was obtained when only the proliferative response of CD4⁺Th2 cells was assessed, without the need to combine the proliferative response of different cell populations.

In summary, in this study we have shown that synthetic analogues from AD-I and AD-II derived from CLV degradation, are efficiently recognised by moDCs from selective-CLV AP with IDHRs. Moreover, these stimulated moDCs can efficiently trigger the proliferation of T cells, mainly of CD4⁺Th2 cells. The combination of synthetic analogues from both ADs significantly increased the sensitivity of moDCs maturation assays and

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LTT, both with high specificity, compared to the sensitivity obtained with CLV itself. These results, although at an early stage, may represent promising approaches to improve the diagnosis of IDHRs after AX-CLV intake, with the advantage of *in vitro* tests can not only differentiate between AP and tolerant subjects, but also specifically identify whether the IDHR is triggered by AX or CLV.

The last objective of this thesis was to evaluate the involvement of autologous mDCs in the evaluation of selective IDHRs to AX and CLV.

As described by others, and in the two previous objectives of this thesis, the analysis of the expression of different markers on moDCs after stimulation with the culprit drug causing the reaction is a useful test to assess the sensitisation to a drug in patients with IDHRs and NIDHRs. In a similar way, the inclusion of drug-stimulated moDCs as APCs in LTT has been reported to increase the sensitivity of LTT mainly in NIDHRs, although little is known about their role in IDHRs.

Although the inclusion of drug pre-primed moDCs serves to increase the sensitivity of LTT compared to C-LTT, its sensitivity remains suboptimal. One possibility for this might could the nature of the APC included in the assay. Most studies use moDCs, mainly because they can be easily transformed *in vitro* from circulant blood monocytes in large numbers. Although moDCs exist in nature, they only appear during inflammatory processes and, after resolution, they disappear. Therefore, other cell populations such as mDCs, true sentinels in blood and peripheral tissues, with great capacity to orchestrate the immune response allowing the interaction between innate and adaptive immune systems, may be more sensitive to drugs, and even more capable of activating autologous lymphocytes in allergic patients (AP), but not in tolerant subjects.

The development of *in vitro* assays with sufficient sensitivity is essential for clinical routine to allow discrimination between allergic patients and tolerant subjects. Nevertheless, they must also have sufficient specificity to identify the concrete drug responsible for triggering the reaction, including between related drugs from the same family and important chemical similarities such as AX and CLV.

For these reasons, herein, we evaluated the recognition of AX and CLV by moDCs and mDCs from 14 selective AP with IDHRs to AX, and from 14 CLV-selective AP. To do so, we measured migration, maturation, and activation markers expression on both DC populations after stimulation with each BL in AP and we compared the results with those

from HC. In addition, we analysed their capacity to trigger the proliferation of specific T cell populations, comparing the results with C-LTT, which consists of the direct stimulation of PBMCs with AX or CLV.

Our results showed that unstimulated mDCs, had higher expression of CCR7 compared to its expression on moDCs, while unstimulated moDCs expressed higher levels of CD40, CD80 and CD86 compared to mDCs. These observations may suggest that, at baseline, mDCs are more ready to migrate to LNs than moDCs, and that the latter DC population is more activated than mDCs in steady conditions. This could have important implications for the latter drug stimulation, as the high activation of moDCs under steady conditions, may reduce their capacity to upregulate these markers because of drug stimulation, since drugs are known to trigger a weak overall activation response.

In accordance with this, the stimulation of mDCs from AX AP with the culprit drug, triggered the upregulation of migration, activation, and maturation markers compared to HC. In contrast, the AX stimulation of moDCs from the same AP only induced the upregulation of CCR7, CD83, and CD86, all to a lesser extent than on mDCs.

Similarly, the stimulation of mDCs from CLV AP with the culprit drug, increased the expression of all the markers analysed, except for CD40 compared to HC. On the other hand, the stimulation of moDCs with CLV of AP with IDHRs to this drug only increased the expression of CD83 compared to HC. These results suggest that mDCs have a greater capacity to be activated by BLs than moDCs, although differences exist regarding the specific BL involved.

This was also confirmed by analysing the % of positive cases. In AX AP, higher positivity was obtained for all markers in mDCs after AX stimulation (47% for CCR7, CD40, and CD83, and 60% for CD80) compared to AX-moDCs (31% for CD40 and CD80, 39% for CD83 and 15% for CCR7), except for CD86 (27% in mDCs and 31% in moDCs). Similarly, the % of positivity was also higher in CLV AP for all markers in CLV stimulated mDCs (43% for CCR7, 50% for CD40, 21% for CD80, and 36% for CD86) compared to that observed in CLV-moDCs (0% for CCR7 and CD40, 8% for CD80, and 13% for CD86), except for CD83 (14% in mDCs and 17% in moDCs).

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Another important feature of these DC maturation assays is their ability to differentiate the culprit drug causing the reaction from other related drugs. For this reason, we stimulated DCs from AX selective AP with the non-culprit drug, in this case CLV, and vice versa. Although mDCs from AX AP stimulated with CLV reported higher maturation index than moDCs, the overall % of positive cases to the non-culprit drug was of 0% for CCR7, CD80, and CD83, of 18% for CD86, and 46% for CD40, while it was of 0% for CD80, 10% for CCR7 and CD40, and 20% for CD83 and CD86 in CLV-moDCs.

The stimulation of DCs from CLV AP with AX, reported overall lower maturation results in mDCs than in moDCs. The % of positivity was of 0% for all the markers analysed, except for CD86 (13%) in mDCs. In contrast, the % of positive cases in moDCs was higher (5% for CCR7, 11% CD40 and CD80, 21% for CD83, and 26% for CD86).

Regarding the capacity of AX pre-primed mDCs from AX AP to stimulate autologous lymphocytes, their inclusion as APCs increased the proliferation index in all the T cell population analysed compared to HC, except in CD8⁺T cells. The inclusion of AX-stimulated moDCs from AX AP only triggered proliferative response of CD4⁺Th2 cells compared to HC, whereas in C-LTT higher proliferation was observed in AX AP compared to HC, independently the cell population analysed, except in CD4⁺Th1 cells.

In CLV AP, CLV pre-primed mDCs triggered a proliferative response in all cell populations analysed, except for CD8⁺T and CD4⁺Th1 cells. No increased proliferative response was obtained for any of the T cell populations analysed, irrespective of the inclusion of CLV-moDCs or the performance of the C-LTT.

Notably, the highest % of positivity in both AX and CLV AP was obtained when mDCs were included as APCs, much higher than the proliferation found after the inclusion of stimulated moDCs or the performance of C-LTT. The % of positivity of CD4⁺Th2 cells, the most relevant cell subpopulation involved in IDHRs was of 67% in AX AP, but only of 25% in CLV AP when pre-primed mDCs were included. Therefore, we hypothesed that mDCs are much more ready-to-act than moDCs and may require a smaller amount of drug to be activated. As we have shown above, due to the high instability of CLV, degradative metabolites with different capacities to interact with proteins can be formed, being only some of them responsible to activate the immune cells from AP. Therefore, their concentration may not be sufficient to activate moDCs but may be sufficient to activate mDCs.

Finally, we determined the capacity of the three LTT approaches to identify the culprit drug responsible for the reaction, by assessing the proliferative response of CD4⁺Th2 cells from AX or CLV AP stimulated with the non-culprit drugs. The % of positive cases when CLV pre-primed mDCs were used as APCs in AX AP was of 0%, whereas it was of 11% in CLV-stimulated moDCs. The % of positive cases when AX pre primed mDCs or moDCs from CLV AP were included as APCs was of 0% in both. However, the % of positivity in C-LTT stimulated with the non-culprit drugs was much elevated compared to the other two approaches (39% in C-LTT from AX AP stimulated with CLV and 21% in LTT from CLV AP stimulated with AX).

Altogether, these data demonstrated the importance of including APCs to increase the sensitivity of cellular *in vitro* test. In particular, we have demonstrated that mDCs efficiently recognise AX and CLV specifically, and therefore, the analysis of activation and maturation markers could be used to assess the sensitisation in patients with IDHRs after AX-CLV reactions. The maturation assay sensitivity found with mDCs was higher than that obtained with moDCs. Moreover, the inclusion of pre-primed mDCs increased the sensitivity of LTT compared to the inclusion of pre-primed moDCs or C-LTT, especially when the proliferative response of CD4⁺Th2 was assessed in AP with IDHRs to BLs, although differences exist regarding the BL involved in the reaction. Nevertheless, more research is needed, although these data give us a more complete picture about the specific mechanism involved in the recognition of AX and CLV by the immune system from AP, which could have implications for the future accurate diagnosis of IDHRs to BLs.

INTRODUCTION

1. ADVERSE DRUG REACTIONS

1.1. Definition

Adverse Drug Reactions (ADRs) were defined in 1972 by the World Health Organisation (WHO) as “a noxious and unintended effect occurring at doses normally used in man”.² Later on, in 1981, Rawlins defined ADRs as: “unintended effects of substances used in the prevention, diagnosis, or treatment of disease”.³ Since then, new and more complete definitions have been proposed, such as the ones suggested in 2000 by Edwards and Aronson which defined ADRs as “an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product”.⁴

1.2. Epidemiology and prevalence

ADRs are a serious healthcare problem, accounting for 3-7% of all hospital admissions.^{2,3,5} In addition, 2-20% of hospitalised patients have experienced an ADR.⁵⁻⁷ These reactions can be life-threatening and cause prolonged stays in hospitals.⁸ The prevalence of ADRs is strongly influenced by diverse and multiple factors such as the nature and frequency of the drug exposure, gender, age, and genetic factors.⁹ Moreover, the risk of suffering an ADR increased with chronic renal failure, longer hospital stays, and number of drugs administered during hospitalisation. Related to this, the risk of suffering an ADR increased by 10% with each different drug administered during hospitalisation.⁷ Therefore, rationality in the administration of drugs during hospitalisation is essential to prevent ADRs.

The drugs most frequently implicated in ADRs are antibiotics (21.2%),⁷ followed by opioids (13.8%), and iodinated contrast media (ICM) (10.6%).⁹ Recent studies have shown that nonsteroidal anti-inflammatory drugs (NSAIDs) are responsible for more than 45% of anaphylaxis (ANA) in Brazil.⁹ Similarly, another study has also indicated that NSAIDs are the most relevant drugs triggering hypersensitivity reactions in children, adults and elderly (58%, 51.5%, and 36.2% respectively). Betalactams (BLs) are the second agents involved (14.3%). These differences between countries and drugs may be explained by different drug consumption patterns, the underdiagnosis of reactions triggered by NSAIDs, as well as overdiagnosis in the case of reactions to BLs.⁹

1.3. Classification

The most common ADR, accounting for more than 80% of all ADRs, are related to drug doses (type A).^{10,11} These reactions depend on the pharmacokinetics and doses of the drug, as well as its interactions with other molecules.¹² Because of this, they are predictable and can occur in all individuals.⁶ The second most common ADR (type B) accounts for about 15% of all ADRs.^{12,13} These reactions are unrelated to the doses or pharmacologic effect of the drug,¹¹ and are also called Drug Hypersensitivity Reactions (DHRs). They are unpredictable, risky, and only some of the subjects exposed to the drug may trigger a DHR.¹⁴ It has been therefore suggested that among other factors, a genetic predisposition may exist, and many studies have investigated this issue in recent decades, although due to the great variability of clinical manifestations, ethnic differences, and drugs involved, further research are needed to elucidate this aspect, although significant progress has been made in recent years. Other less common reactions are those related to the doses and time (type C), mainly triggered by cumulative toxic effects, caused by long-term exposure to the drug. These reactions can be divided into time-related reactions (type D), where the effects appear later as consequence of drug administration; and withdrawal (type E), related to rebounds or relapses.⁴ The last ADR included in the classification are reactions related to the unexpected treatment failure.⁸ The classification of ADRs, the mechanism involved, the main features and clinical examples are shown in Table 1.

Type	Mechanism	Feature	Examples
A	Pharmacological toxic drug reactions	– Common – Predictable – Low mortality	– Drug overdoses – Side effects due to cytostatic drugs
B	Hypersensitivity reactions	– Uncommon – Unpredictable – High mortality	– Immunological reactions – Idiosyncratic reactions
C	Cumulative toxic effects	– Uncommon – Long-term exposure	– Nephropathy from analgesics
D	Late-onset adverse effects	– Uncommon – Dose-related	– Carcinogenesis
E	Therapy withdrawal	– Uncommon – Rebound – Relapse	– Corticosteroid/opiate withdrawal
F	Treatment failure	– Common – Dose-Related	– Antibiotic resistance

Table 1. ADRs classification. Modified from *Edwards & Aronson, 2000* and *Bircher, 2022*.

2. DRUG HYPERSENSITIVITY REACTIONS

2.1. Epidemiology

Despite the human health implications, there are few data to elucidate the actual incidence of DHRs.¹⁵ The estimated prevalence of DHRs ranges from 2.8% to 20% in the general population,¹⁶ although only 4.4% to 6.9% of those subjects are believed to have a real DHR.¹⁵ However, one thing seems clear, the incidence of DHRs is increasing in both adults and children in the United States (US) and Europe over the past few decades.⁵

BLs are the most frequent drugs responsible for triggering DHRs, in both adults,^{5,17} and children.^{18,19} This high prevalence could be explained because BLs are also the most frequent prescribed drugs for the treatment of bacterial infections in both children,^{19,20} and adults,²¹ ranging from 3.6g to 23g per 1000 people per day,²⁰ mainly because of their low cost, reduced side effects and high effectiveness.¹⁸

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DHRs to BLs have been associated with an increased risk of treatment failure, antibiotic resistance, higher rates of ADRs, and complications due to *Clostridium difficile* infections.^{18,22} Moreover, they are also associated with longer hospital stays,²² higher number of readmissions within 12 weeks,²² ambulatory visits,²³ and even higher mortality.¹⁸ It is, therefore, an important health problem for national health systems.

Although the real prevalence of DHRs caused by BLs in the general population is unknown,²⁴ it is estimated to range from 0.7% to 10%, and the incidence of life-threatening reactions ranges from 0.004% to 0.015%.²⁵ Nevertheless, up to 20% of hospitalised patients claim to be allergic to penicillin, although less than 5-18% are confirmed through appropriate diagnostic techniques.^{17,25,26}

This significant overdiagnosis in DHRs to BLs may be explained by the diversity of clinical entities, concurrence of viral and infectious rashes, or the underperformance of adequate selection of diagnostic tests.¹⁷ This overdiagnosis has important implications for the health of patients and healthcare systems, because the impairment of using BLs may imply the administration of second-line antibiotics, mainly vancomycin or fluoroquinolones (FQs), usually less effective, with more side effects, and associated with higher risk of antibiotic resistant infections.^{17,21,27,28} In addition, they are more expensive treatments. It is estimated that BL allergic patients have to pay between 1.82 and 2.58 more than the non BL allergic subjects for antibiotic treatment.²⁹ In particular, a study carried out in Europe showed that penicillin DHRs increased the length of hospitalisation by 8.7%, increasing costs by up to 9.1%.¹⁷ Assuming that only 10% of allergic individuals are actually allergic, a correct delabelling of BL allergic patients could save more than 1.9 million USD and decrease the number of hospital and ambulatory admission in 3,863 cases.¹⁷

The incidence of DHRs to concrete BLs changes over time. In 1992 in Spain, the most frequent BL triggering a DHR in children was penicillin (35%), although the incidence decreased to 13.6% in 2005 and to 0% in 2015.³⁰ This is consistent with other recent studies, which reported a prevalence of DHRs to penicillin ranging from 0.6% to 5.6%.^{18,22} Nevertheless, due to the difficulty in establishing a concrete prevalence of this type of reactions, and the differences between countries, there is a large variability between studies. In contrast to penicillin, DHRs caused by AX have been increasing, reaching 57.4% at present, and due to AX-CLV to 17%.³⁰ These differences between BLs

and years could be explained by changes in drug prescription patterns,³¹ as AX has gradually replaced penicillin in recent years, accounting for 91.6% of all BLs dispensation in the US in 2014.⁹

Following BLs, NSAIDs are the drugs most frequently involved in DHRs regardless the clinical manifestation (cutaneous, nasal, ocular, bronchial, cardiovascular, or gastrointestinal), with a higher prevalence in women than in men, as it has been reported by Gomes and colleagues in an adult Portuguese population.⁵

Other drugs such as FQs or ICMs are also implicated in a high number of DHRs, the latter in up to 8% of patients, but their incidence has been decreasing in recent years to less than 1% at present.²⁶ By contrast, DHRs caused by chemotherapeutic agents have been increasing in recent years, mainly due to the increasing incidence of cancer and the consequent administration of chemotherapy, ranging from 1% to 27%, depending on the drug involved and number of doses administrated.²⁶ The estimated incidence of DHRs caused by monoclonal antibodies (mAbs), used to treat cancer, autoimmune, and inflammatory diseases,³² ranges between 0.1% and 10% of treated patients.²⁶

DHRs in children (<14 years) are much less frequent than in adults, although an important overdiagnosis exist.³⁰ The estimated DHR prevalence in children ranges from 3.8% to 4.4%, whereas in adults, it ranges between 8.4% and 10%.³⁰ Although not completely understood, it has been suggested that the differences between adults and children may be explained by differences in their immune systems and the lower exposure to drugs in children than in adults.^{30,33}

In children, skin eruptions during treatment with BLs are common, ranging from 1% to 7.3%.^{34,35} However, in most cases, the reaction is caused by other factors different to the BL treatment, such as viral infections.²⁰ Most DHRs to BLs in children are those with mild/moderate symptoms, although more severe reactions can occur.^{19,36} Most of these children are labelled as “allergic”, without a complete allergological study, with important implications for their future health and also for the costs of healthcare systems, with an estimated increase of around 30%-40%.²⁰ In fact, after a complete allergological study, only 6.8% of children with DHRs to BLs have positive results, with more than 90% being tolerants.^{19,20} Likewise, another study showed that only 14.7% of those children with skin rash during the treatment with AX were confirmed to have a real DHR.³⁷

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In a study carried out by Orhan *et al.* in urban schoolchildren a parent-reported prevalence of DHRs was found to be 2.8%, mainly to BLs (59.3%-81%), followed by sulphonamides (11.1%), or NSAIDs (9.9%-16%).^{30,38} In another study carried out by Erkocoglu and colleagues, the self-reported incidence of DHRs in children was of 7.87%, although after a complete diagnostic study, only an incidence of 0.11% could be established.¹⁵ Similarly, Lange and colleagues reported a parent-reported prevalence of 7.5% in German children.³⁹ Interestingly, 44% of parents whose children were clearly labelled as nonallergic by clinical tests, maintained the label of their own children as “allergic”.³⁹

This important overdiagnosis in children is mainly based on the elevated viral skin infections with dermatologic symptoms during childhood, which mimic an allergic reaction, which without a proper allergological study, are misdiagnosed as allergic.¹⁵ A similar overdiagnosis appears to occur in adults, as estimates suggest that only about 18% of patients labelled as “penicillin allergic”, are actually allergic.²⁵

2.2. Models for drug interaction with the immune system

Three different models have been proposed to explain how drugs interact with the immune system: the hapten model, the p-i model, and the altered peptide model.^{12,40-42}

2.2.1. Hapten model

It is based on the principle that drugs and/or their metabolites are too small molecules to be immunogenic by themselves.^{12,13} Therefore, to interact with the immune system, drugs and/or their metabolites must covalently bind to peptides or proteins carriers, generating a new antigen (hapten-protein complex) (Figure 1).^{12,43,44} Drugs can be haptens, or prohaptens, depending on their need of a previous metabolization process to be able to bind to carriers.⁴⁵ An example of prohaptens is sulfamethoxazole (SMX) which is inert in its native form, and to become immunogenic, it must previously be metabolised in the liver, leading reactive metabolites.⁴⁴

The drug or metabolite can modify the structure of proteins, such as albumin, transferrin, immunoglobulins, selectins, or integrins.⁴⁶ This new antigen is recognised by the immune system and may trigger a DHR (antigenicity).¹³ The first stages of drug-

protein interactions constitute a fast process, mainly composed by electrostatic interactions, hydrogen bonds or van der Waals forces. Afterwards, covalent links are formed between specific available amino acids from the protein and the drug (haptization). Interestingly, these covalent links are strong enough to persist after cell processing. After haptization, hapten-protein complexes are processed by antigen presenting cells (APCs), and presented on human leukocyte antigens (HLA) context to T cells through T cell receptors (TCRs).⁴⁷ It is important to note that after hapten-protein complex processing, smaller peptides can be generated, interacting with different HLA.¹² Therefore, the presentation is not restricted to a single HLA molecule, if not that involves a very diverse and complex immune process.

2.2.2. p-i model

This model has been mainly described for NIDHRs, and it indicates that some drugs, although small enough to elicit an immune response on their own, can interact directly with protein receptors such as TCRs, triggering direct T-cell activation, without processing (Figure 1).^{44,48} This model has been validated mainly by sulphamethoxazole (SMX).¹³

Other drugs can interact directly with HLA, changing the protein conformation, and triggering T cell activation.^{45,48} Schnyder and colleagues proposed a model of this interaction in 1997. They demonstrated that SMX was efficiently recognised by T CD4⁺ and T CD8⁺ clones without the need for antigen processing by APCs (Figure 1). Although to be activated, T cell clones needed to be cultured with APCs, even they were fixed, and thus unable to process the drug.⁴⁴ These findings support the model that SMX can bind directly to MHC complexes on the surface of APCs, being independent of intracellular processing. Similarly, Atabecestat, a BACE inhibitor, and its metabolites can interact directly and reversibly with HLA class II molecules, without prior cellular processing.⁴⁹

Unlike hapten-protein interactions, p-i bonds are formed in a non-covalently way, composed mainly by hydrogen bindings, electrostatic interactions and Van der Waals forces.⁴⁸ Moreover, no second and complementary signals are needed to trigger T-cell activation.¹³

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HLA polymorphisms in the population is extremely high (>14,000 HLA alleles), with between 14 and 20 different individual HLA alleles in each person.¹³ This heterogeneity may explain why only some individuals have a potential HLA variants that can interact with drugs, triggering a DHR.¹² However, not all individuals who carry the allele and are exposed to the concrete drug develop a DHR, so this model cannot fully explain elicitation of DHRs.

Since p-i model suggests that drugs interact directly with some specific cells, the number of stimulated cells at the beginning of the reaction is very restricted, and only after their expansion and migration, clinical manifestations occur.¹² Because of this, the p-i model has been described mainly for NIDHRs and may explain some gaps that cannot be explained by the hapten model. However, more research is needed regarding how the sensitisation occurs in allergic patients, or to describe the specific mechanisms involved in the absence of costimulatory molecules.

2.2.3. Altered peptide model

This is the last interaction model proposed. It was described for specific drugs as abacavir and specific HLA*57:01 restricted T cells.⁵⁰ In presence of abacavir, the drug binds to the F pocket of HLA*57:01, altering the biochemical and biophysical properties, mainly by modifying the amino acid sequences of the peptides.⁴⁵ Consequently, this modification was identified as exogenous, and triggering the T cell activation, and the development of the immune response (Figure 1).^{14,45,50}

Interestingly, in this model, no “true” drug recognition was produced, but rather recognition of the presence of autologous peptides that are not present prior the interaction with abacavir. More recently, this model has also been validated for HLA-B*15:02 and carbamazepine-10, 11-epoxide, the major metabolite from carbamazepine degradation.^{45,51}

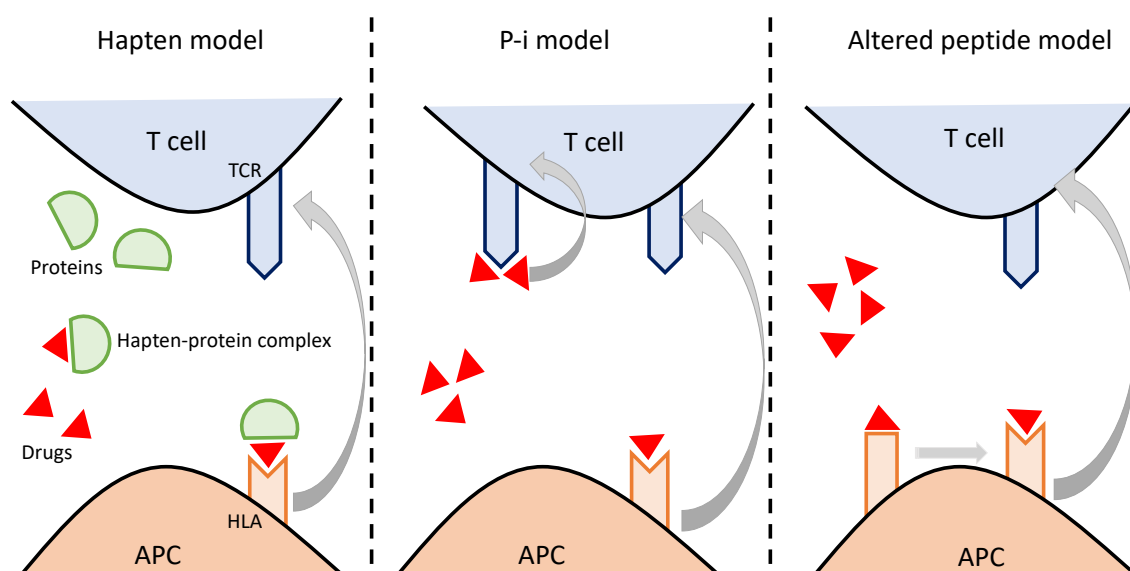


Figure 1. Drug-immune system interaction. Three different models have been proposed, the hapten, p-i, and altered peptide model. Abbreviations: APC, antigen presenting cell; HLA, human leukocyte antigen; TCR, T cell receptor.

Each of the proposed interaction mechanism has its strengths and limitations (Table 2).⁵² Furthermore, although some drugs appear to be specifically involved in one or the other mechanism, the same drug may interact with the immune system through different pathways, or even more than one simultaneously.⁵²

	Hapten model	p-i model	Altered peptide model
Interaction	Covalent	Non-covalent	Non-covalent
Clinical entities	URT/ANG/ANA DRESS/MPE/SJS/TEN	SJS/TEN/DRESS	SJS/TEN/DRESS
Time interval	Immediate Non-immediate	Non-immediate	Non-immediate
Binding site	Proteins/Peptides	HLA/TCRs	MHC Peptide binding groove
Cell processing	Yes	No	No
Drug removed by washing	Yes	No	No
HLA variants associated	Yes	Yes	Yes

Table 2. Similarities and differences of hapten, p-i and altered peptide models. Modified from Pichler et al. 2016¹² and Yun et al. 2016.⁴¹ Abbreviations: ANA, Anaphylaxis; ANG, Angioedema; DRESS, Drug reaction with eosinophilia and systemic symptoms; HLA, Human leukocyte antigen; MPE, Maculopapular exanthema; MHC, Major histocompatibility complex; SJS, Stevens-Johnson syndrome; TCR, T cell receptor; TEN, toxic epidermal necrolysis; URT, Urticaria

2.3. Classification

The classification of DHRs is a complex process, due to the great variability of clinical manifestations, the different immunological mechanisms and cells involved, which are not fully understood,⁴⁰ and to some extent, the existence of overlaps between classifications. Because all of this, there is not an unique and universally validated classification.¹² Therefore, the most used classifications, based on clinical aspects and mechanism involved are discussed here.

2.3.1. Classification based on clinical aspects

There is a broad consensus that it is important to establish a classification of DHRs to facilitate their diagnosis and management.⁵³ Based on this idea, a classification based on the time elapsed between drug administration and the onset of symptoms has been proposed. Thus, DHRs can be classified as immediate (IDHRs) or non-immediate (NIDHRs).

2.3.1.1. IDHRs

IDHRs are reactions in which symptoms appear within minutes to one hour after drug administration (Figure 2), and are characterised by URT, angioedema (ANG), bronchospasm, ANA or anaphylactic shock (AS).^{9,40,53-55} Nevertheless, symptoms in IDHRs can also appear within the 6 hours from drug administration and symptoms onset, with an overlap existing between IDHRs and NIDHRs.

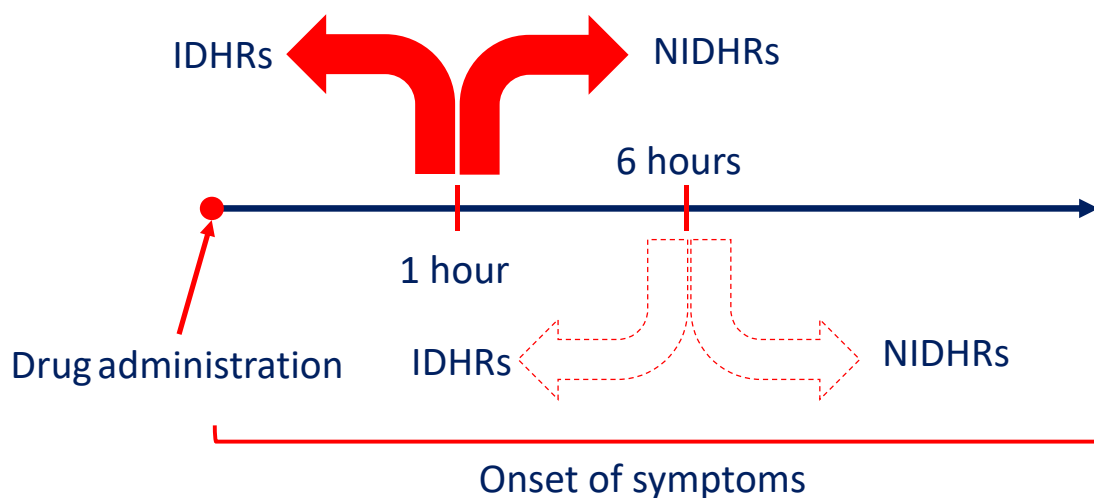


Figure 2. Classification based on clinical aspects. Differences between IDHRs and NIDHRs based on the time elapse between drug administration and onset of symptoms.

2.3.1.1.1. Anaphylaxis (ANA)

Anaphylaxis (ANA), is an acute, life-threatening systemic immune reaction caused by the release of inflammatory mediators affecting multiple organs.^{26,56-58} Symptoms appear rapidly, within 5 to 30 minutes after drug administration. Clinically, it is characterised by diffuse erythema, pruritus, bronchospasm, hypotension or cardiac arrhythmias, and in the most severe reactions, cardiovascular collapse leading to AS may occur.^{11,24,59} The incidence of ANA ranges from 0.05% to 3.1%, with a mortality of approximately 0.65%.^{54,56} There is clear evidence that the incidence of ANA has increased in recent years,⁶⁰ with a worsening of symptoms.¹⁶

Between 0.7% and 10% of all cases of ANA are caused by penicillins, with fatal cases of ANA due to these drugs estimated at 0.0015-0.002%.²⁶ Cephalosporins are also important drugs triggering ANA, although with a lower incidence than penicillins (0.0003% to 0.002%). Nevertheless, their use has increased in recent years, so the incidence may now be rising.²⁶ Other drugs such as FQs, NSAIDs, NMBAs, ICMs, or gadolinium derivatives are also common drugs implicated in ANA.²⁶ Recently, new drugs such as chemotherapeutic agents and monoclonal antibodies have been reported to trigger ANA, being the third most frequent drug involved in the disease in US.²⁶

The immunological mechanism of ANA is complex, mainly because of the diversity of cell populations involved, variability of mediators and immunoglobulins involved. In addition, due to its unexpected nature and rapid development, obtaining samples during the reaction is not always easy.

The classical and most studied mechanism that explains basophil and mast cell activation in ANA is mediated by specific immunoglobulin e (sIgE).⁵⁹ It involves the activation of basophils and mast cells by cross linking between drug-sIgE, and high affinity receptors, FcεRI, triggering their degranulation, and the consequent release of inflammatory and lipid mediators, such as histamine, prostaglandins, platelet activating factor (PAF), and leukotrienes (Figure 3).^{57, 58}

Nevertheless, other pathways may also exist, as indicated by different mouse models.^{57-59,61} An alternative non-IgE dependent ANA can be mediated by sIgG.⁶² sIgG forms immunocomplexes with drugs, which binds to the low-affinity receptors, FcγRIII, present on mast cells, basophils, macrophages, neutrophils, or platelets,^{61,62} with the subsequent release of PAF (Figure 3).^{57,59} Due to the lower affinity of IgG to FcγRIII,

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compared to that between IgE and FcεRI, the amount of antigen required to trigger ANA by sIgG is much higher than by sIgE-mediated mechanism. Therefore, sIgG-mediated ANA is only possible with high doses of allergen, such as in the case of therapeutic doses of drugs and antibodies, but is unlikely for venom stings or food allergy.^{57,63} Moreover, IgG has a dual role, which depends mainly on antigen/antibody concentration. If the sIgG concentration is high, but the amount of antigen available is low, sIgG antibodies will intercept the antigen, before sIgE binds, preventing sIgE-mediated activation.⁶³ Conversely, if the concentration of antigen and antibody is high, sIgG mediated ANA can occur. This is the case for NMBAs, which have been demonstrated to be involved in sIgG-ANA and in the formation of immunocomplexes that activate circulating neutrophils.⁵⁶ Although the mechanism is completely different between sIgE- and sIgG-mediated ANA is indistinguishable in terms of kinetics, severity, organs involved or challenge doses.⁶¹

Another possible activation pathway is the so-called complement activation. It is mediated by anaphylatoxins such as C3a and C5a, which can trigger basophil and mast cell degranulation, as has been shown for peanut allergy (Figure 3).⁶⁴ Nevertheless, it is unclear whether C3a and/or C5a can activate effector cells on their own, or whether they are factors that exacerbate ANA reactions together with other activating mechanisms.⁵⁷ Mast cells and basophils can also be directly activated by drugs by non-immunologic mechanisms (Figure 3), such as the direct drug interaction with the Mas-related G-protein coupled receptor member X2 (MRGPRX2 on mast cells) or by inhibition of enzymes such as the cyclooxygenase.^{65,66} Non-immunologic reactions will be explained in depth in the following section: classification based on mechanisms involved.

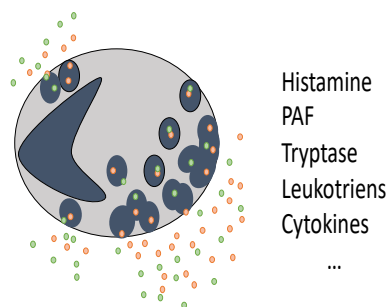
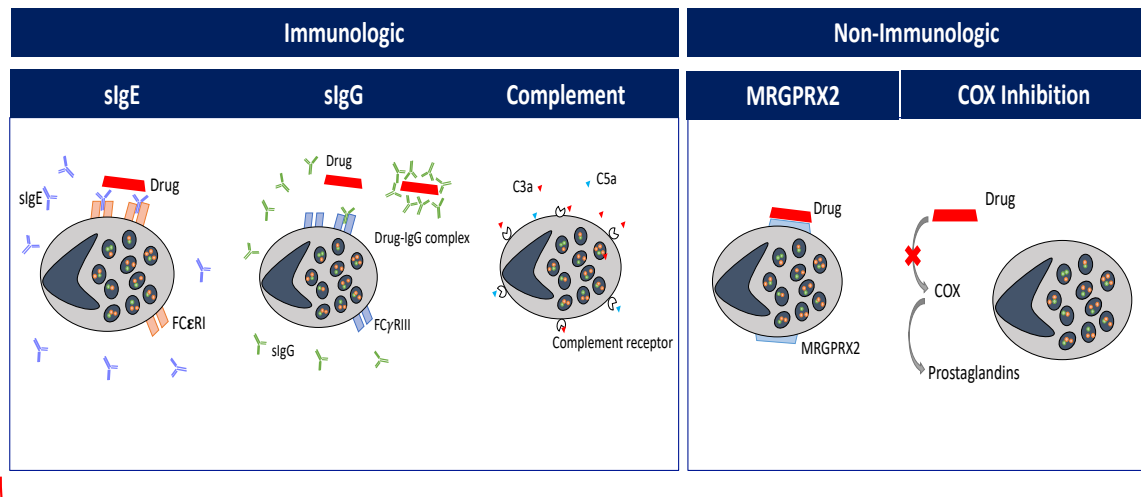


Figure 3. Mechanisms in ANA. Abbreviations: COX, cyclooxygenase; MRGPRX2, Mas-related G-protein coupled receptor member X2; PAF, Platelet activating factor; sIgG, specific immunoglobulin G; sIgE, specific immunoglobulin E.

2.3.1.1.2. Urticaria

Urticaria with or without angioedema is characterised by the rapid appearance of lesions following drug administration, but also resolves rapidly after treatment interruption.¹¹ It is characterised by transient and pruriginous wheals, on different parts of the body, but always confined to the skin (Figure 4).²⁴ Despite being different clinical entities, it can sometimes be difficult to differentiate between ANA and URT/ANG, mainly in the early stages of symptoms onset.²⁴ The estimated incidence of URT/ANG is of 4.9% of the general population.¹⁶ It is driven by the release of inflammatory mediators by basophils and mast cells,¹⁶ mediated mainly by sIgE, but other mechanisms, such as the formation of immunocomplexes may occur.⁶⁷ URT/ANG can also occur following topical contact with drugs or other allergens, such as latex, which is termed allergic contact URT.⁶⁷



Figure 4. Clinical manifestation of URT.

2.3.1.2. NIDHRs

NIDHRs are hypersensitivity reactions in which symptoms appear more than 1 hour, days, and even weeks after drug administration (Figure 2),^{55,68} NIDHRs affect to 2-3% of hospitalised patients,⁶⁹ with the skin being the most common target organ involved.⁵⁵ Because of the wide variety of immune mechanisms and the different cells involved, the clinical manifestations of NIDHRs are also very diverse, ranging from mild-symptoms reactions such as maculopapular exanthemas (MPE), or delayed-URT, to systemic reactions such as AGEP, DRESS and life-threatening reactions such as SJS/TEN.^{14,34,43,53,68,70}

2.3.1.2.1. MPE

MPE is the most common NIDHR, characterised by diffuse erythematous macules and papules without systemic involvement (Figure 5).^{11,54} It is accompanied by mild/moderate symptoms usually appearing after 7-12 days after drug administration,¹³ and resolves rapidly after drug treatment suspension.⁷¹ BLs, NSAIDs, allopurinol, sulphonamides, and anticonvulsants are the most common drugs implicated in MPE.¹¹



Figure 5. Clinical manifestation of MPE.

To better understand the immunological mechanism involved in MPE, Fernandez and colleagues showed an increased expression of interferon gamma (IFN- γ) and tumour necrosis factor alpha (TNF- α) in skin and CD4⁺T cells, together with a high expression of chemokines such as CXCL9, CXCL10, CCL20, and CCL27 in skin, and homing chemokine receptors CCR6, CXCR3, and CCR10.⁷² Similarly, Naisbitt and colleagues cloned T cells after stimulation with lamotrigine in a patient with MPE.⁷³ Most of the drug-specific clones were CD4⁺T cells, although some CD8⁺T cell clones were also detected. High levels of IFN- γ , corresponding with a type 1 cytokine pattern were also observed, together with perforin release and moderate amounts of IL-5. In addition, high CLA expression was observed in specific clones (Figure 6).⁷³

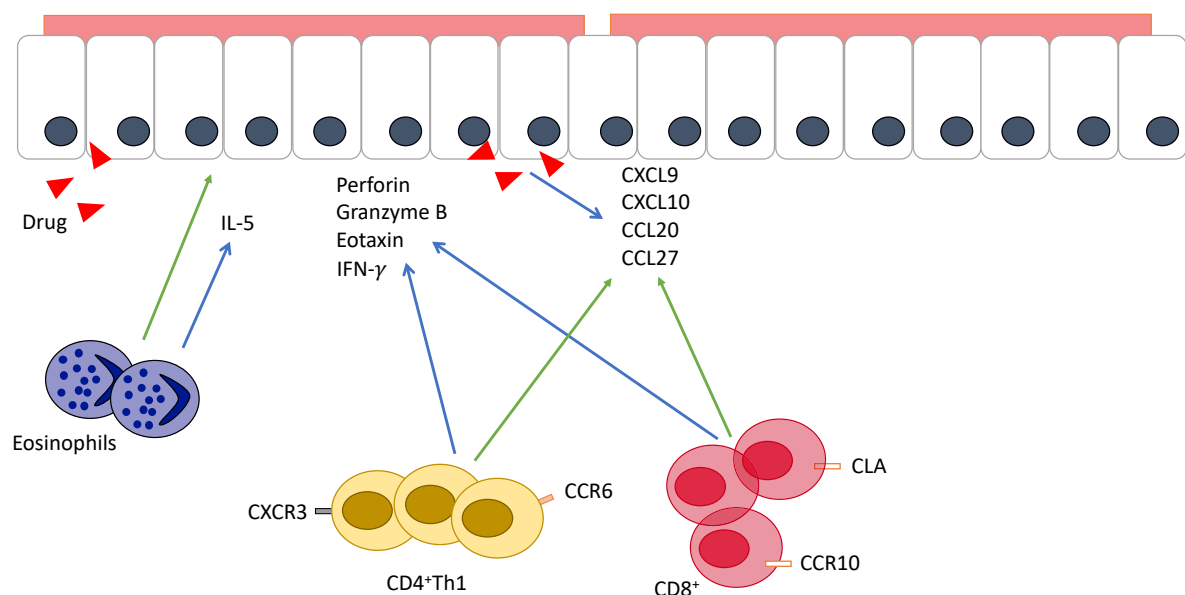


Figure 6. Mechanisms in MPE.

2.3.1.2.2. AGEP

AGEP is characterised by the appearance of pustules on erythematous background, fever, and neutrophilia (Figure 7).¹⁴ Although the incidence is low, ranging from 1-10 cases per million people per year, mortality associated with AGEP is estimated in 4%.⁵⁴ The symptoms usually appear between day 3-5 after drug administration.¹³ BLs and FQs are strongly associated with AGEP.⁵⁴



Figure 7. Clinical manifestation of AGEP.

Keratinocytes and $CD4^+$ T cells produce large amounts of CXCL8 (previously named as IL-8), essential for the recruitment of neutrophils to the skin, main feature of AGEP (Figure 8). Moreover, $CD4^+$ Th1 cells are also recruited through CXCR1, which release $IFN-\gamma$ and granulocyte-macrophage colony-stimulating factor (GM-CSF), contributing to increase the inflammation in the damaged tissues. More recently, the presence of $CD4^+$ Th17 cells has been detected in AGEP lesions, attracted through CCR6-CCL20 released by keratinocytes.¹⁴

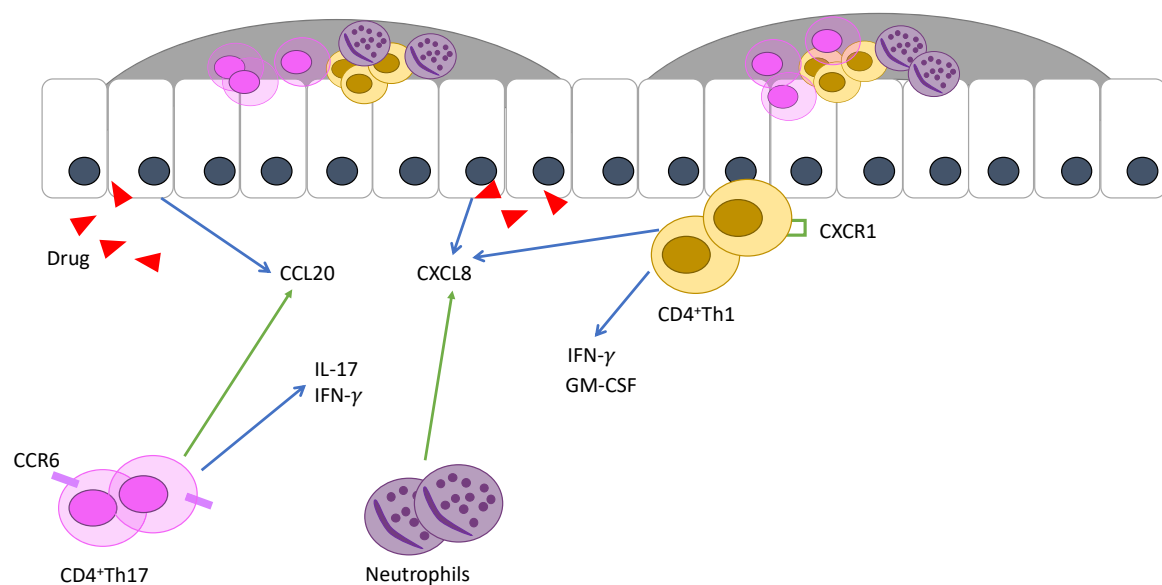


Figure 8. Mechanisms in AGEP.

2.3.1.2.3. DRESS

DRESS, is an acute NIDHR characterised by skin rash, lymphadenopathy and leucocytosis with eosinophilia, accompanied with fever and systemic involvement.^{54,74-76} Symptoms usually appear within 2-8 weeks,^{13,75,77,78} with anticonvulsants, sulphonamides, antibiotics, vancomycin, abacavir, and allopurinol being the most commonly drugs involved.^{69,76} The mortality rate ranges from 1-10% per million people per year.^{14,54,75,79} After the interrupt of treatment with the culprit drug, symptoms of DRESS can persist for weeks or even months.⁸⁰

The immune mechanism is mainly based on IL-5, released by CD4⁺Th2 cells in the damaged skin, which is responsible for the recruitment, maturation, activation, survival, and adhesion of eosinophils.⁷⁷ CCL17, released by keratinocytes, CD11⁺ DCs and monocyte derived dendritic cells (moDCs) triggers the recruitment of CCR4⁺ cells, mainly CD4⁺Th2 cells, which produce high amount of cytokines such as IL-4, IL-5, or IL-13 (Figure 9).⁷⁴ Furthermore, the involvement of CD8⁺T and CD4⁺Th1 cells have also been demonstrated to be important in DRESS, due to the high levels of IFN- γ and TNF- α found in the damage tissues, mainly related to the severity of the disease.⁶⁹ Recently, innate lymphoid cells type 2 (ILC2) have been proposed to be involved in the pathogenesis of DRESS. IL-33 is highly expressed in the skin, mainly produced by monocytes in response to cell damage, responsible for the recruitment of ILC2 through their ST2 receptor.^{14,79} ILC2 release a large number of Th2-type cytokines, such as IL-5 and IL-13, which contribute to eosinophil recruitment.⁷⁹

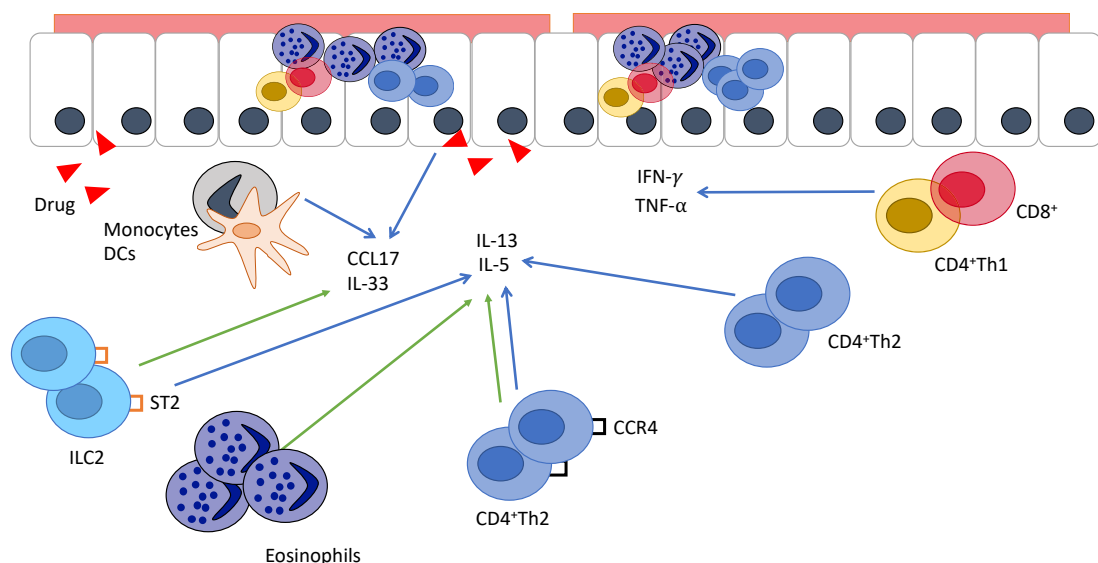


Figure 9. Mechanisms in DRESS

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During the development of DRESS, reactivation of viruses has been reported, although the mechanism is not completely understood.^{69,81,82} After the interruption of drug administration which triggers the initial reaction, it is frequent the appearance of relapses as exacerbation of exanthemas or eosinophilia. They may be related to viral infections, but also to the administration of new drugs, previously well tolerated.⁸⁰

The mechanism involved in the sensitisation to a previously tolerated drug during DRESS is unknown, although in many cases, patients after the recovery phase were able to tolerate the second drug again (non-immunogenic mediated). These reactions are thought to be caused while the second drug is administrated when the immune system is still activated by the first drug which cause the real DRESS.⁸³ This new interaction exacerbates the symptoms of the first reaction, or even triggers new ones, but because of the reduced exposure to the second drug, there is no real stimulation of the immune system for a long time, resulting in no real T cell expansion, and therefore without a real sensitisation to the new drug.⁸³

On the other hand, other patients may trigger a multiple drug hypersensitivity syndrome (MDH), so patients must avoid the use of very different drugs, affecting their health and quality of life. T cell activation in MDH is not caused by cross reactivity between drugs, because in most cases, the chemical structure of these drugs is completely different.^{83,84} The most common clinical manifestations in the former reaction are mainly DRESS and severe exanthemas, while the latter reactions can be more heterogeneous.⁸³

Jörg and colleagues showed that half of DRESS patients have at least one relapse, which 39.1% are related to new drugs. Of those with drug-related relapses, 17.4% have a proved sensitisation.⁷⁸ Similarly, another study showed that second-sensitisation to antibiotics related to non-antibiotic-causing DRESS is frequent, with 53% of positive antibiotic sensitisation, confirmed by patch tests (PT).⁸⁵ Interestingly, most cases of drug-relapses were triggered by high doses of BLs, but also ICMs, proton pump inhibitors and analgesics.⁷⁸ These sensitisations could explain the persistence of symptoms, as well as the occurrence of exacerbations after culprit drug interruption, which are frequently found in patients with DRESS.⁸⁵

2.3.1.2.4. SJS/TEN

SJS/TEN, although with low incidence (0.93–12.7 per million people per year),⁵⁴ is one of the most severe NIDHRs, with a mortality ranging from 10 to 50%.^{11,14,86,87} It is characterised by a rapid blistering exanthema of macules with skin detachment (Figure 10).^{54,88} It is also accompanied by other symptoms such as fever, myocarditis, myocardial infarction, hepatitis, or respiratory and gastrointestinal compromises.⁸⁸ SJS/TEN is considered the same disease, with different degrees of severity. SJS is considered when less than 10% of skin is affected, while TEN is considered when more than 30% of the skin affected.^{14,88} Therefore, there is an overlap between 10-30% of the skin affected, and most studies consider both, SJS and TEN to be the same clinical entity.⁸⁹ Symptoms usually appear between the first 10-24 days from drug administration.¹³ The most common drug involved in SJS/TEN are antibiotics, allopurinol, NSAIDs, anticonvulsants,⁸⁶ and sulphonamides.¹⁴



Figure 10. Clinical manifestation of SJS/TEN.

The immunological mechanism of this clinical entity is not fully understood. During the first days from the reaction, fluid accumulates under the affected epidermis, with high infiltrates of monocytes, macrophages, and lymphocytes.⁸⁹ In addition, the important role of cytotoxic T lymphocytes (CTLs), mainly CD8⁺T cells,⁹⁰ together with NK cells, have been demonstrated (Figure 11).^{90,91} Moreover, elevated levels of IL-17 and IFN- γ are found in SJS/TEN lesions, and also CD4⁺T producing IL-17, mainly those also

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expressing CLA and CCR4, markers highly associated with T cell homing to the affected skin.⁹² This suggests the participation of CD4⁺Th17 which produces high amount of pro-inflammatory cytokines such as IL-6 and TNF- α , or chemokines such as CXCL8.^{14,92} In addition, a recent study showed that elevated levels of IL-15 in serum and blisters correlate with the diseases progression and severity, which may be considered a biomarker, and a potential target for future therapies.⁹³

Nevertheless, the high keratinocyte mortality, characteristic of the disease, may suggest the involvement of different mechanisms to trigger the keratinocyte apoptosis. Granule-mediated apoptosis by release of perforin, granzyme B,⁸⁹ granulysin,^{14,88} or through interaction between FAS-FASL has been proposed, although controversy exists regarding the latter. Granulysin not only triggers the apoptosis of keratinocytes, a study has shown that, at low concentrations, this molecule also functions as chemoattractant and activator of monocytes, CD4⁺ and CD8⁺ memory T cells, NK and moDCs, triggering the release of cytokines and chemokines at the site of inflammation.⁹⁴ Additionally, the involvement of other death-receptors ligands, such as TNF- α , TWEAK or TRAIL, has been suggested to be involved in the progress of the disease.^{14,89} Concretely, CTLs produce a large amount of IFN- γ , responsible for the activation of monocyte, macrophages, and DCs, the main sources of TWEAK and TRAIL. Then, NK, T cells, monocyte and macrophages which express the receptor of these ligands, are the main responsible for the death of keratinocytes.

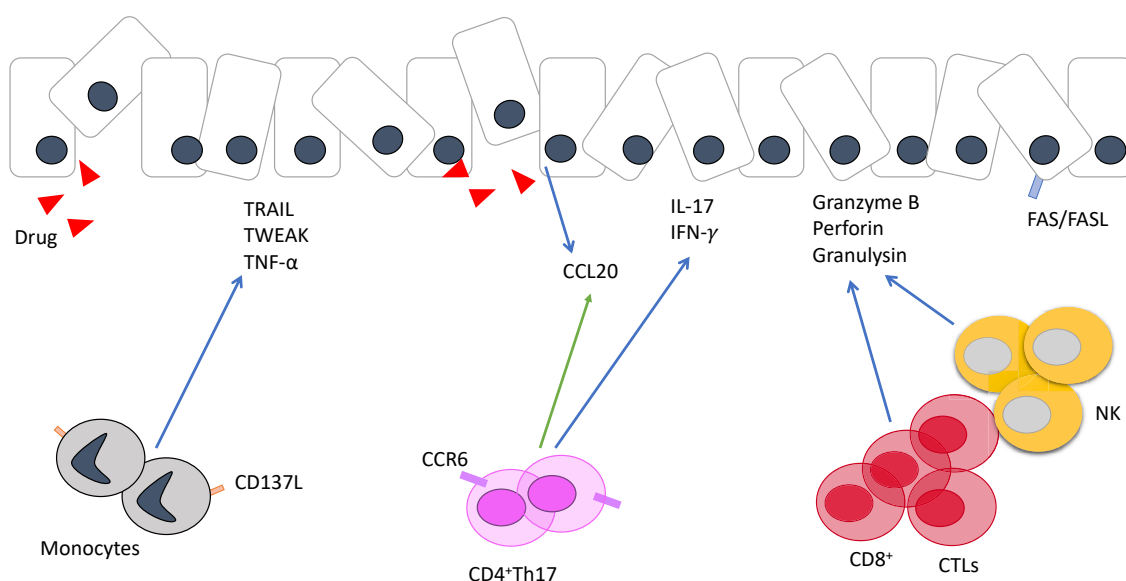


Figure 11. Mechanisms in SJS/TEN.

Due to the great heterogeneity of mechanisms involved in NIDHRs, the search for biomarkers and the study of the specific cell populations involved in each clinical entity may be particularly relevant. Even though this classification has demonstrated to be very useful for clinical practice, there is some controversy about its validity, mainly because the overlap between IDHRs and NIDHRs. In addition, this classification does not consider the nature of the drug, the immunological mechanism involved, susceptibility or drug doses.¹²

2.3.2. Classification based on the mechanism involved

DHRs can be classified according to the mechanism involved into allergic and non-allergic reactions.⁵³ Non-allergic, also called pseudo-allergic reactions, are those in which the involvement of specific immunological mechanisms has not been demonstrated,⁴³ and are produced by the direct interaction of the drug with receptors or enzymes of immune cells. From these, NSAIDs are the most frequently implicated drugs. NSAIDs-induced non-allergic reactions are based on over-inhibition of specific enzymes such as COX-1, which triggers an exacerbated release of leukotrienes,⁹⁵ leading the direct activation of basophils and mast cells.⁹ Another non-immunological activation mechanism involves the off-target occupation of receptors in mast cells such as the MRGPRX2, described mainly for NMBAs and FQs.⁶⁵ Allergic and non-allergic reactions cannot be differentiated clinically because they share the same symptoms after mast cell or basophil activation, although non-allergic reactions are limited to specific drugs, and are produced by higher drug dose.¹²

Allergic reactions are those DHRs that are mediated by known immunological mechanisms. They can also be subclassified according to the traditional Gell and Coombs system (Table 3).^{6,53,54} Type-I reactions, or immediate-type reactions, are those mediated by the interaction between IgE and high-affinity receptors (FcεRI) on basophils and mast cells. Type II are cytotoxic reactions, mediated by specific immunoglobulin G (IgG) or immunoglobulin M (IgM), which trigger cell death. Type III are reactions mediated by IgG and the formation of drug-antibody immunocomplexes, leading to activation of the complement system. Type IV are non-immediate reactions, mainly mediated by T cells.^{6,54,55} Among all reactions, type-I and II reactions are the most frequent.

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Reaction	Type I	Type II	Type III	Type IV
Timing of reaction	Immediate	Variable	Weeks	>1 hour
Mediators	Histamine, Leukotrienes, Prostaglandins	IgM, IgG Cytotoxic	IgM, IgG Precipitating	Cytokines, Effector cells
Mechanism	sIgE bound to FcεRI from basophils and mast cells	IgM/IgG bound to cell surface proteins	Formation of immune complexes	APCs and T cell activation
Clinical entities	URT/ANG/ANA	Agranulocytosis Thrombocytopenia	Vasculitis Serum sickness	AGEP/MPE/DRESS

Table 3. DHRs classification. Based on Gell and Coombs system.

There is agreement between the classification based on clinical aspects and mechanism involved, as IDHRs are mostly sIgE-mediated, although non-allergic reactions like those mediated by complement activation, COX-1 inhibition, and off target interaction of MRGPRX2 can be also produced, while NIDHRs are mostly T-cell mediated. For the purpose of this thesis and for facilitate reader's understanding, the classification based on clinical aspects will be used, differentiating between IDHRs (IgE mediated) and NIDHRs (mediated by cells), considering only those reactions with known immunological mechanism.

3. IMMUNOLOGICAL MECHANISMS OF IDHRs

3.1. Sensitisation

Drugs/metabolites or drug-carrier conjugates are recognised by APCs, with dendritic cells (DCs) being the most potent APCs in the body, although other cell populations, such as B cells or monocytes are also considered APCs.

After drug recognition, APCs, mainly DCs, capture the antigens, and process them. During this process, DCs undergo a complex maturation process, in which they express costimulatory molecules on their membrane, such as CD40, CD80, CD83, CD86, as well as C-C chemokine receptor type 7 (CCR7). Due to the increased expression of CCR7, DCs migrate to lymph nodes (LNs), organs rich in naïve B- and T-cells. There, DCs

present the processed antigens on the MHCs context to TCRs on naïve T cells, supported by costimulatory molecules and cytokine release. Naïve T cells differentiate into CD4⁺T helper cells (Th), mainly with a type-2 cytokine pattern (Th2), characterised by release of interleukins (IL)-4, IL-5, IL-9, and IL-13 (Figure 12).

The interaction between CD4⁺Th2 and naïve B cells via CD40-CD40L and cytokines such as IL-4, induces IgE isotype class switching, proliferations of antigen-specific B cells, transforming them into plasmablasts and plasma cells that leave LNs and release drug-sIgE into the bloodstream and tissues.

Although this model is well validated, in recent years, thanks to technical advances, different studies have shown that most IgE producing B cells are derived from IgG, mainly IgG1 and immunoglobulin A (IgA) B cells, rather than IgM or immunoglobulin D (IgD) B cells.^{40,96} This may suggest that drug-sIgE is produced mainly by ascending antigen-specific B cells, rather than from naïve B cells.⁹⁶

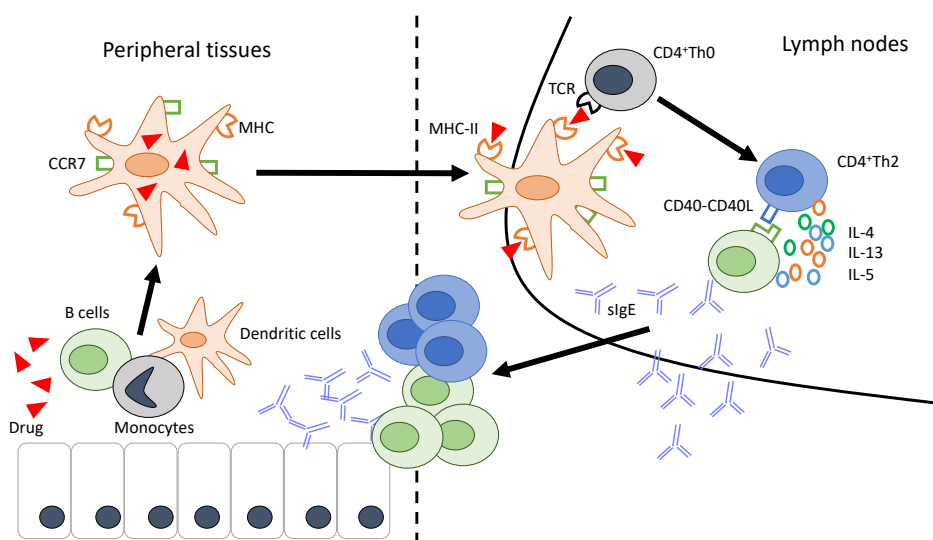


Figure 12. Sensitisation phase in IDHRs.

3.2. Effector phase

The most studied effector phase in IDHRs is that mediated by sIgE. If re-exposure to the same drug follows sensitisation, the drug, metabolite, or drug-protein conjugates interact with sIgE from at least two adjacent FCεRI (cross linking). The signal is transduced, triggering p38MAPK phosphorylation and calcium influx, leading to degranulation and release of inflammatory mediators, such as histamine (Figure 13).⁹⁷

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More recently, alternative activation pathways involving sIgG have been described. Drugs can interact with sIgG from low-affinity receptors (FC γ RIII) or form drug-IgG immunocomplexes, which can also trigger de activation and degranulation of basophils, mast cells and neutrophils. Nevertheless, this mechanism is mainly produced with high doses of drugs, and most studies have been conducted in mouse models, so further studies are needed to validate this model in humans.

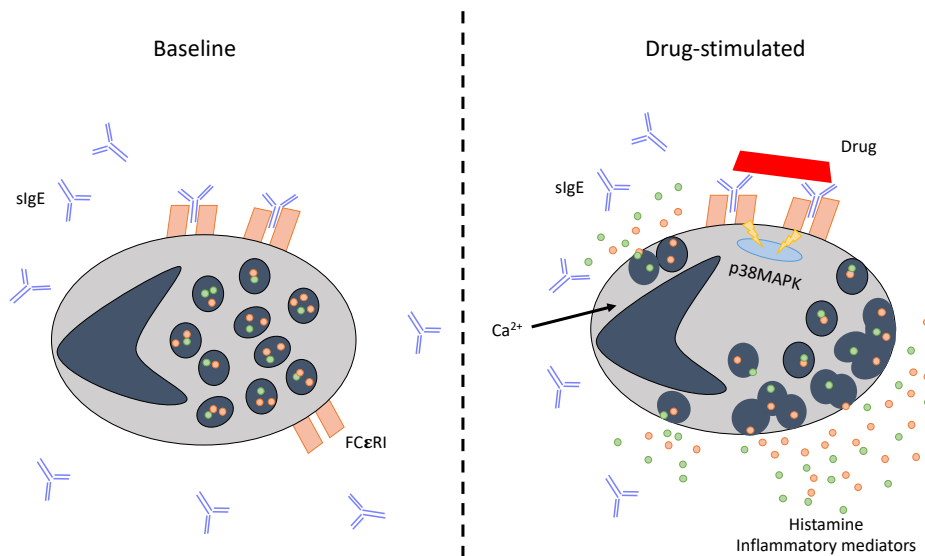


Figure 13. Effector phase in IDHRs

4. IMMUNOLOGICAL MECHANISMS OF NIDHRs

4.1. Sensitisation

The sensitisation phase in NIDHRs is not as well studied as in IDHRs. Drug recognition, processing, and presentation by DCs may be similar to the one described for IDHRs. The main difference between NIDHRs and IDHRs is that DCs in the formers promote the differentiation of naïve T cells into CD4⁺Th1 cells, rather than CD4⁺Th2, characterised by the release of cytokines such as IL-2, IL-12, TNF- α , or IFN- γ (Figure 14).⁴⁰ Nevertheless, depending on the clinical entity, the involvement of different T cell populations, such as CD4⁺Th2, CD4⁺Th17, or CD8⁺T cells has been also demonstrated. This great diversity makes the study of these reactions a complex process. In addition, due to the different drug recognition mechanisms (hapten, p-i, or peptide altered model), different drugs may interact with APCs, or directly with T-cell populations, with

or without the need of processing or presentation steps, making it difficult to understand the specific immunological mechanism involved.

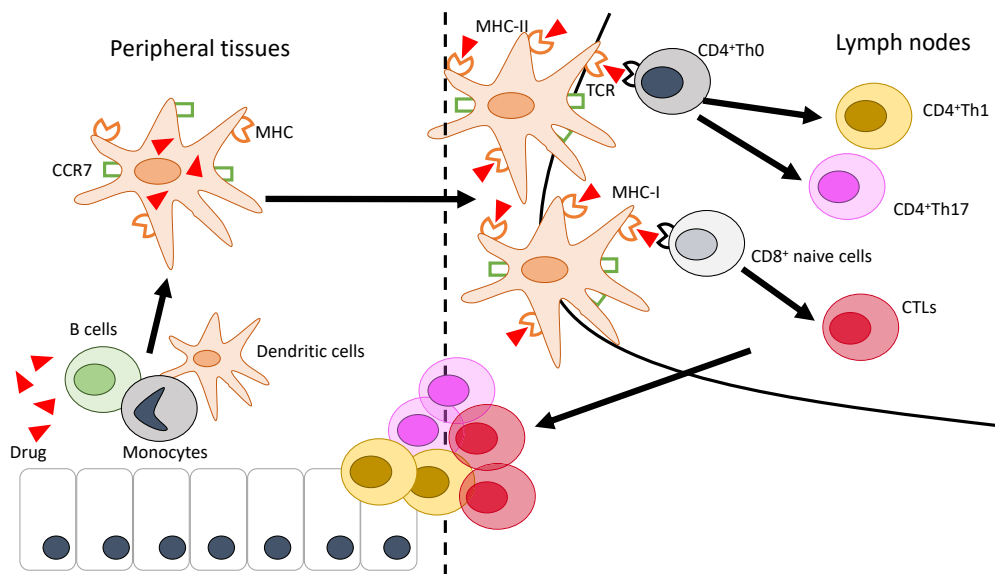


Figure 14. Sensitisation phase in NIDHRs

4.2. Effector phase

Following drug sensitisation, with re-exposure, drug-specific $CD4^+$ and $CD8^+$ T cells are activated and mainly migrate to the skin, the most frequent target organ in NIDHRs, through specific homing receptors, such as cutaneous lymphocyte antigen (CLA), CCR10, CCR6, and CXCR3, attracted by the release of their ligands, such as e-selectin, CCL27, CCL20, CXCL9, and CXCL10 into the skin (Figure 15).⁵⁵ In the skin, activated T cells, mainly cytotoxic T lymphocytes (CTLs), $CD4^+Th1$, $CD4^+Th17$, and $CD4^+Th2$ cells release a large number of cytokines and mediators such as IL-12, IFN- γ , TNF- α , or IL-17, which promote the recruitment of activated T cells, CTLs, monocytes, DCs, NK cells neutrophils or eosinophils, responsible for inflammation or massive keratinocytes death through the release of cytotoxic mediators such as perforin, granzyme B and granulysin.^{14,40}

The fact that patients previously sensitised to a concrete drug can react against the same drugs years after sensitisation may suggest that drug specific tissue resident memory T cells (T_{rm}) persist for long time in tissues.⁹⁸ T_{rm} are found in a wide variety of peripheral tissues, including the skin, and are characteristic for being not recirculating.⁹⁹ It has been proposed that T_{rm} are derived from circulating precursor cells,¹⁰⁰ which migrate to tissues through CXCR3 ligands. In the skin, these cells rapidly express CD69,

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which promotes their retention in tissues.¹⁰⁰ Other markers such as CD103 are also related to the permanence in tissues and cell survival, only expressed on T_{rm} , but not on circulating T cells.^{99,100} On this basis, during acute phase of DRESS an increased number of both $CD4^+$ and $CD8^+$ T cells were found in affected skin, and after resolution, the frequency of $CD69^+$ and $CD103^+$ expressing T cells in the skin increased significantly,⁹⁸ supporting the presence of stable non-recirculating population mainly composed by $CD4^+$ cells.⁹⁸

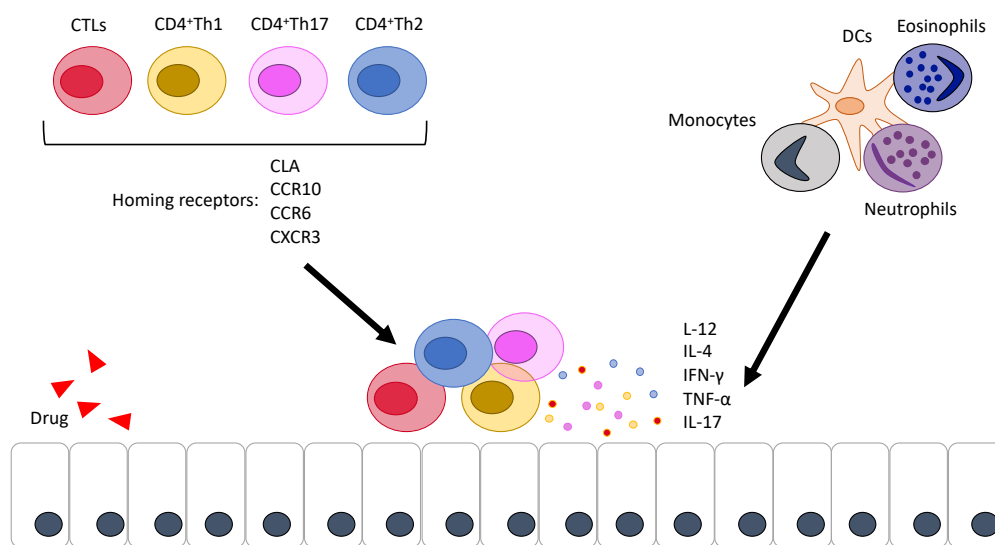


Figure 15. Effector phase in NIDHRs

5. RISK FACTORS

The reason why only some subjects become sensitised to a drug and develop a DHR, whereas others, after the administration of the same drug, tolerate it, as well as the reason why some patients develop an IDHR and others a NIDHR remain unknown. Some factors have been associated with an increased risk to suffer a DRHs, such as the altered role of T and B regulatory cells (Tregs and Bregs), genetic predisposition, early exposure to antibiotics, history of family DHRs, physiological characteristics such as gender or age and environmental factors (Figure 16).



Figure 16. Risk factor associated with suffering a DHR.

5.1. T and B regulatory cells

Tregs and Bregs, are important cell populations involved in the tolerance response to drugs and antigens. Tregs are mainly characterised as CD4⁺CD127_{low}CD25⁺FoxP3⁺ cells and can modulate the response of immune effector cells, through the expression of some receptors and molecules on their surface such as ICOS, CTLA-4, PD-1, and PD-L1 and through the release of cytokines and mediators such as IL-10, IL-35, Gal-9, or TGFβ (Figure 17). Dysregulation in terms of numbers or mechanism of regulatory cells impairs the control of inflammatory responses,¹⁰¹ as it has been associated with DHRs.^{42,52,102} In relation to this, several studies have observed altered activity of Tregs during DRESS or SJS/TEN, so that impaired control of effector T cells may trigger the development of DHRs.¹⁴ Moreover, patients with human immunodeficiency virus (HIV) appear to be more predisposed to DHRs, probably because these patients also present significant Treg defects.⁴² Bregs are an heterogeneous group of cells which may also be important in regulating the tolerogenic response to drugs and allergens, through the expression of surface molecules such as CD40, FAS-L, CD80/CD86, PD-1, and PD-1L, and by the release of IL-10, IL-35, or TGFβ.¹⁰³ They promote the induction of Tregs, cell apoptosis and exhaustion, as well as by decreasing the activity of DCs and monocytes, T and B cell activation, or cytokine production (Figure 17).

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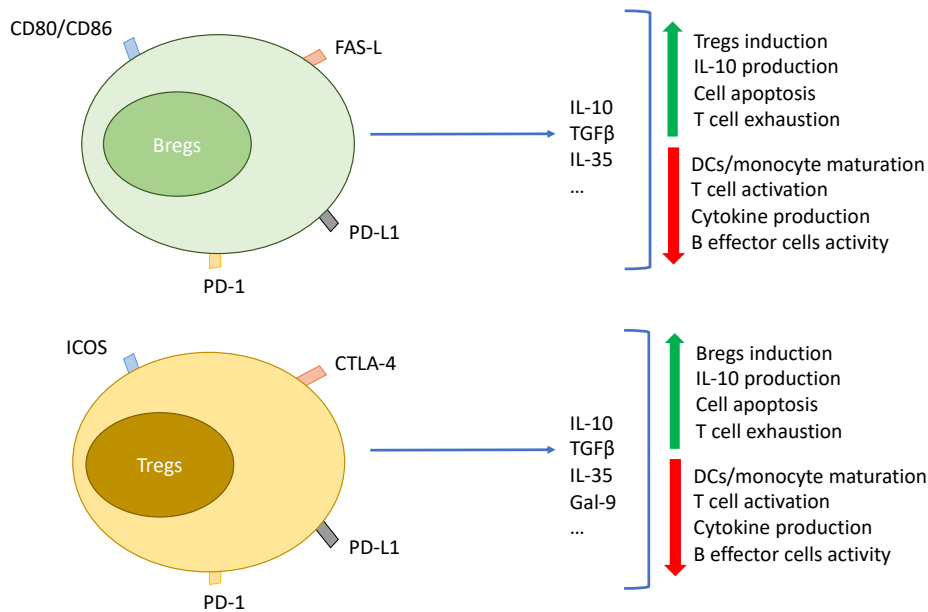


Figure 17. Role of T and B regulatory cells in DHRs.

5.2. Genetic factors

It is well known that some genetic factors are associated with an increased risk for developing DHRs. Many studies have attempted to address this complex and difficult issue, by conduction genome-wide associations research.¹⁰⁴ Thanks to the advance in technology, it is now possible to detect more than 500,000 single nucleotide polymorphisms.¹⁰⁴ Moreover, transcriptomic analysis are also useful for identifying candidate genes associated to specific pathomechanisms in patients with DHRs.¹⁰⁴

Most studies have focused on the study of HLA variants in severe NIDHRs.^{40,105} This high polymorphic system plays a central role in drug recognition and presentation to T cells.⁵¹ Some of the most representative associations between HLA variants and NIDHRs are presented in the Table 4

Allele	Drug	DHR	Population	Size	References
HLA-B*57:01	Carbamazepine	SJS/TEN	Europeans	28	86
	Abacavir	NIDHRs	North Americans	18	106
HLA-A*31:01	Carbamazepine	DRESS	Europeans	10	86
HLA-B*58:01	Allopurinol	NIDHRs	Han Chinese	51	107
		SJS/TEN	Japanese	58	108
		SJS/TEN	Thai	27	109
		SJS/TEN	Europeans	31	110
HLA-A*32:01	Vancomycin	DRESS	North Americans	23	75
HLA-A*24:02	Lamotrigine	MPE	Koreans	50	111
		NIDHRs	Norwegians	86	112

Table 4. Relevant HLA variants and its association with NIDHRs

Although most genetic associations have been made between severe NIDHRs and HLA variants, some studies have attempted to make associations between genetic variants and an increased predisposition to IDHRs. These associations are linked to genes related to antigen presentation, class switch recombination, or IgE receptors on effector cells.^{104,113,114} Moreover, associations between HLA variants or gene polymorphisms and intolerance to SNAIDs have also been made.^{113,115-117}

Despite many efforts to address this issue, genetic predisposing to suffer a DHR cannot exclusively explain a drug sensitisation, mainly because most patients who develop a DHR do not have a strong genetic predisposition. Additionally, only few patients who have the specific allelic variant and are exposure to the specific drug, develop a DHR, suggesting that other factors might be involved.^{106,111,118,119}

Because of the variability of HLA molecules, the small number of patients included in genetic studies, and the relevant differences in frequency according to ethnic populations, make these studies very difficult and, in some cases, controversial, as the high risk in an ethnic population cannot extend these findings to other ethnicities.^{45,105-107,120} Because of this, more and extensive studies are required to confirm these data.

5.3. Family history of DHRs

It has long been believed that the presence of relatives with DHRs predisposes to a similar reaction, although no clear reasons or mechanisms have been proposed. Related to this, two siblings with DHR to allopurinol has been described, suggesting that

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familiar genetic predisposition might be possible.¹²¹ Nevertheless, other studies have shown that there is not such positive association, at least for DHRs.¹⁵

5.4. Early exposure to antibiotics in children

In children, early exposure to antibiotics during the first 2 years of life increased the risk of fever, eczema, or allergies.¹²² This could be explained by the fact that antibiotics administration affects gut microbiota of children, which may influence in increased risk to suffer allergies.¹²³ However, other factors such as the exposure to protective factors like breastfeeding may also influence this susceptibility.¹²² The early use of antibiotics is a cause of concern, not only because of the increased incidence of DHRs, but also because the emergence of mutations and antibiotic resistance. In this regard, antibiotics administration to hospitalised neonates was of 34.9%, ranging from 14.2% in Africa, and 68% in South America, compared to children (28.3%), from 14.5% in Africa to 48.9% in South America. The parental administration is the most frequent via of administration, in Asia (88%), South America (81%), and in Europe (67%).¹²⁴ This gives us an idea of the importance of the rational administration of antibiotics in infants.

5.5. Gender and age

Higher rate of ANA appears to be related to gender, being women more susceptible to develop an ANA, which can be explained by the higher drug consumption, or immune cells-hormones interaction,¹²⁵ although controversy exists regarding this point.⁵⁶ Older people have also been implicated with a higher frequency of drug-induced ANA, an also a higher risk of having more severe reactions,¹²⁶ mainly related to comorbidities and the use of great number of drugs.^{56,125}

5.6. Environmental factors

Although not exclusively about DHRs, it is important to highlight the high prevalence of allergic diseases in the Western World. Factors that can explain this high incidence are related to the environment, food, pollution, or exposure to allergens. In the Western World, the exposure to pathogens has been reduced, while the exposure to chemicals has been increasing, mainly during the childhood (hygiene hypothesis). This

may reduce the natural stimulation of toll like receptors (TLRs), leading to a weaker immune response, as well as imbalance between Th1 and Th2 responses.¹⁰⁴ Simultaneously, the use of detergents and chemicals can trigger the damage and disruption of the epithelial barrier, which has an essential role preventing allergies.

6. DIAGNOSIS OF DHRs

The diagnosis of DHRs is difficult, mainly due to multiple factors, such as the fact that clinical history is often poorly documented, the reaction may have occurred a long time ago, almost any drug can trigger a DHR, and a wide variety of immunological mechanisms and cells involved, being necessary different and specific tests depending on each reaction.⁹⁷ Moreover, in some cases, the concrete epitope responsible for the reaction is unknown, and the combination of different drugs (comedication), and comorbidity are also common.⁹⁷ Therefore, there is a lack of consensus on diagnostic procedures,⁵ although many efforts have been made in recent years to reach an agreement on diagnostic procedures for DHRs. Nevertheless, an accurate diagnosis is essential for the future management of the patients to make accurate drug prescriptions and recommendations. Moreover, about 10% of the general population self-reported a DHR, although only between 5% and 15.9% are confirmed by diagnostic procedures.^{9,22} Similarly, less than 10% of children with cutaneous reactions are truly confirmed as allergic.^{19,20} This overdiagnosis is detrimental and associated with higher costs, worse side effects due to second-line treatments, higher morbidity, and mortality.^{9,22} Therefore, both overdiagnosis and underdiagnosis have important implications for modifying the real incidence of DHRs, reducing the treatment options and increasing associated costs, or increasing the risk for mislabelled patients, mainly those with severe DHRs.¹²⁵

The first step in the diagnostic procedure is the study of the clinical history, regardless of the reaction or mechanism involved.^{6,31,127} It is essential for clinicians to decide the most appropriate diagnostic tests to perform, or if necessary, the reintroduction of the drug.⁶ During this first phase, different aspects of the clinical history should be evaluated, such as the drug/drugs involved, the time between the reaction and the onset of symptoms, and between the reaction and the study, organs involved, presence of comedication, or previous sensitisation to the culprit drug.⁶ After

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careful study of the clinical history, different available *in vivo* and/or *in vitro* tests can be selected. It is important to differentiate between IDHRs and NIDHRs, as the best biomarker is different depending on the immunological mechanism involved (Table 5). In the following sections we will discuss the most commonly used and validated *in vivo* and *in vitro* test for the diagnosis of DHRs.¹⁹

IDHRs	NIDHRs
slgE	Drug-Specific cell populations % Cell subpopulations
Basophil activation markers ◆ CD203c ◆ CD63	Cytokine-producing cells
Inflammatory mediators ◆ Histamine ◆ Chymase ◆ Tryptase Lipid mediators ◆ PAF ◆ LTE ◆ Periostin Cytokines ◆ IL-4 ◆ IL-13	Cytokines ◆ IFN-γ ◆ IL-17 ◆ IL-2 ◆ IL-4 ◆ IL-15 ◆ IL-12 Chemokines ◆ CCL27 ◆ CCL20 ◆ CXCL8 ◆ Eotaxin Cytotoxic markers ◆ Perforin ◆ Granzyme B ◆ Granulysin
Genetic biomarkers ◆ HLA variants ◆ GNAI2 variants	Genetic biomarkers ◆ HLA variants ◆ Polymorphisms
Gene expression ◆ Periostin ◆ CNKSR ◆ SPTBN2 ◆ ADORA3	Post-transcriptomics regulators ◆ mRNA

Table 5. Biomarkers in IDHRs and NIDHRs. Modified from Ogulur *et al.* 2021.¹²⁸

6.1. *In vivo* tests

Following the European Network on Drug Allergy (ENDA) recommendations, the general procedure for evaluating DHRs should be the study of the clinical history, followed by the performance of different *in vivo* tests (Table 6).

<i>In vivo</i> tests
Skin prick test (SPT)
Patch test (PT)
Intradermal test (IDT)
Drug provocation tests (DPT)

Table 6. *In vivo* tests for the diagnosis of DHRs.

From the available *in vivo* test for evaluating DHRs, STs are the most used and validated tests involved in the diagnosis of DHRs. Among them, STs can be divided into skin prick test (SPT), patch test (PT), and intradermal test (IDT), based on the different ways of drugs exposure.

6.1.1. SPT

This test consists of the application of the drug barely into the skin, using lancets, and is mainly performed for evaluating IDHRs. After 10/15min from the drug exposure, readings to detect the appearance of wheals caused by the culprit drug are done. A negative test with saline and histamine as positive control should always be performed. The papules formed from each drug should be compared with the histamine control, and when the diameter is at least half the histamine papule, the result is considered positive. One of the main advantages of SPT is that it is considered safer than other *in vivo* techniques such as IDT, although SPT also shows lower sensitivity.¹²⁹

6.1.2. PT

PT is mainly performed for the evaluation of NIDHRs. It consists of using commercialised drug compounds, dissolved in appropriate solvent, mainly petrolatum,¹³⁰ and applying it on the back of patients for 48 hours. Readings are done

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then and after another 24h and 48h. However, patients should be warned about possible reactions several days after the last reading.⁶⁸

Similarly, to SPT, PT can be performed with any commercially available drug,⁵⁵ and it is considered a safe technique. In contrast, PT sensitivity is lower than IDT,¹³⁰ ranging from 10.8 to 37.5%,^{68,131} mainly because of the poor penetration of the drug through the skin, the not inclusion of a drug metabolites which caused the reactions, concomitant factors, or the vehicle used.^{53,68} Specifically, the sensitivity of PT to BP was of 7.5%, and of 37.3% for AX and AMP, while in children the sensitivity is even lower (5.9%).³⁷

6.1.3. IDT

IDT consists of injecting intradermal solution of the drug. The reading should be done immediately and between 24 and 72 hours after the test, because it is useful to evaluate both IDHRs and NIDHRs.⁶⁸ Nevertheless, reactions after 72 hours can occur, so additional readings are recommended. The appearance of an erythema with a diameter greater than 5mm is considered a positive result. In addition, the morphological description of the erythema should be taken into consideration.

IDT requires injectable form of the drug, not available for most drugs. Moreover, there is a lack of detailed and validated protocols.^{129,132} The ideal drug concentration is the highest possible without causing skin irritation. The need to use such high concentration can lead to false-positive reactions.¹²⁹ There is the possibility that some drugs can be irritants, with false positivity being relatively frequent. This is the case of NMBAs, because of this, each drug must be tested at different concentrations in healthy donors to establish the maximum non-reactive concentration of the drug that can be used in IDT.¹³³

The sensitivity of IDT seems to be higher than SPT or PT, although IDT can trigger potential side effects, and may be not recommended in severe NIDHRs.^{53,68} Because of this, SPT or PT are usually performed first, and if negative, IDT is performed. Nevertheless, a study carried out by Trubiano and colleagues in 2019 showed that IDT represents a safe procedure, at least as safe as PT for evaluating severe NIDHRs to different antibiotics, although the sample size was too small to obtain definitive conclusions.¹³²

For BLs, the use of penicilloyl-polylysine (PPL) was a major breakthrough for the diagnosis of DHRs by STs. Subsequently, the inclusion of minor determinants of penicillin (MDM) was also helpful to increase sensitivity of STs, although they are not available in all countries. The full panel of penicillin determinants, including PPL, MDM, and AX, appears to increase the sensitivity of ST by up to 15%. Nevertheless, since 2013, the inclusion of benzylpenicilloyl octa-L-lysine (BP-OL) as the major determinant and benzylpenilloate (penilloate) (DM) as the minor determinant have been included in STs in place of PPL and MDM, being the formers purer, more stable and with improved sensitivity and specificity.¹³⁴⁻¹³⁸ Moreover, in a study carried by Macy and colleagues in 2013 showed that a wheal bigger than 5 mm in BL-ST is more suitable than others using a wheal larger than 3mm, because it reduces the number of false positive tests.¹³⁹ In addition of these determinants, if possible, the inclusion of the suspicious concrete BL, such as CLV or the specific cephalosporins must be included.^{24,140}

Following ENDA recommendations, the combination of IDT and PT with PPL, MDM, BP, ampicillin (AMP), and AX besides the suspected drug must be performed.⁶⁸ For IDT, the sensitivity was of 12% for BP and MDM, and of 39% for AX and AMP, with 100% of specificity.¹⁴¹ The inclusion of minor determinants of AX, amoxicilloic acid, and diketopiperazine do not appear to increase the sensitivity found with AX itself.¹⁴²

The usefulness of IDT is controversial in children, due to the difficulty of performing it, and its low sensitivity and positive predicted value (PPV) in mild NIDHRs,^{19,34} most common type of reactions in children. A recent study reported that IDT is only useful to assess mild-NIDHRs in 2% of cases,³⁶ in line with previous studies, where no positive cases were detected in children with delayed IDT, although 14.5% has positive DPT.^{20,143}

The sensitivity of ST to BLs is in any case higher than 70%.⁵³ Since IDHRs caused by BLs frequently occur after the intake of the combination of AX and clavulanic acid (CLV), it is important to include this drug in the ST, especially when AX tests are negative. To cephalosporins, sensitivity ranges from 30-70%, with the need of including penicillins and other cephalosporins to rule out possible cross reactivity.⁵³ STs to pyrazolones, one of the most common drugs responsible for IgE-mediated reactions is around 50%.^{144,145} The number of positive cases for ICM detected by ST is of 62.5%.¹⁴⁶

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Because of the low negative predictive value of ST for evaluating DHRs, mainly NIDHRs, a negative result cannot rule out a reaction,¹⁴⁷ so it is necessary to use complementary confirmatory tests.⁴³ Conversely, if a patient tested positive, it is highly probable that the subject is truly sensitised to the drug. Furthermore, although not many studies have been conducted to determine the real value of ST, several research seem to indicate that the sensitivity of these tests for evaluating NIDHRs is much lower than previously thought.⁵⁵ In addition, systemic reactions after the performance of ST are possible,¹⁴⁸ so they should be carried out with caution.

6.1.4. DPT

This test consists of administering of a drug in increasing doses to the suspected patient. Because other *in vivo* and *in vitro* tests do not have an optimal sensitivity, or are not possible for all drugs, DPT is currently necessary, and considered the “gold standard”, essential sometimes to confirm the diagnosis or assessing the tolerance to related drugs.¹⁴⁶ For instance, it is estimated that up to 30% of patients cannot be confirmed as allergic to BLs if DPT is not performed.²⁴

The usual procedure is to perform a single-blind placebo controlled DPT, in which the drug is administrated in progressively increasing doses at 30/60 minutes intervals to evaluate IDHRs, and if well tolerated, continue until the full therapeutical doses of the drug is reached.²⁴ For NIDHRs, the concentration of the administrated drug should be increasing until reach 1/5 of therapeutical dose, then, if no symptoms appear, at least 48h later, increasing doses should be administered, until the therapeutic dose is reached.²⁴ Administration of the full dose for a few consecutive days may also be recommended, as NIDHRs can be affected by the cumulative effect of the drug.²⁴

Nevertheless, DPT is time-consuming and not risk exempt for patients,¹⁴⁹ and can only be performed in specialised centres with close monitoring by trained professionals, and it is not recommended for most severe reactions.¹²⁹ Therefore, it should be carried out after carefully balance the risk/benefit for each patient.¹²⁹

It is important to note that after a reaction, if the culprit drug is not administered again, the clearance of sIgE with time become *in vivo* and *in vitro* tests negative. Loss of sIgE can even allow drug administration in patients with previous negative ST and/or DPT. Nevertheless, the administration of the drug may result in a re-sensitisation which

is estimated to occur at a rate of 0 to 27.9%.¹⁵⁰ In a recent study, the re-sensitisation appears to occur in 21.3% of patients with IDHRs to BLs, mostly in ANA patients, with higher risk in older patients, and after the fifth week from the initial evaluation.¹⁵⁰ These data suggest the need to include retesting for evaluating IDHRs to BLs after an initial negative study.

ENDA guidelines indicate that DPT with NIDHRs to BLs should only be performed after a negative ST.^{141,151} However, a recent study carried out in 710 patients (children and adults) with NIDHRs to BLs demonstrated that DPT is a safe alternative for assessing mild/moderate NIDHRs to BLs, without the need to carry out previous STs with a 5-day challenge protocol.¹⁵²

In the case of children, DPT is even more important because of the low sensitivity and PPV of ST, although it is not risk-free and is time-consuming.³⁴ Moreover, since in most cases skin rashes in children during BL treatment are associated with viral infections, but not with NIDHRs, DPT is essential as confirmatory tool to avoid BLs-NIDHR overdiagnosis.^{20,37} Nevertheless, lack of consensus exists regarding the most suitable protocol to performance DPT in children.^{34,36,153} Several studies have demonstrated that single-dose DPT is a safe procedure, even though previous STs have not been performed in children with mild NIDHRs to BLs,^{19,20,34} although DPT should be performed as soon as possible from the original reaction.^{34,152} This new work-up might make the process easier and less time-consuming, while still being safe.¹⁹ Nevertheless, some concerns arise regarding the best protocol for the timing and number of doses, as there may be a possibility of not detecting all allergic subjects with short protocols.¹⁹ Nevertheless, most studies seem to indicate that performing DPT without prior ST in children with mild/moderate NIDHRs is a safe alternative.¹⁴³

6.2. *In vitro* tests

Because of the limited sensitivity of STs for evaluating DHRs, or the fact that they cannot be performed for all drugs, the use of DPT is necessary to confirm the DHR diagnosis or assess the good tolerance to other drugs. Indeed, it has been estimated that up to 5% of patients with IDHRs to BLs who tested negative in ST to PPL, BP, and MDM, have positive DPT. Due to this, the use of *in vitro* tests may represent useful alternatives to assess DHRs in a safe manner to reduce the need of performing DPT.¹⁵⁴ From the

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available *in vitro* tests, some of them are orientated to evaluate the acute phase of the reaction, while others are orientated to assess the responsible drug involved in the DHR (Table 7).

<i>In vitro</i> tests	
Tests for assessing the acute phase	Tryptase and inflammatory mediators
	Immunohistochemistry
Tests for assessing the responsible drug	Serum specific immunoglobulin E (sIgE) determination
	Basophil activation test (BAT).
	Lymphocyte Transformation Test (LTT)
	Cytokine release
	Enzyme-linked Immunospot Assay (ELISpot).
	Cell markers upregulation.

Table 7. *In vitro* tests for the diagnosis of DHRs.

6.2.1. Tests for assessing the acute phase

6.2.1.1. Tryptase and inflammatory mediators

Tryptase is one of the most commonly used biomarkers to confirm a diagnosis of ANA.¹⁵⁵ Nevertheless, samples must be collected during the reaction, and compared with normal levels at baseline, which makes sample obtaining difficult in most cases. The peak of serum-tryptase after ANA varies from 0.5 to 2 hours after the reaction. Afterwards, tryptase levels decrease rapidly.⁶ Similarly, histamine can also be used, since it is highly increased in the acute phase. But like tryptase, it decreases rapidly within the first hour from the reaction. To note, 43.3% of patients with IDHRs referred to the emergency department showed higher levels of plasma histamine (>10 nmol/L), and 20.6% of them with high levels of total serum tryptase.¹⁵⁶

Higher histamine levels are associated with URT, exanthemas, and wheezing, whereas higher levels of histamine are associated only with patients with URT.¹⁵⁶ Other serum biomarkers, such as PAF, can also be measured during acute phase. These mediators, together with prostaglandin D₂ can also be measured in urine samples, up to 24 hours from the reaction to assess basophil and mast cells degranulation.⁶

6.2.1.2. Immunohistochemistry

Because of the skin is the organ most commonly involved in NIDHRs, determining the specific cell populations involved in the reaction can help to better understand the mechanism involved or discriminate between the different type of reactions.^{98,157} However, immunohistochemistry does not give any information about the drug involved.⁵⁵ Furthermore, acute phase samples are required, which makes this technique difficult in most cases, mainly because the allergic study is usually performed during the resolution phase of the disease. For instance, Salas and colleagues found that infiltrates of CXCR3⁺ T cells, are a characteristic marker of Th1 response associated with MPE to CLV.¹⁵⁸ Hari and colleagues demonstrated that elevated number on CD4⁺T cells was observed in MPE lesions, whereas in bullous diseases, such as AGEP, mainly and exclusively CD8⁺T cells were observed in skin lesions.¹⁵⁹ It is believed that CD8⁺T cell-mediated reactions may be more severe than the ones mediated by CD4⁺T cells such as MPE, mainly because of the greater cytotoxic potential of CD8⁺T cells.

6.2.2. Tests for assessing the responsible drug involved

6.2.2.1. Serum specific immunoglobulin E (sIgE) determination

The measurement of serum sIgE by immunoassays is the most widely used and validated *in vitro* test to study IDHRs.⁵³ Despite the fact that it has been mainly used to detect the presence of serum sIgE to proteins and macromolecules, it can also be used to measure sIgE to drugs.⁶ Nevertheless, due to the low concentration of drug-sIgE in blood, immunoassays for its detections must have excellent sensitivity.¹²⁹

Because of the low molecular size of drugs, they have to be conjugated to carriers, such as poly-L-lysine (PLL) and the drug-carrier conjugates are coupled to solid phases. After incubating serum with these solid phases, drug-sIgE recognises the drug-carrier conjugates, and can be detected by using a secondary human anti-IgE, with a radioisotope (RIA), or fluorescent enzyme (FEIA).¹²⁹

Among commercially available immunoassays, the FEIA ImmunoCAP® is the most validated for the detection of sIgE. However, it is only available for a few numbers of drugs, mainly for BLs, cephalosporins, NMBAs, and opiates.

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ImmunoCAP® has been widely used mainly for the evaluation of IDHRs to BLs,³¹ although with good specificity, its sensitivity is not optimal, ranging from 0% to 52.4% in function of the specific drug involved.^{31,160,161} Moreover, the number of false positive with penicillin suggests that ImmunoCAP® for the detection of BP-sIgE has a reduced diagnostic value due to its low sensitivity and limited specificity.¹⁶⁰ The selectivity of the patients is also an important factor in the sensitivity of immunoassays being much higher for cross reactors, compared to AX selective patients.¹⁶² In addition to the limited sensitivity, it is only available for some drugs and, for instance, the detection of sIgE to CLV is not possible.¹⁵⁸

For NMBA, the detection of sIgE to quaternary ammonium morphine (QAM) by ImmunoCAP® is possible, with an overall sensitivity of 84.2% and 94.6% of specificity.¹⁶³ For morphine and mixtures containing pholcodine (PHO) the ImmunoCAP® sensitivity ranges from 65% to 68%, whereas for suxamethonium, the sensitivity was of only 39%.¹⁶⁴

On the contrary, radioimmunoassay (RIA) is an in-house method, which although useful due to its versatility, the lack of standardisation, as well as the use of radiolabelled reagents have made this methodology fall into disuse for diagnostic purposes. For BLs, the sensitivity ranges from 42.9% to 75%, although its specificity is not good enough (66.7%-83.3%).¹⁶¹

For the detection of sIgE to FQs, only in-house methods are available, mainly by conjugation of FQ with Sepharose as solid phase. Sensitivity ranges from 31.57% to 54.5%,^{165,166} with high variability between drugs,¹⁶⁵ although only a low number of studies have been performed to evaluate it, and few data are available regarding the specificity of the test. Nevertheless, since FQs and NMBAs can trigger the release of inflammatory mediators from the direct interaction with basophils and mast cells, the detection of sIgE to these drugs is important to differentiate the immune mechanism involved in each reaction. For other drugs such as pyrazolones, no detectable sIgE was observed.¹⁴⁵ Sensitivity and specificity of both RIA and FEIA immunoassays with different drugs are presented in Table 8.

In recent years, advances in the field of nanotechnology may indicate that the use of nanomaterials, such as nanoparticles, can be efficiently conjugated with different drug-carriers, such as dendrimers, which may increase the sensitivity and specificity of

immunoassays for the detection of serum-sIgE.^{167,168} In a recent study carried out by Gil-Ocaña and colleagues, it was shown that the use of BLs-dendrimers coupled to magnetic nanoparticles have as much sensitivity as the one found with ImmunoCAP® for the detection of AX-sIgE.¹⁶⁸ Moreover, it also improved significantly the sensitivity and specificity for the detection of benzylpenicillin (BP)-sIgE, allowing differentiation between selective and cross reactors patients, which is not possible with sufficient sensitivity and specificity with ImmunoCAP®.¹⁶⁸

Drug Family	Technique	Sensitivity	Specificity	References
BLs/Ceph	FEIA	0%-52.4%	83.3%-100%	160-162
NMBA	FEIA	84.2%	94.6%	163
Opioids	FEIA	39%-68%	-	164
BLs	RIA	42.9%-75%	66.7%-83.3%	161
FQs	RIA	31.57%-54.5%	-	165,166

Table 8. Sensitivity and specificity of sIgE determination. Abbreviations: BL, betalactam; Ceph, cephalosporin; FEIA, fluorescent enzyme immunoassay; FQ, fluoroquinolone; NMBA, neuromuscular blocking agents; RIA, radioimmunoassay.

6.2.2.2. Basophil activation test (BAT)

During last years, BAT has been demonstrating a reliable *in vitro* technique to study IDHRs.¹⁶⁹ Despite basophils being a cell population with low frequency in blood (0.2-1% of peripheral blood leukocytes),^{58,154} the use of a very sensitive technology, flow cytometry, allows the detection of enough events to obtain representative samples.¹⁶⁹ The technique consists of the *in vitro* stimulation of basophils with suspected drugs, to later measure activation markers expressed on the basophil surface as a consequence of their activation (Figure 18). This test has been widely used for evaluating IgE-mediated hypersensitivity reactions, and it has shown be useful when no other *in vitro tests* are available.

Different approaches have been used to identify basophils. Anti-IgE has traditionally used,^{144,170} because IgE is very abundant on basophil surface.¹⁵⁴ Nevertheless, as it is not specific for basophils, and due to its high variability between subjects, it is not currently used. Therefore, other more suitable basophil identification markers can be included such as CRTH2, CCR3,¹⁷¹ CD203c, or the combination between them.¹²⁹

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Two different pathways of basophil degranulation can occur, which are closely related to the severity of the reaction.⁵⁴ Anaphylactic degranulation is based on the fusion of histamine-containing granules with the plasma membrane, with the subsequent release of histamine and other inflammatory mediators. CD63 is the most validated activation marker,¹⁷¹ related to the anaphylactic degranulation (Figure 18).^{144,172} This 52kDa protein is expressed on plasmatic granules of resting basophils, and after degranulation, this molecule is highly expressed on cellular surface, being able to assess basophil activation.^{97,144,154} Piecemeal degranulation consists of the formation of small vesicles from histamine-containing granules, which rapidly fuse with basophil plasma membrane. This mechanism has been associated with the upregulation of CD203c (Figure 18), and with the most severe and rapid reactions such as AS.^{54,172}

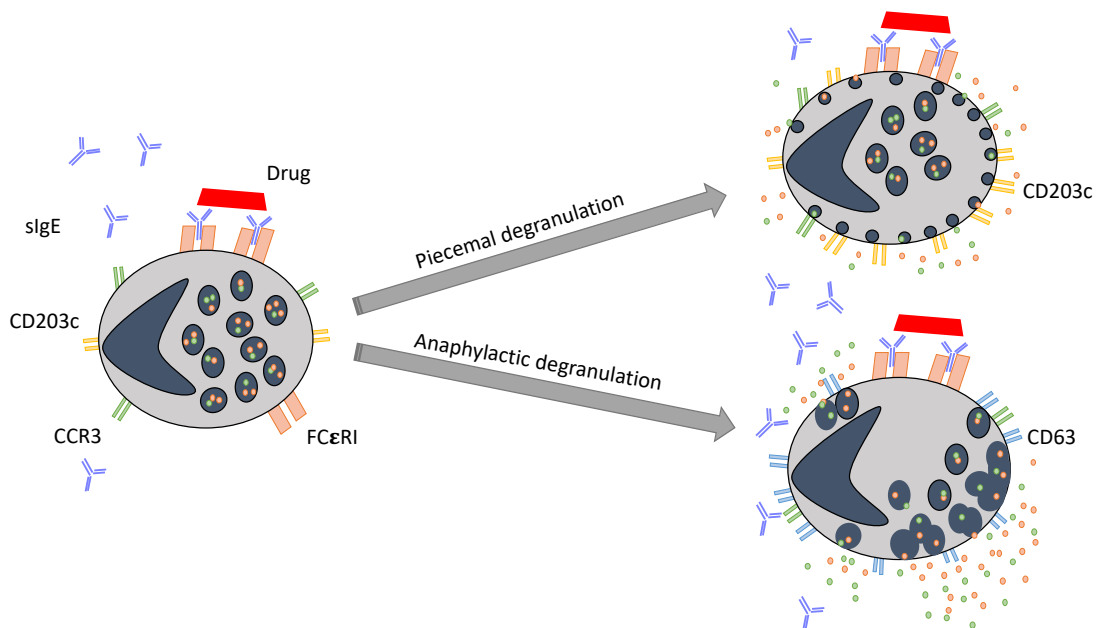


Figure 18. Basophil degranulation pathways.

An important advantage of BAT compared to other *in vitro* tests, is that BAT can be performed with both, conjugated and free drugs, so almost all drugs, as well as drug-specific antigenic determinants can be included and evaluated as soon as they are described.¹⁶⁹ Moreover, this technique is even more important when other *in vitro* tests are not available, as in the case of CLV-sIgE detection.¹²⁹ However, unlike food and inhalant allergens, activation of basophils with drugs is much less pronounced, so that, although with good specificity (80-100%), its overall sensitivity is not optimal.

The usefulness of BAT is highly dependent of the specific drug involved in the reaction (Table 8). For BLs, sensitivity ranges from 48.6% to 50% with good specificity (80%-93.3%).^{154,162,171} BAT is a good alternative for evaluating IDHRs to CLV, because the detection of serum-sIgE is not possible, and the positive BAT results can be considered an indirect way to measure the presence of CLV-sIgE,¹⁵⁸ mainly when wortmannin (WTM), a potent inhibitor of the IgE signalling (phosphoinositide 3-kinase enzymes) demonstrated that the pathway is IgE mediated, and that the basophil activation is not triggered by the pharmacologic feature of the drug.¹⁵⁸ Nevertheless, its sensitivity is not optimal (41%), but the inclusion of different CLV metabolites can help to improve BAT sensitivity up to 69% with 88% of specificity.¹⁷³ It is important to perform the test as soon as possible from the reaction, as it has been demonstrated for BAT with AX, which become negative earlier than other *in vitro* test such as immunoassays.¹⁷⁴

For NMBAs, sensitivity ranges from 36.1% to 64% with a specificity between 93% and 100%.^{175,176} Concretely to rocuronium, BAT showed a sensitivity of 91.7% with 100% of specificity.¹⁷⁵ In addition, BAT with NMBAs is as reliable technique to identify cross-reactivity between NMBAs, in contrast to other *in vivo* tests.¹⁷⁵

In the case of NSAIDs, two different mechanism of basophils activation have been established: immunological and non-immunological mediated, being the latter the most common,^{97,177,178} although controversy exists about the usefulness of BAT in the evaluation of non-immune mediated reactions to NSAIDs.¹⁷⁷⁻¹⁷⁹

It has also been shown that some NSAIDs can trigger sIgE-mediated reactions, such as pyrazolones,¹⁴⁵ and specifically, metamizole, with a sensitivity of 37.5%-52.4%, and a specificity close to 100%.^{95,180}

BAT with FQs reported sensitivities between 17.9% to 88.3% depending on the drug, with 88.9-94.4% of specificity.¹⁸¹ Importantly, drug photodegradation should be taken into consideration.¹⁸¹ Furthermore, moxifloxacin appears to induce preferentially the expression of CD203c after stimulation, whereas ciprofloxacin induces the upregulation of CD63.¹⁷² The upregulation of one activation marker or the other is not only influenced by the drug but also by the severity of the reaction, being AS more associated with CD203c upregulation, and ANA with CD63 expression.¹⁷²

Specific clinical entities, the immunological mechanism involved, the nature of the drug, the presence of metabolites derived from drug degradation, the time interval

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between the reaction and the test, differences regarding methodologies and technical issues may explain differences in the usefulness of BAT for evaluating IDHRs between studies. Nevertheless, big effort has been doing in recent years from the European Academy of Allergy and Clinical Immunology (EAACI) to homogeneity procedures and results interpretation, essential for the clinical validation of BAT in the drug allergy field.

Drug family	Sensitivity	Specificity	Reference
NSAIDs*	11.7%-52.4%	74.1%-100%	177-179
NSAIDs	37.5%-54.9%	85.71%-100%	95,180
NMBA	36.1%-91.7%	93%-100%	175,176
BLs	48.6%-55%	80%-91.3%	162,171
FQs	17.9%-88.3%	88.9%-94.4%	172,181
ICM	62.5%	100%	146

Table 9. Sensitivity and specificity of BAT. *Non-allergic reactions. Abbreviations: BL, betalactam; FQ, fluoroquinolone; ICM, iodinated contrast media; NMBA, neuromuscular blocking agent; NSAID, Non-steroidal anti-inflammatory drug.

Traditionally, before the advent of flow cytometry, other related techniques were evaluated. They consist of PBMCs stimulation with the culprit drugs. Because of drug stimulation, basophils degranulate and release great variety of inflammatory mediators. The quantification of such mediators could be important biomarkers to be taken into consideration for diagnostic procedures, nevertheless, the low number of studies, and lack of standardisation and that no commercial kits available make difficult determining their potential value for the diagnostic routine.

The utility of the cysteinyl-leukotriene release test (CAST), consist of the stimulation of leukocytes with the culprit drugs, and measure the release of leukotrienes, mainly evaluated by NSAIDs,¹⁸²⁻¹⁸⁴ and BLs, together with histamine release test (HRT).^{149,185} However, with the advent of flow cytometry, a much more sensitive and powerful technique, these assays have fallen into disuse.

6.2.2.3. Lymphocyte transformation test (LTT)

The most widely used and validated *in vitro* assay for evaluating NIDHRs is LTT, although it can be used also for evaluating IDHRs.¹⁸⁶ It consists of stimulating lymphocytes with the suspected drugs, and analysing their specific proliferation.¹²⁹ It is

based on the assumption that although most subjects were exposure to drugs, only T cells from of those with hypersensitivity reactions will proliferate as a result of drug simulation (Figure 19).⁴³

The proliferation can be revealed by determining the thymidine incorporated into the genome during cell division, so the inclusion of tritiated thymidine (^3H -thymidine) can be used to assess the proliferative response by measuring the radioactivity in a liquid scintillation counter system.^{43,129} This method has traditionally been used.⁴³ More recently, to avoid the use of radioactivity, ^3H -thymidine has been substituted by fluorescent molecules such as carboxyfluorescein succinimidyl ester (CFSE) that can be measured by flow cytometric methods. CFSE binds to intracellular lysine residues, and other amine groups through its succinimidyl group. CFSE is highly stable, after labelling, if lymphocyte proliferation occurs, the fluorescent signal drops by half at each cell division. This method shows a high sensitivity, and due to the safety of not using radioactivity, and the possibility to identify and assess the proliferation of different cell populations have made this method a good alternative. Nevertheless, there is a lack of studies comparing both methodologies.

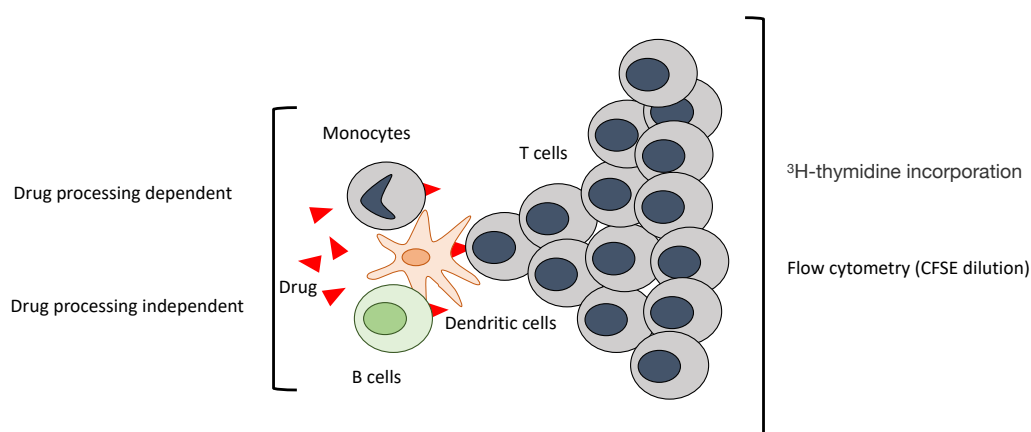


Figure 19. Principle of LTT

The sensitivity of LTT for BLs, ranged between 33.3% to 63.7% in mild NIDHRs such as MPE and URT/ANG in children with a specificity higher than 90%.^{34,36} Interestingly, differences between BLs were observed, as sensitivity of LTT for AX was of 63%, while for CLV was of only 33%.³⁴ These data suggest that although a negative LTT result cannot exclude an NIDHRs, its high PPV may indicate a true reaction if the test is

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positive.³⁶ The sensitivity of LTT values are similar in adults (48.6%-55%) with a specificity ranging from 80% to 91.3% (Table 10).^{162,171}

LTT for sulphonamides showed a sensitivity of 75% with 100% of specificity, although with a high variability of clinical entities and drugs tested,¹⁸⁷ similarly to other study (67%) with variable drugs and clinical entities.¹⁵⁹ In another study, the sensitivity of LTT for MPE to different drugs, mainly BLs and NMBAs was 75%, with 100% of specificity.¹⁸⁸ In a study with NIDHRs to antituberculosis drugs, only an overall sensitive of 14.9% was obtained (Table 10).¹⁸⁹

For a long time, LTT has been thought to be more sensitive for evaluating mild/moderate reactions. Nevertheless, more recent studies suggested that LTT also has a good sensitivity for evaluating severe reaction.^{190,191} Confirming this would be of great importance, as the use of other tests for evaluating severe reactions are much more limited than for mild/moderate reactions.

Family	Clinical entities	Method	Sensitivity	Specificity	Reference
BLs	MPE, URT/ANG	CFSE dilution	33.3%-63.7%	100%	34
BLs	MPE, URT	³ H-thymidine	52%	92%	36
Sulph.	MPE/AGEP/DRESS	³ H-thymidine	75%	100%	187
Anaesth.	URT/MPE	³ H-thymidine	60%	-	131
Various	MPE/AGEP	³ H-thymidine	67%	-	159
Various	SJS/TEN	³ H-thymidine	27%	100%	192
Various	MPE	³ H-thymidine	55%	100%	188
Antitub.	Various	³ H-thymidine	14.9%	-	189

Table 10. Sensitivity and specificity of LTT. Abbreviations: AGEP: acute generalised exanthematous pustulosis; Anaesth: anaesthetic drug; Antitub.: antituberculosis drug; BL, betalactam; DRESS, drug reaction with eosinophilia and systemic symptoms; MPE, maculopapular exanthema; SJS/TEN, Stevens-Johnson syndrome/toxic epidermal necrolysis; Sulph: sulphonamide; URT, urticaria.

Although LTT is a useful assay to safely evaluate drug sensitisation, it is considered research tool rather than a diagnostic one, mainly because it requires trained-professionals, sterile cell culture, long assay duration, variability of sensitivity reported, and difficult to standardise protocols between laboratories.¹⁹³

6.2.2.4. Cytokine release

Upon activation, T cells release large number of cytokines, such as IL-5, IL-2, IL-10, IL-13, or IFN- γ .¹⁹⁴⁻¹⁹⁶ They can be measured in the supernatants of drug-stimulated cell cultures stimulate, mainly by enzyme-linked immunosorbent assays (ELISAs). Increased production of these cytokines in cell supernatants after drug stimulation can be associated with cell activation. The sensitivity of measuring IL-2 and IL-5 was of 38%, and 55% for IFN- γ for evaluating SJS/TEN patients.¹⁹² In the case of MPE, Sachs and colleagues found that when measuring the release of IFN- γ in cell supernatants stimulated with different drugs, the sensitivity was of 92% for IL-5, 50% for IL-10, and 36% for IFN- γ , with 100% of specificity for the first two cytokines, and 60% for IFN- γ .¹⁸⁸ Similarly, measurement of IL-5, IL-10, and IFN- γ in supernatants of drug-stimulated cells showed a sensitivity of 79% with 100% of specificity.¹⁹⁷

In an attempt to reduce the use of radioactivity in traditional LTT, detection of specific cytokine-producing cells by flow cytometry is also possible after drug-stimulation. The same authors obtained a sensitivity of 75% and a specificity of 100%,¹⁹⁷ with the added advantage that specific cell subpopulations can be studied simultaneously, depending on the immunological mechanism involved in each reaction. Interestingly, the combination of both cytokine producing measures allowed the identification of all the 28 patients with DHRs, with 100% of specificity.¹⁹⁷

6.2.2.5. Enzyme-linked Immunospot Assay (ELISpot).

ELISpot is a similar technique that the described above, although it analyses the number of cells producing cytokines, immunoglobulins or cytotoxic mediators following stimulation with the culprit drug.¹⁹⁸ It has been demonstrated to be useful for the diagnosis of DRESS to vancomycin, with a sensitivity of 86% when IFN- γ production was measured.⁷⁵ The sensitivity of ELISpot in SJS/TEN patients for granzyme B was of 33% and for granulysin of 40%.¹⁹² Despite its potential uses, more research is needed, mainly because of the low number of studies addressing this issue, the lack of standardised protocols and reduced commercial supplies.

6.2.2.6. Cell markers upregulation

In response to a drug, APCs and T cells are activated, and different markers on their surface are upregulated. Culturing these cells with the culprit drug and analysing their expression compared to non-stimulated cells can be used to assess drug recognition and cell activation. Accordingly, Beeler and colleagues demonstrated in 2007 that measuring CD69 on T cells after their stimulation with culprit drugs represents a useful and specific technique for probing a NIDHR.¹⁹³ It appears to be as effective as LTT with the advantage that results can be provided in only 48 hours.¹⁹³ Similarly, HLA-DR was upregulated in both CD4⁺ and CD8⁺ T cells in acute phase of MPE and bullous diseases.¹⁵⁹ The analysis of maturation markers, such as CD80, CD40, CD83, and CD86 on DCs seems to be useful for assessing drug desensitisation in different studies.^{158,199-202} Although promising, more research is needed to establish its potential clinical use for evaluating NIDHRs.

A review conducted by Mayorga and colleagues nicely showed that the combination of most relevant *in vivo* and *in vitro* test can improve the overall sensitivity for diagnosing DHRs to 75.1%/75.9% with a specificity of 91.8%/97.8%, depending on the specific test combination and the type of reactions involved.¹²⁹

7. FACTORS TO IMPROVE *IN VITRO* TESTS

7.1. Usefulness of DCs as APCs in DHRs

DCs are the most potent APCs in the body, located in all tissues.^{203,204} They patrol and act as “sentinels”, ready-to-act against intra and extra cellular antigens.²⁰⁵ They have the capacity to initiate and modulate the immune response,²⁰⁶ by the activation with naïve T cells, constituting a link between innate and adaptative immune systems, essential to modulate the immune response. In addition to their capacity to activate T cells, DCs can also produce several chemokines such as CCL17, CCL18, and CCL22, having an important role in the trafficking of memory T cells, regulating the specific immune response in the inflammation sites.²⁰⁵

7.1.1. DCs biology and diversity

DCs represent a heterogeneous population,²⁰⁷ they were firstly classified as “migratory tissue DCs”, and “LN resident DCs”.^{204,206} Nevertheless, more robust classifications have been proposed based on lineage of DCs correlated with the expression of interferon regulatory factors (IRF), IRF-4 and IRF-8.²⁰⁶ This classification allows differentiating between plasmacytoid DCs (pDCs),²⁰⁸ and classical DCs (cDCs), which correspond to myeloid DCs (mDCs).²⁰⁵ cDCs, or mDCs can also be subdivided into cDC1 (CD141⁺) and cDC2 (CD1c⁺).²⁰⁶ This is a more robust classification than those only based on phenotypic markers, as markers are shared by different cell subsets, and the expression of some of them are not homogenously expressed within the same cell population depending on the tissue they are found or the inflammatory status.²⁰⁷

pDCs do not express myeloid antigens and have the ability to respond rapidly to viral infections by producing interferons and cytokines.²⁰⁶ mDCs, which account for 0.5% to 1% of blood circulating mononuclear cells, are rapidly responsive to stimuli.²⁰⁹ In this sense, after consequential challenging with allergens in asthmatic patients, mDCs from blood migrate through the expression of CCR6 to the airway in the 6 to 24 hours after the administration of the allergen.²⁰⁹

cDC1 recognises mainly viral and intracellular antigens, presenting them to CD8⁺T cells (cross presentation),²⁰⁴ and triggering an immune response based on Th1 cytokine pattern.²⁰⁶ cDC1 are less frequent in blood than cDC2 (1:10) and have been characterised by the expression of CD141 (BDCA-3), although some studies indicated that this marker can also be expressed on cDC2 and monocytes in some tissues and *in vitro* cultures.²⁰⁶

cDC2 are the most abundant DC subpopulation in blood, tissues, and lymphoid organs.^{206,210} Besides myeloid antigens, they also express CD1c, CD2, CD1, and CD33. A recent study has shown that cDC2 have higher expression of HLA-DR compared to cDC1, in both, tissue and LNs. In addition, cDC2 highly express CCR7, which is responsible for trafficking these cells to LNs.²¹⁰ Moreover, these characteristics are maintained over time, compared to cDC1, indicating the important “sentinel” role during all life, detecting and processing antigens.²¹⁰ They have a strong ability to detect and present extracellular antigens. Consequently, these cells respond well to lipopolysaccharide

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(LPS). They trigger the activation of CD4⁺T cells, including Th1, Th2, and Th17, but also CD8⁺T cells, triggering a wide range of immune responses.²⁰⁶

Langerhans cells (LCs) are a subpopulation of DCs located in the skin that express CD1a.²¹¹ This subpopulation is clearly important during skin inflammation because they locally produce cytokines and migrate to LNs. After maturation, they mainly present antigens to CD8⁺T cells.²⁰⁶ Thus, they are important cells for maintaining the epidermal health, tolerance to commensals, and response to intracellular pathogens in the skin.²⁰⁶

Monocyte-derived DCs (moDCs), is a cell population that appears when inflammation occurs, remain in the injured tissue, rather than migrate to LNs, although to a lesser extent, they can also express CCR7.²⁰⁶ After inflammation, they disappear.

The great variability of DC populations and the different markers expressed, make the DC classification process difficult, and new DCs subsets are still being described.²⁰⁷ For example, a new subpopulation of migratory DC (CD141^{high}) appears to be different from cDC1 in skin, with a higher capacity to stimulate CD8⁺T cells.²⁰⁴

The mononuclear phagocyte system can be divided into three major groups of cells: macrophages, DCs, and moDCs.²⁰⁷ There is much controversy regarding the differentiation between DCs and moDCs. It has previously thought that both groups of cells derived from a common precursor. Nevertheless, recent investigations have identified that DCs are originated from a common DC progenitor (CDP), which hypothetically gives rise to two different precursors, pre-pDCs, give rise to the final pDCs, and pre-cDCs, which give rise to both cDC1 and cDC2 (Figure 20).²⁰⁷ On the other hand, moDCs derive from a different common monocyte progenitor (cMop). Interesting findings have revealed that tissues-resident monocytes do not derive from hematopoietic stem cells (HSCs) but develop from embryogenic precursors.

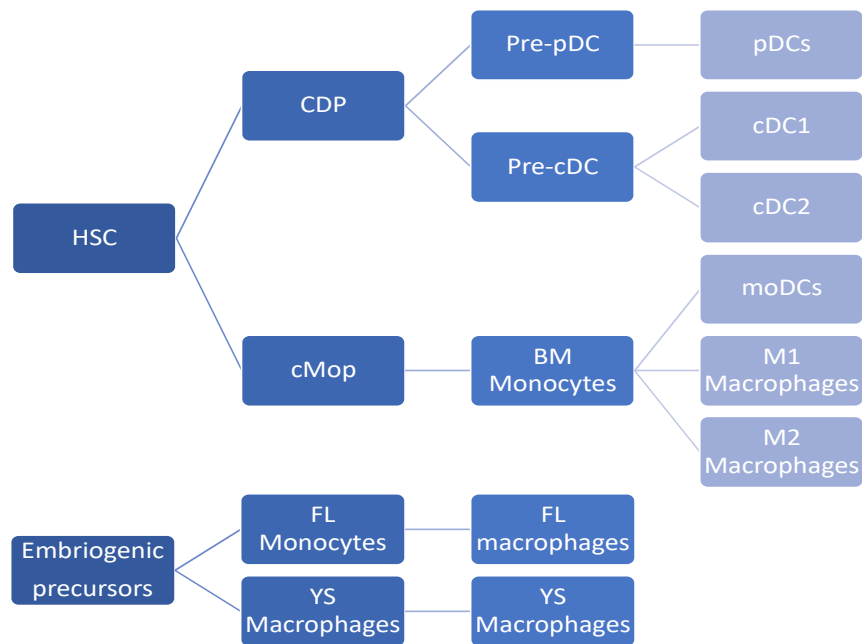


Figure 20. Development and classification of the mononuclear phagocyte system. Modified from Guilliams et al. 2015. Abbreviations: BM, Bone-Marrow; cDC, conventional dendritic cell; CDP, common DC progenitor; cMop, common monocyte progenitor; FL, fetal liver; HSC, hematopoietic stem cells; moDCs, monocyte derived dendritic cells; pDC, plasmacytoid dendritic cell; YS, yolk sac.

7.1.2. DC maturation

Immature DCs have a high endocytosis capacity, which allow DCs to efficiently detect and capture antigens.²¹² In contrast, during this steady-phase, DCs have a low antigen presenting capacity. Therefore, this phase has been linked with tolerogenic responses.⁵⁵

After detection and uptake, the antigens are processed inside the cells, which undergo phenotypical maturation changes. The expression of migration markers such as CCR7, is greatly increased, allowing DCs to efficiently migrate to LNs in order to present the antigens to naïve T cells, responsible for the later effector response.^{207,209} During this process, DCs gain antigen presenting capacity, while reducing their capacity of endocytosis,²¹³ by upregulating the expression of MHC (I/II), and costimulatory molecules such as CD40, CD80, CD83 and CD86 (Figure 21).²¹² This process is known as maturation, and it is mainly mediated by the phosphorylation of p38 MAPK.²¹⁴

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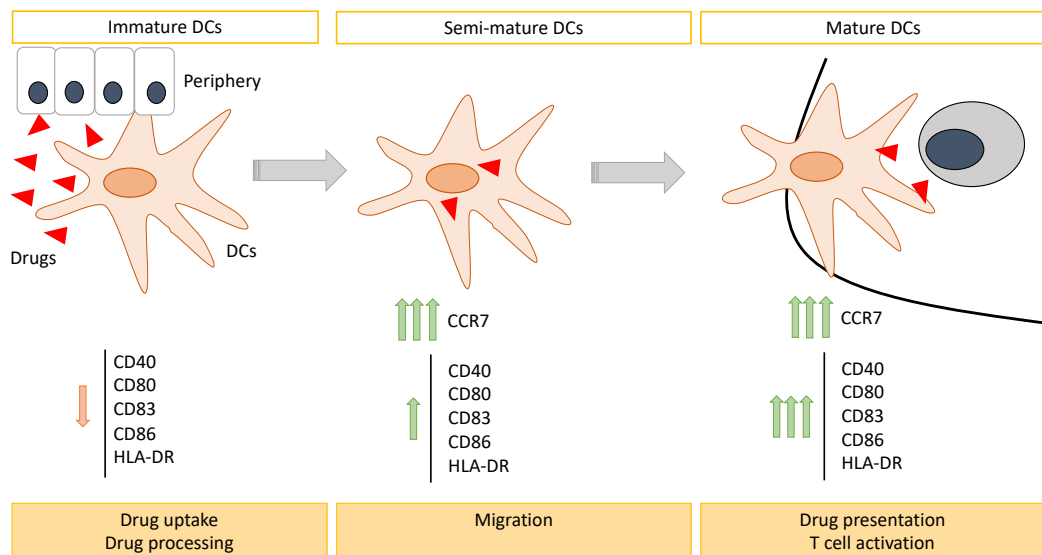


Figure 21. DCs maturation process

Maturation of DCs can be triggered by wide range of different stimuli, including danger signals, mainly those related to exogenous pathogens, such as pathogen-associated-molecule patterns (PAMPs).^{55,215} These molecules interact with toll-like-receptors (TLRs) on DCs, mainly LPS, agonist of TLR-4.^{213,216} The PAMP-TLR interaction triggers the activation cascade, mainly dependent of mitogen-activated protein kinases,⁵⁵ upregulating the expression of costimulatory molecules, cytokine and chemokine production resulting in T cells activation (Figure 22).²⁰³

DCs are the most commonly used APCs to evaluate *in vivo* targeting and *in vitro* antigen exposure.²¹⁶ These cells have been used to assess the sensitisation to drugs, by measuring cytokine production and/or phenotype modification on their surface as a consequence to a specific stimulus, in this case a drug. For instance, higher upregulation of CD40, CD80, CD83 and/or CD86 have been shown on DCs from allergic patients to drugs after stimulation with the culprit drugs, but not in healthy donors (Figure 22).^{158,199,202,217} Moreover the capacity of DCs to mature can be modulated by statins,²⁰³ or drugs such as rapamycin, which impair the antigen uptake by DCs via the two most important pathways, impairing the micropinocytosis and the mannose receptor-mediated endocytosis.²¹⁸ Altogether these data suggest that the analysis expression of maturation markers on drug-stimulated DCs, as well as their potential inclusion as APCs in LTT may present useful approaches to specifically study the sensitisation to a drug in allergic patients.

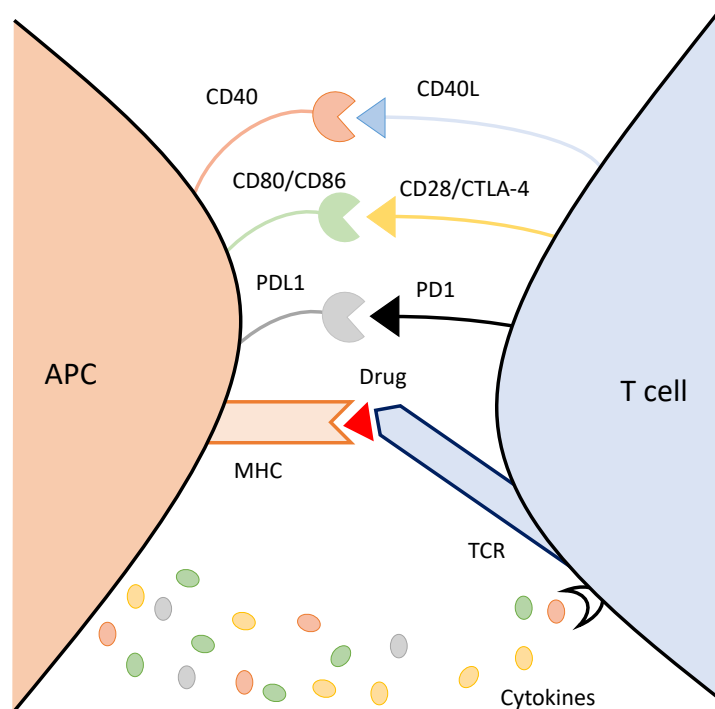


Figure 22. Interaction between mature DCs and T cells

7.2. Evaluation of the specific cells involved in the DHR.

DHRs are heterogeneous reactions, with a great variability of clinical manifestations and immunological mechanisms involved. Therefore, there are different effector cell populations, depending on the nature of the reaction. Because of this, the search for specific biomarkers involved during specific reactions can be very useful. Similarly, the assessment of the concrete role of specific cell populations and the study of their *in vitro* response after drug stimulation may serve as useful tools to increase the usefulness of currently available cellular *in vitro* tests.

Despite the fact that basophils and mast cells are the main effector cells in IDHRs, CD4⁺Th2 cells are essential during the sensitisation phase to promote the production of drug-sIgE.²¹⁹ In relation to this, the participation of CD4⁺Th2 cells, mainly characterised by the release of IL-4 or IL-13 is much more relevant than CD4⁺Th1 cells, mainly producers of IFN- γ or IL-2, as it has been demonstrated by different studies.²²⁰⁻²²⁵

The high heterogeneity of mechanisms in NIDHRs, much diverse than in IDHRs, make these reactions more complex to study, due to the high and different molecules such as chemokines, cytokines, inflammatory and cytotoxic mediators involved, as well as the differential role of diverse cell populations depending on the specific mechanism

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involved.¹⁴ A common feature of all NIDHRs, is the relevant contribution of CD4⁺Th1 cells, which release high amount of IFN- γ , IL-2, and IL-12.^{199,225-227} Nevertheless, other cell populations are specifically implicated in each concrete clinical entity.

In SJS/TEN reactions, cytotoxic NK and T cells, characterised by high production of cytotoxic mediators such as granzyme B, granulysin and perforin are involved.²²⁷⁻²³¹ In DRESS reactions, although a major contribution of CD4⁺Th1 cells has been described, an important role of CD4⁺Th2 has also been also demonstrated, together with NK cells, eosinophils and ILC2.^{69,74,77,79,200,232} In AGEP lesions, high amount of IL-22 and IL-17 have been measured, mainly released by CD4⁺Th17 and CD4⁺Th1 cells, with a significant involvement also of monocytes and neutrophils.^{14,231,233,234} In MPE, besides the role of CD4⁺Th1 cells, high contribution of CD4⁺Th2 has been also observed^{72,73,235}

Altogether, these findings demonstrated the differential involvement of specific cell populations depending on the clinical entity and the immune mechanism involved. Therefore, the study of these specific cell populations may be relevant to improve the available current cellular *in vitro* tests. Nevertheless, further studies in this direction are warranted to better understand the pathophysiologic mechanisms involved in DHRs, by identifying the main cell population involved in each reaction.

7.3. Involvement of drug metabolites in the evaluation of DHRs.

Some drugs are not reactive in their native form, requiring prior metabolisation to allow the recognition by the immune system. Others, such as CLV or metamizole, have low stability in solution, so they are rapidly and spontaneously degraded.^{95,173,236-239} Therefore, several studies have analysed the recognition of different drug metabolites by the immune system from allergic patients, mainly in *in vitro* test.

Ariza and colleagues used BAT to assess the recognition of metamizole, most common NSAIDs witch cause sIgE-mediated reactions, and its metabolites: 4-methylamino-antipyrine (MAA), 4-aminoantipyrine (AA), 4-acetylamino- antipyrine (AAA) and 4-formylamino-antipyrine (FAA). The BAT sensitivity for metamizole was 37.5%, whereas the inclusion of MAA increased BAT sensitivity to 50%, with all metamizole-positive cases tested also positive to MAA (Table 11).⁹⁵

Similarly, we have demonstrated that two synthetic metabolites from CLV degradation (Clav2 and Clav3) are efficiently recognised by the immune system, and

their inclusion in BAT, increased the sensitivity from 41% with the native drug, CLV, to 69% with the combination of CLV, Clav2, and Clav3.¹⁷³ These two synthetic metabolites derived from a potential antigenic determinant of CLV degradation (AD-I), while metabolites from other AD (AD-II) seemed not to trigger the activation of basophils from allergic patient in a more efficient way than those from AD-I.

It has also been demonstrated that, although carbamazepine,⁴⁵ and its main metabolite, carbamazepine-10, 11-epoxide (EPX), can bind to HLA-B*15:02, only EPX can modify peptides from the HLA F-pocket, by interacting with tyrosine and serine from position 74 and 77 respectively.⁵¹ Taken together, these observations may indicate that not only HLA alleles and TCRs, but also the products from drug metabolism play an important role in drug recognition and the development of DHRs.⁵¹

It has been investigated that DHRs to sulphonamides, and specially to sulfamethoxazole require a prior step of metabolism by cytochrome P450 to allow the formation of highly reactive hydroxylamine compounds,⁹¹ mainly responsible for interacting with proteins, and forming the hapten-protein complex, necessary for their recognition and the development of the DHR.

BACE inhibitors, such as atabcestat can directly and reversibly interact with HLA class II molecules without the need of cellular processing.⁴⁹ Nevertheless, its metabolites, atabcestat epoxide (mediated by cytochrome P450), and especially diaminothiazine (DIAT) (mediated by carboxylesterase) have a greater capacity to activate CD4⁺T cells compared to the whole drug by HLA p-i interaction.⁴⁹

Taken together, these data indicate the need for performance *in vitro* assays with different drug metabolites to determine their recognition by the immune cells from allergic patients and healthy subjects, such as by DC maturation assays, as well as their ability to trigger lymphocyte proliferation (LTT).

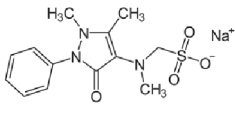
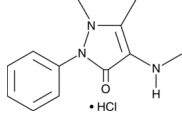
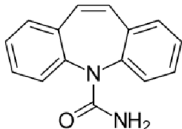
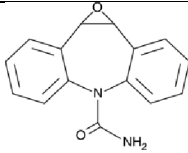
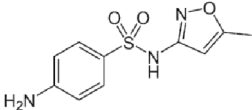
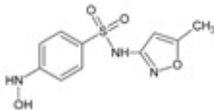
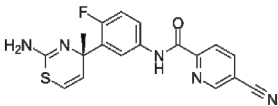
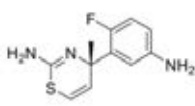
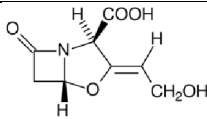
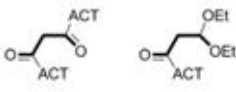
Family	Drug	Metabolite	Technique	Sensitivity improvement
NSAIDs	 Metamizole	 4-methylamino-antipyrine (MAA)	BAT	From 37.5% to 50%
Anticonvulsants	 Carbamazepine	 Carbamazepine-10, 11-epoxide (EPX)	HLA Modification	X
Sulphonamides	 Sulfamethoxazole	 Sulfamethoxazole hydroxylamine	Protein interaction	X
BACE inhibitors	 Atabecestat	 Diaminothiazine	HLA interaction	X
BLs	 Clavulanic acid	 Clav2 Clav3	BAT	From 41% to 69%

Table 11. Drug metabolites used to evaluate DHRs

7.3.1. Chemical structure of BLs and haptization

Based on their chemical structure, BLs can be divided into two groups, penicillins and cephalosporins, and into 4 different minor subclasses, monobactams, carbapenems, oxacephems, and clavams.²⁴ The common chemical structure of BLs consists of a 4-member betalactam ring, in penicillins the BL ring is condensed with a thiazolidine ring (5 members), whereas cephalosporins has a dihydrothiazine ring (6 members) (Figure 23).^{24,240} Moreover, penicillins have only one side chain, whereas cephalosporins have two.²⁴

Under physiological conditions, penicillin spontaneously degraded to different reactive metabolites,²⁴¹ which act as haptens, interacting with proteins or peptides to form the drug-hapten complex which stimulate the immune system. Concretely, the

immunogenicity of BLs is related to the capacity of the carbonyl group, which act as alkylating agent with amine groups from proteins.²⁴⁰ Moreover, its thiazolidine ring increases the tension of the molecule, resulting in high reactivity.²⁴⁰

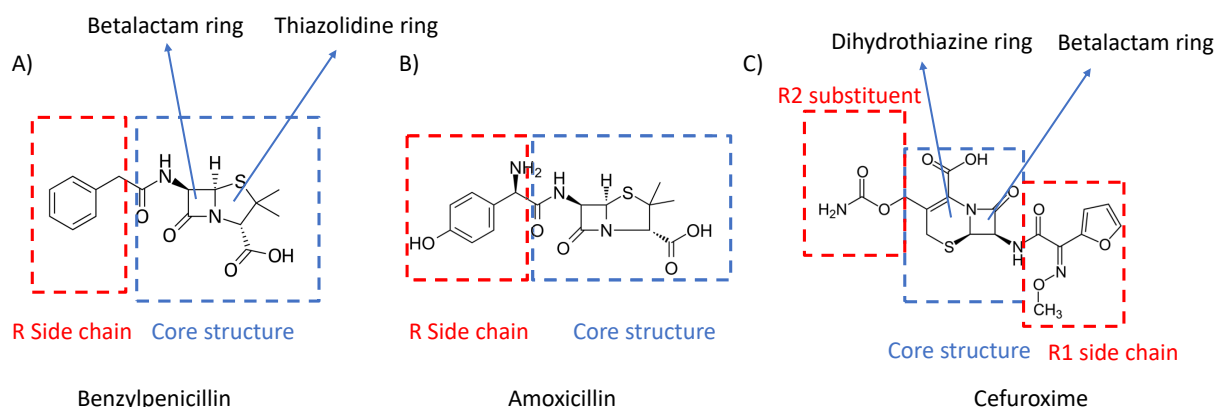


Figure 23. Chemical structure of BLs. Chemical structure of two penicillins, a) benzylpenicillin and B) amoxicillin; and one cephalosporin C) cefuroxime. Modified from Goh et al. 2021

The hapten process is the best-studied recognition model for BLs.^{31,47} Reactivity of BLs is linked to the nature of their BL ring, which undergoes nucleophilic attacks by free amine groups of proteins and peptides,²⁴² mainly those from lysine residues, leading to the opening of the BL ring,⁴⁷ and the formation of penicilloyl adducts on lysine residues and free amine moieties.¹⁵⁷ The kinetics of the process is fast, and generates drug-carrier conjugates in a concentration-dependent manner.²⁴ In addition, some factors such as the redox status and oxidative stress can influence the capacity of the drug to bind to proteins.^{242,243}

A recent review carried out by Goh and colleagues nicely described the different ways in which BLs interact with proteins, and how they are processed and presented by APCs.¹⁵⁷ The first pathway is the one in which the drug enters in the APC, where it interacts with intracellular proteins, and after proteasomal degradation, different drug-peptide complexes are generated. These hapten-peptide complexes are coupled to HLA class I and/or II, entering in the endoplasmic reticulum through transporter associated with antigen processing (TAP). These complexes are wrapped into vesicles within the Golgi apparatus, and transported to the plasma membrane, where they are exposed to the outside for further interaction with lymphocytes. Another related pathway consists

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of drugs interacting with HLA-peptides, previously formed inside the cells, directly on the surface of APCs. Alternatively, drugs may interact with proteins in the extracellular environment. Then hapten-protein conjugates are then degraded inside the APC and attached to HLA molecules. Finally, a new pathway has been proposed in which intracellular-haptenised proteins can be exported to APCs through the production of exosomes,²⁴⁴ which are processed in a similar way to the other pathways. It is not completely clear which is the most common pathways of interaction, and presumably different ways can occur in the same cells, with the same drug, and simultaneously. Nevertheless, more research related this important issue is required.

The first and best studied haptenisation model has been described for BP. More than 95% of penicillin is spontaneously and under physiological conditions degraded to benzyl penicilloyl, the most important antigenic determinant of penicillin (major antigenic determinant),²⁴¹ which is stable enough to be characterised and purified.²⁴⁰ The remaining 5% is degraded to other antigenic determinants such as benzylpenicilloic and benzylpenilloic acid (minor antigenic determinants). Similarly, cephalosporins are able to interact with proteins, but slowly and less efficiently than penicillins.²⁴ Opening of the BL ring causes cephalosporins to lose their R2, being only the core structure and R1 important for the hapten formation.

Regarding the chemical structure of BLs, two different recognition patterns are possible, some patients preferentially recognise the side chain of the drug, developing a selective reaction. In contrast, there are patients who recognise the central structure of the BL ring, reacting to different BLs (cross reactors). Cross reactivity between penicillins and cephalosporins is estimated in 10%, and increases to 30% when the cephalosporin and penicillin shared the same side chain.^{24,245} Interestingly, cross reactivity exclusively between cephalosporins has been also proved, when cephalosporins shared the same R1 chain.²⁴

Since human serum albumin (HSA) is the most abundant protein in human serum, it is likely to be closely related to the interaction with BLs, and the formation of new adducts. In fact, most studies which evaluate BL haptenisation are based on HSA models. Very early, three potential HSA lysine residues have been identified as modified by BP.¹⁵⁷ More recently, It has been identified that 51/59 lysine residues from HSA are occupied by BP at basic pH, and 21/59 at physiological pH, some of them specifically

shared in allergic patients.⁴⁷ Specifically, the study showed that two BP-modified peptides (Lys 159 and 525) are important sites for the hapten-protein complexes recognition.⁴⁷ In addition, other serum proteins such as transferrin, or the light and heavy chains of immunoglobulins can also be protein target for BLs haptensisation.^{46,246}

In a study carried out by Jenkins and colleagues, they evaluated the reactivity of piperacillin, composed by a BL and thiazolidine ring, meropenem, similar to piperacillin, but with a carbon instead of sulphur in the thiazolidine ring, and aztreonam, a monobactam with only a BL ring in patients with multiallergic syndrome.²⁴⁷ They nicely observed that each drug modifies the lysine residues from the HSA in a different way, and that T cell reactivity does not exist among the T cell pool.²⁴⁷ In other words, each T cell clone generated specifically recognises each drug-carrier conjugate, with no cross-reactivity, but there may be different T cell priming against the different antigenic determinants.²⁴⁷

7.3.2. Clavulanic acid

CLV is a small molecule belonging to BLs. Despite its reduced antibacterial activity, it is a potent inhibitor of the enzyme betalactamase, and because of this, it is often prescribed alongside AX, with great antibacterial activity.^{248,249} This drug derived from 6-aminopenicillanic and instead a thiazolidine ring, like penicillins, it has an oxazolidine ring. Besides, it has not got side chain (Figure 24).²⁴⁸

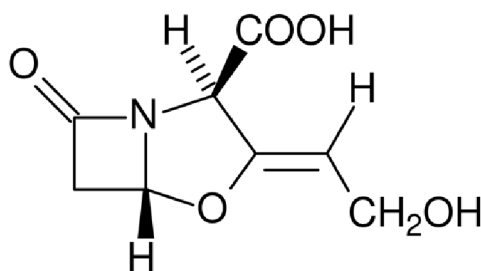


Figure 24. Chemical structure of CLV

Initial studies reported that CLV has not immunogenicity,²⁵⁰ nevertheless in 1995, the description of two patients with selective IDHRs to CLV indicated the contrary.²⁴⁸ Moreover, the incidence of IDHRs to CLV are increasing nowadays, being now more frequent than IDHRs to penicillin.¹⁵⁸

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The recognition of CLV by the immune system is assumed to be like the rest of BLs. It requires the nucleophilic attack of the carbonyl group from the BL ring by amines from lysine residues, leading to the formation of CLV-carrier complexes.^{239,249} Nevertheless, the chemical structure of CLV is much more unstable than that of other BLs. Previous studies have shown that degradation of CLV occurs 5-10 times faster than for AX or penicillin.^{237,238} Moreover, this degradation is self-accelerated, and degradation products can be detected within minutes.^{236,251,252}

In addition, the instability of the reactive intermediates makes the study of these structures, and their interaction with the immune system very difficult. To address this difficult issue, our group undertook a study to identify these potential CLV degradative metabolites, as well as to identify the recognition of each of them by the immune system, and the potential sites for interaction between CLV and HSA.¹⁷³ In this study we also revealed that metabolites derived from CLV degradation, concretely of AD-I, have the ability to modify Lys 475 and Lys 195 from HSA.¹⁷³ Same residues were modified by other drugs such as piperacillin, BP, and flucoxacillin,²⁵³⁻²⁵⁵ suggesting that the haptenisation process occurs in a similar way than in other related drugs.²⁵⁶ Interestingly, although CLV metabolites were detected to be capable to modify HSA, no the complete CLV structure was found,¹⁷³ in agreement with the high degradation rate of CLV.

Even though AX and CLV belong to the same family, because of important differences in their chemical structures, a patient can be sensitised to AX or CLV after the administration of both drugs together.²⁵⁷ The reasons why a patient becomes sensitised to one drug or the other, remains elusive at present, but preliminary data suggested that cross reactivity between both drugs is not often.^{250,252,258} Therefore, determining the specific drug that triggers the reaction following AX-CLV administration is of importance, as selective patients to CLV can tolerate other BLs, including AX, increasing the therapeutic options in these patients. However, a study conducted by our group has shown that the same patient can be simultaneously sensitised to both AX and CLV, as demonstrated in 3 patients with IDHRs and 1 patient with NIDHR.¹⁵⁸ Although not frequent, this study highlights the importance of including determinants for both BLs in the diagnostic procedures, thus the confirmation of a DHR to either AX or CLV cannot completely rule out hypersensitivity to the other drug.¹⁵⁸

JUSTIFICATION AND HYPOTHESIS

DHRs are an important health problem because, despite not being a high prevalent disease in the general population,^{5,26} they have important implications for the patients and healthcare systems. They are associated with higher costs due to longer stays in hospitals, more hospital and ambulatory admissions, and increased risk of infections during hospital stays.¹⁷ Moreover, patients diagnosed with DHRs require second-line treatments, which are usually less effective, with more associated side-effects and higher costs.^{28,259} Additionally, the prevalence of these reactions are increasing in the last decades.^{5,9,15,39}

The diagnosis of DHR is a complex process because of the great variability of drug-immune system interactions,^{12,54} clinical manifestations,^{10,54,55,260} immune mechanisms, and cells involved.^{14,56,190,200,261-263} Nevertheless, there are important differences between IDHRs, whose symptoms appear within the first hour, eventually between 1-6 hours after drug administration, and NIDHRs, whose symptoms appear after more than one hour, commonly days or even weeks after drug exposure, that will strongly affect their evaluation and diagnosis.^{264,265}

NIDHRs are mediated by T cells and have very heterogeneous clinical manifestations, ranging from mild/moderate reactions, such as MPE or delayed-URT, to severe reactions such as SJS/TEN or DRESS. The diagnostic procedure is complex, and relies on *in vivo* tests, mainly STs (PT and delayed IDT).^{68,130} However, their sensitivity is not optimal, so DPT is sometimes the only test available to evaluate or confirm the diagnosis. Nevertheless, DPT is not risk-free for patients, is not recommended in patients with severe reactions, or cannot be performed for all drugs. Moreover, since symptoms in NIDHRs may appear days or even weeks after drug administration, determining the concrete drug that triggers the reaction is sometimes difficult, especially when patients use different drugs during the period.

Given the limited and variable sensitivity of *in vivo* tests, the use of *in vitro* tests could represent useful and safe alternatives to improve the diagnosis of NIDHRs. Different biomarkers and techniques have been evaluated, such as ELISpot,²⁶⁶ measurement of cytokine production,^{77,188} upregulation of markers on immune cells,^{158,193,199,201,202} and immunohistochemistry,⁷² although the most studied *in vitro* method to evaluate NIDHRs is LTT.^{34,36,43,187,191} Nevertheless, its variability between studies and/or its limited sensitivity, as well as the need to use radioactive materials, have made this technique more related to the research field than to clinical routine.

In contrast, IDHRs are less heterogeneous, with most of them being mediated by sIgE. BLs are the most frequent drugs responsible for triggering IDHRs, namely AX. Nevertheless, due to the high betalactamase activity of CLV, it is frequently prescribed alongside AX. Following administration of both drugs, patients may become specifically sensitised to one drug or the other,^{248,257,267,268} with cases in which a patient is sensitised to both drugs simultaneously being rare.¹⁵⁸ Thus, determining the specific drug responsible for the reaction has important implications, because CLV selective patients may tolerate other BLs, including AX, although it is sometimes difficult to determine the specific drug that triggers the reaction.

The diagnostic procedure for AX-CLV IDHRs is mainly based on the study of clinical history, followed by *in vivo* testing (SPT and IDT).²⁶⁹ If STs are negative, it is necessary to perform DPT, which can be dangerous for patients, and for some drugs, as in the case of CLV, it is not possible, since there are not available products, so a CLV IDHR has to be indirectly proved by tolerance of AX. To reduce the need for DPT, *in vitro* assays have been evaluated. For BLs, sIgE detection is the most widely used *in vitro* test, although its sensitivity and specificity are very limited and variable, not being available for the detection of CLV sIgE.^{160,239} Other methods such as serum tryptase or histamine measurements can be useful to assess a reaction at the acute phase, however, it is not possible to obtain information of the responsible drug or immune mechanism involved.^{156,270}

BAT is a useful *in vitro* alternative for evaluating IDHRs to both AX and CLV.²⁶⁷ Nevertheless, its sensitivity is not optimal, mainly for evaluating CLV induced IDHRs.¹⁷³ CLV, like other BLs, needs to bind to serum proteins or peptides to be immunogenic.^{252,271} Nevertheless, the low sensitivity of *in vitro* test with CLV could be explained by the high degradation rate of this drug,^{237,238} and the consequent generation of different metabolites,²³⁹ with variable capacities to bind to proteins and therefore with different ability to be recognised by sIgE on basophil surface. Because of this heterogeneity, only some metabolites can efficiently interact with the immune system, but they may not be included in sufficient concentration in the different *in vitro* tests, which may explain their reduced sensitivity.

To prove a sensitisation to a drug, the *in vitro* use of DCs, specially moDCs, which can be easily transformed from circulant monocytes in high number, has been evaluated with different drugs involved in DHRs.^{186,201,217} It is based on the fact that upon drug stimulation, moDCs mature, upregulating the expression of migration, activation and maturation markers, and release of cytokines only in patients with DHRs, but not in tolerant subjects. Furthermore, their capacity to present the drug and stimulate lymphocytes in response to specific drugs can be used for evaluating both NIDHRs and IDHRs.^{217,272} Nevertheless, moDCs, are only found under inflammatory processes in nature, so they are not the true “sentinels” in blood or tissues. Therefore, other APCs may be more effective to be included in these approaches.

Altogether, and with the aim of increasing the *in vitro* diagnostic sensitivity of DHRs, we hypothesised that the determination of maturation, activation, and migration markers on DCs could be a useful assay to assess the sensitisation to a concrete drug. Furthermore, the inclusion of pre-primed DCs with the drugs in LTTs may help to improve their sensitivity in both NIDHRs and IDHRs, which have been shown to be suboptimal. In addition, the inclusion of other factors such as the assessment of the proliferative response of specific cell populations depending on the concrete immunological entity involved, as well as the inclusion of antigenic determinants derived from drug degradation in *in vitro* cellular assays may help to increase their sensitivity.

OBJECTIVES

GENERAL OBJECTIVE

To improve the sensitivity of different cellular *in vitro* diagnostic tests for evaluating both NIDHRs and IDHRs, by using different approaches including the use of moDCs and mDCs as APCs to increase lymphocyte proliferation in LTT, the assessment of the response of specific cell populations involved in each reaction, and the inclusion of specific antigenic determinants derived from the degradation of drugs that might be involved in the reaction, to improve its *in vitro* immunological recognition.

OBJECTIVE I

To assess the added value of including moDCs as APCs in the specific lymphocyte proliferation induction to improve the evaluation of NIDHRs with different clinical entities and drugs.

- ◆ To study the overall sensitivity and specificity of LTT after including drug pre-primed moDCs as APCs (dDC-LTT) versus the conventional LTT (C-LTT).
- ◆ To compare the sensitivity and specificity of C-LTT and dDC-LTT according to the clinical entity involved.

OBJECTIVE II

To analyse the usefulness of assessing the proliferative response of specific cell populations to improve the sensitivity of dDC-LTT in different clinical entities, using flow cytometry technology.

- ◆ To determine the proliferative response of different cell populations in dDC-LTT, depending on the clinical entity involved in NIDHRs.
- ◆ To Identify the best combination of proliferative results of different cell populations, depending on the clinical entity.

OBJECTIVES

OBJECTIVE III

To evaluate the recognition of CLV and its different antigenic determinants (ADs) by moDCs, as well as their capacity to stimulate lymphocytes from IDHRs patients with a selective response to CLV.

- ◆ To assess the capacity of CLV and different synthetic analogues from two potential ADs derived from CLV degradation (Clav1-3 from AD-I, and Clav4-7 from AD-II) to induce the activation, maturation, and migration of moDCs from CLV-selective patients with IDHRs.
- ◆ To analyse the ability of CLV and its ADs to induce the activation of moDCs and their ability to trigger the proliferation of different cell populations and find the best combination of synthetic analogues to achieve the best LTT sensitivity and specificity.

OBJECTIVE IV

To study the recognition of AX and CLV by two different populations of DCs (moDCs and mDCs) and to assess their ability to present the drug and activate different lymphocyte subpopulations in patients with both AX- or CLV-selective IDHRs compared to healthy donors.

- ◆ To assess the expression of migration, activation, and maturation markers on mDCs and moDCs in response to AX and CLV.
- ◆ To analyse the ability of AX or CLV pre-primed mDCs and moDCs to activate different lymphocyte subpopulations, comparing both methods with direct drug stimulation of all PBMCs.
- ◆ To determine the capacity of mDCs and moDCs to differentiate between AX and CLV reactions based on patient selectivity

MATERIAL AND METHODS

FIELD OF STUDY

The experimental work detailed in the following sections was carried out between the years 2019-2022. The allergic patients and healthy controls selection, as well as the samples obtaining was done in the Allergy Unit, belonging to the Hospital Regional Universitario de Málaga (HRUM). Cellular *in vitro* experiments were performed in the research laboratory from the Instituto de Investigación Biomédica de Málaga (IBIMA), from the Hospital Civil (HRUM). The chemical synthesis and characterisation of compounds were performed in the Nanostructures for Diagnosing and Treatment of Allergic Diseases Laboratory, from the Centro Andaluz de Nanomedicina y Biotecnología (BIONAND).

ALLERGIC PATIENTS AND HEALTHY CONTROL SELECTION

Biological samples for this thesis were obtained from patients confirmed with both NIDHRs to different drugs, and IDHRs to BLs (selective to AX or CLV). The clinical evaluation of the patients was performed by the Allergy Unit of HRUM. Patients with suggestive compatible history of NIDHRs to different drugs or IDHRs to BLs were prospectively evaluated by this unit during the period 2013-2019.

The diagnosis of DHRs was confirmed following ENDA and EAACI guidelines.^{127,151,260,273-275} Specifically, the diagnosis of NIDHRs to different drugs was based on STs (PT or IDT) and, if negative, DPT. If the reaction was severe, and DPT was contraindicated, the diagnostic confirmation was based on the casualty Algorithm of the Spanish Pharmacovigilance System (ASPS).^{190,191,276} This algorithm evaluates factors such as the relationship between the drug and the reaction, rechallenge drug effects, alternative causes, or the chronology of the reaction among others. This algorithm allows the classification of reactions based on different scores, classifying reactions as unlikely, conditional, possible, probable, or definite. The assessment of AX- and CLV-selective IDHRs, after the intake of AX or AX-CLV, were evaluated following the International Consensus on Drug Allergy (ICON), ENDA, and EAACI guidelines and recommendations.^{127,151,259,260,273-275} The allergological work-up was mainly based on STs (SPT and IDT), followed by DPT if STs were negative, or to demonstrate good tolerance to alternative BLs.

A sex- and age- matched group of subjects with no reported DHRs to any drug was included. All participants were properly orally informed and signed an informed consent to participate in the study. The research projects on which this thesis is based were approved by the Ethics Committee of Málaga and were conducted in accordance with the guidelines of the Declaration of Helsinki, General Assembly, Fortaleza, Brazil, 2013 and their future updates and the ICH on PCBs CPMP /ICH / 135/95.

ALLERGOLOGICAL WORK-UP

STs

For evaluating NIDHRs, PTs were performed according to the European Society of Contact Dermatitis (ESCD) using 1/3 of the therapeutic dose of the commercialised drug, diluted in petrolatum, and applied to the back.²⁷⁷ Readings were done 20min after removal of the strips, and 48, 72, and 96 hours after the first reading. Positive results were considered when patchy erythema with or without infiltration, papules, or bullous reactions appeared. IDT was performed with the available drugs in 0.9% sodium chloride (NaCl). Readings were done 20min after the test was performed, and 24, 48, and 72h after the first reading. A positive result was considered when an infiltrated erythema greater than 5mm appeared, with negative and positive response to control saline and histamine respectively, following EAACI guidelines.^{273,278}

For IDHRs to BLs, SPT was performed first, and if negative, IDT was done. Benzylpenicilloyl octa-L-lysine (BP-OL) at 0.04mg/mL, equivalent to 8.64×10^{-5} molar (M), MDM at 0.5mg/mL, equivalent to a concentration of 1.5×10^{-3} M, AX and CLV at 20mg/mL were used. The readings were done after 20min after the performance of the test. Positive results were considered when an infiltrated erythema bigger than 3mm was observed, with positive response to the histamine positive control and negative to the control saline solution, following EAACI guidelines.^{151,275} IDT was performed as previously described for NIDHRs, but readings were done 20min after the performance of the test.^{273,278}

DPTs

In the case of NIDHRs, placebo-single-blinded DPT with the culprit drug was carried out if STs were negative, but only in mild/moderate reactions, after balancing the risk/benefit for each patient. It was initiated by administrating 1/100 of the therapeutic dose. In case of tolerance, a 1/10 dose was administrated from 3 days to a week later, depending on previous data regarding the specific drug or the time interval between drug administration and onset of symptoms. If drug tolerance persisted, the full therapeutic dose was administrated at the same interval. DPTs were performed at least 4 weeks after the reaction, to avoid interference with the acute phase, and after medications were stopped, according to EAACI guidelines.²⁷⁹ In case of symptoms onset during DPT, the procedure was immediately interrupted.

For IDHRs, if STs were negative to both AX and CLV, single-blind placebo controlled oral DPT was performed with AX. AX was administrated at 50mg in the first phase. If well tolerated, increasing doses (100, 150, and 200mg) were administrated until reaching the cumulative therapeutical dose of 500mg. Afterwards, patients were instructed to take AX for two days at home every 8h to confirm good tolerance. If patients had positive STs to CLV, DPT with AX was performed as described above. Patients with positive STs or DPT to AX, DPT with PV was performed to assess the selectivity of the reaction, and the good tolerance to other BLs.

Patients were monitored during DPT, and it was considered positive if cutaneous and/or respiratory symptoms appeared during the test, then, the procedure was interrupted and symptoms were evaluated and treated.^{150,280} A complete cardiopulmonary resuscitation equipment was always available.¹⁵⁰

MATERIALS

Consumables

- ◆ 12-well clear plate (Corning Inc., New York, US).
- ◆ 96-well clear plate, U bottom (Corning Inc., New York, US).
- ◆ Cryogenic Tubes 1ml (Thermo Fisher Scientific, Waltham, US).
- ◆ Heparinised tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, US).
- ◆ LD columns (Miltenyi Biotec, Bergisch Gladbach, Germany).
- ◆ LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany).
- ◆ MS columns (Miltenyi Biotec, Bergisch Gladbach, Germany).
- ◆ Pasteur pipettes (Sigma-aldrich, San Luis, US).
- ◆ Polystyrene tubes 12mL (Akralab S.L. Alicante, Spain).
- ◆ Serological pipettes 1mL, 5mL, 10mL, 25mL, 50mL (Corning Inc., New York, US).
- ◆ Syringes 5,10,20mL (Becton Dickinson, Franklin Lakes, US).
- ◆ Whatman® Puradisc 30 syringe filters, 0.2 µm, sterile (Whatman plc, Maidstone, UK).

Reagents

- ◆ 3,3-diethoxypropanoic acid (Sigma-Aldrich, San Luis, US).
- ◆ 3-acetamidopropanoic acid (Sigma-Aldrich, San Luis, US).
- ◆ Anti-biotin μ beads (Miltenyi Biotec, Bergisch Gladbach, Germany).
- ◆ Benzylpenicilloyl octa-L-lysine (BP-OL) (Diater Laboratorio de Diagnóstico y Aplicaciones S.A., Madrid, Spain)
- ◆ Bovine Serum Albumin (BSA) (Sigma-Aldrich, San Luis, US).
- ◆ Butylamine (Sigma-Aldrich, San Luis, US).
- ◆ Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen™, Thermo Fisher Scientific, Waltham, US).
- ◆ CD14 μ Beads (Miltenyi Biotec, Bergisch Gladbach, Germany).
- ◆ CD19 μ beads (Miltenyi Biotec, Bergisch Gladbach, Germany).
- ◆ CD1c (BDCA-1)-Biotin μ beads (Miltenyi Biotec, Bergisch Gladbach, Germany).
- ◆ Dichloromethane (Sigma-Aldrich, San Luis, US).
- ◆ Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, San Luis, US).
- ◆ Ethanol (EtOH) (Sigma-Aldrich, San Luis, US).
- ◆ Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, San Luis, US).
- ◆ FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany).
- ◆ Fetal Bovine Serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, US).
- ◆ Gentamycin (Laboratorios Normon S.A., Madrid, Spain).
- ◆ Granulocyte-macrophage colony-stimulating factor (GM-CSF) human recombinant (R & D Systems Minneapolis, US).
- ◆ Hydrazine hydrate (Sigma-Aldrich, San Luis, US).
- ◆ Hydrochloric acid (HCl) (Sigma-Aldrich, San Luis, US).
- ◆ Interleukin 4 (IL-4) human recombinant (R & D Systems Minneapolis, US).
- ◆ Isobutylchloroformate (Sigma-Aldrich, San Luis, US).
- ◆ Isopropanol (Sigma-Aldrich, San Luis, US).
- ◆ L-Glutamine (Sigma-Aldrich, San Luis, US).
- ◆ Lymphoprep™ (STEMCELL Technologies, Vancouver, Canada).
- ◆ Magnesium sulphate (MgSO₄) (Sigma-Aldrich, San Luis, US).
- ◆ Malonic acid (Sigma-Aldrich, San Luis, US).

MATERIAL AND METHODS

- ◆ Methyl 3-(methylthio)propionate (Sigma-Aldrich, San Luis, US).
- ◆ Mix of minor determinants from benzylpenicillin (MDM) (Diater Laboratorio de Diagnóstico y Aplicaciones, S.A., Madrid, Spain).
- ◆ N-hydroxysuccinimide (Sigma-Aldrich, San Luis, US).
- ◆ N-Methyl morpholine (Sigma-Aldrich, San Luis, US).
- ◆ Penicillin V (PV) (Diater Laboratorio de Diagnóstico y Aplicaciones, S.A. Madrid, Spain).
- ◆ Penicilloyl-polylysine (PPL) (Diater Laboratorio de Diagnóstico y Aplicaciones, S.A. Madrid, Spain).
- ◆ Phosphate buffered saline (PBS) (Gibco, Thermo Fisher Scientific, Waltham, US).
- ◆ Phytohemagglutinin-L (PHA-L) from *Phaseolus vulgaris* (Roche, Basel, Switzerland).
- ◆ RPMI 1640 Medium without L-Glutamine (Lonza Group, Basel, Switzerland).
- ◆ Sodium chloride (NaCl) (Sigma-aldrich, San Luis, US).
- ◆ Streptomycin (Laboratorios Normon S.A., Madrid, Spain)
- ◆ Trypan blue (Sigma-Aldrich, San Luis, US).
- ◆ Ultrapure lipopolysaccharide from *E. coli* K12 (LPS) (InvivoGen, San Diego, US)

Media and buffer solutions

- ◆ Culture medium: RPMI 1640 supplemented with L-glutamine (2mM), gentamycin (50ug/ml), streptomycin (5mg/mL), and 10% of decompemented FBS.
- ◆ R40 medium: RPMI 1640 supplemented with L-glutamine (2mM), gentamycin (50ug/ml), streptomycin (5mg/mL), and 40% of decompemented FBS.
- ◆ Freezing medium: RPMI 1640 supplemented with L-glutamine (2mM), gentamycin (50ug/ml), streptomycin (5mg/mL), 40% of FBS, and 20% of DMSO.
- ◆ Separation buffer: phosphate-buffered saline (PBS) at pH of 7.2, supplemented with 0.5% BSA, and 2 mM EDTA

Drugs

Table 12 shows the different drugs, sources, forms and concentration of the different drugs used in *in vitro* tests.

Drug	Source	Form	Concentrations
Amoxicillin	Sigma-Aldrich (San Luis, USA)	Powder	
Allopurinol	Normon (Madrid, Spain)	Pill	
Cefepime	Fresenius (Bad Homburg, Germany)	Powder	250µg/mL,
Cefixime	Merck (Darmstadt, Germany)	Oral capsule	125µg/mL,
Clavulanic acid	Sigma-Aldrich (San Luis, USA)	Powder	25µg/mL
Diazepam	Roche (Basel, Switzerland)	Injectable solution	
Sulfamethoxazole	Almofarma S.L. (Barcelona, Spain)	Powder	
Trimetoprim	Almofarma S.L. (Barcelona, Spain)	Injectable solution	
Iobitridol	Guebert (Villepinte, France)	Injectable solution	5mg/mL,
Iodixanol	GE Healthcare (Chicago, USA)	Injectable solution	1mg/mL,
Iomeron	Bracco (Milan, Italy)	Injectable solution	0.1mg/mL
Ciprofloxacin	Sigma-Aldrich (San Luis, USA)	Powder	100µg/mL, 10µg/mL, 1µg/mL
Iron supplement	Medix (Madrid, Spain)	Oral solution	250µg/mL, 125µg/mL, 25µg/mL, 5µg/mL
Carbamazepine	Ken Pharma (Barcelona, Spain)	Pill	50µg/mL, 10µg/mL, 1µg/mL
Phenobarbital	Normon (Madrid, Spain)	Injectable solution	

Table 12. Drugs, sources, forms, and concentrations used in *in vitro* assays. The solvent used to dilute each drug was culture medium.

Antibodies

The complete list of monoclonal antibodies used in these experiments can be consulted in Table 13. Isotype controls are shown in Table 14.

Assay	Antibody	Fluorochrome	Clon	Company
mDCs characterisation	CD19	PECy7	HIB19	BD Pharmingen™
	CD14	FITc	MφP9	BD™
	CD8	FITc	SK1	BD™
	CD16	FITc	CLB/FcGran1	BD™
	CD1c	AF647	L161	BioLegend®
	HLA-DR	VB605	L243	BioLegend®
Monocytes/moDCs characterisation	CD1a	APC	HI149	BioLegend®
	CD1a	PECy7	HI149	BioLegend®
	CD16	PE	B73.1	BD™
	HLA-DR	FITc	L243	BD™
	CD14	PerCP-Cy5.5	63D3	BioLegend®
mDCs/moDCs maturation	CCR7	PerCPCy5.5	150503	BD Pharmingen™
	CD40	PE	5C3	BD Pharmingen™
	CD80	PECy7	L307.4	BD Pharmingen™
	CD83	APC	HB15e	BD Pharmingen™
	CD86	FITc	2331	BD Pharmingen™
LTT	CD3	PerCP	SK7	BD™
	CD4	APC	SK3	BD™
	CXCR3	PE	1C6/CXCR3	BD Pharmingen™
	IFN- γ	PECy7	B27	BD Pharmingen™
	CD4	PerCP	SK3	BD™
	CRTH2	AF647	BM16	BioLegend®
	IL-4	PECy7	MP4-25D2	BioLegend®
	CD56	PE	MY31	BD™
	Perforin	AF647	δG9	BD Pharmingen™
	CD4	PECy7	SK3	BD™
	CCR5	PE	3A9	BD Pharmingen™
All	Live/Dead	near IR	-	-

Table 13. Monoclonal antibodies used in the study.

Antibody	Specie	Isotype
FITc	Mouse	IgG2b, k
FITc	Mouse	IgG2a, k
FITc	Mouse	IgG1, k
PE	Mouse	IgG1, k
PE	Mouse	IgG2a, k
PerCP	Mouse	IgG1, k
PerCPCy5.5	Mouse	IgG1, k
PerCPCy5.5	Mouse	IgG2a, k
PECy7	Mouse	IgG1, k
PECy7	Rat	IgG1, k
APC	Mouse	IgG1, k
AF647	Mouse	IgG1, k
AF647	Rat	IgG2a, k
VB605	Mouse	IgG2a, k

Table 14. Isotype controls used in the study.

SYNTHESIS OF CLV ADs AND SYNTHETIC ANALOGUES

The synthesis and purification of CLV ADs and their synthetic analogues was performed in the Nanostructures for Diagnosing and Treatment of Allergic Diseases Laboratory, in collaboration with the Biomimetic Dendrimers and Photonic Laboratory, led by Prof. Perez-Inestrosa, both from BIONAND, using the procedures described by Barbero and colleagues,¹⁷³ or purchased at Sigma-Aldrich, San Luis, US; and Merck KgaA (Darmstadt, Germany). The chemical characterization of the pure final structures was assessed by nuclear magnetic resonance (1H-NMR, 13C-NMR) and High Resolution Mass Spectrometry (HRMS), both in agreement with those published by Barbero and colleagues.¹⁷³

Two potential CLV ADs were proposed, following the possible degradation pathways of CLV (Figure 25).^{173,239} AD-I corresponds to an aldehyde derived from oxopropamide (N -protein, 3-oxopropanamide), while AD-II to an amine derived from β -Alanine (N -protein, 3-aminopropanamide) (Figure 25).

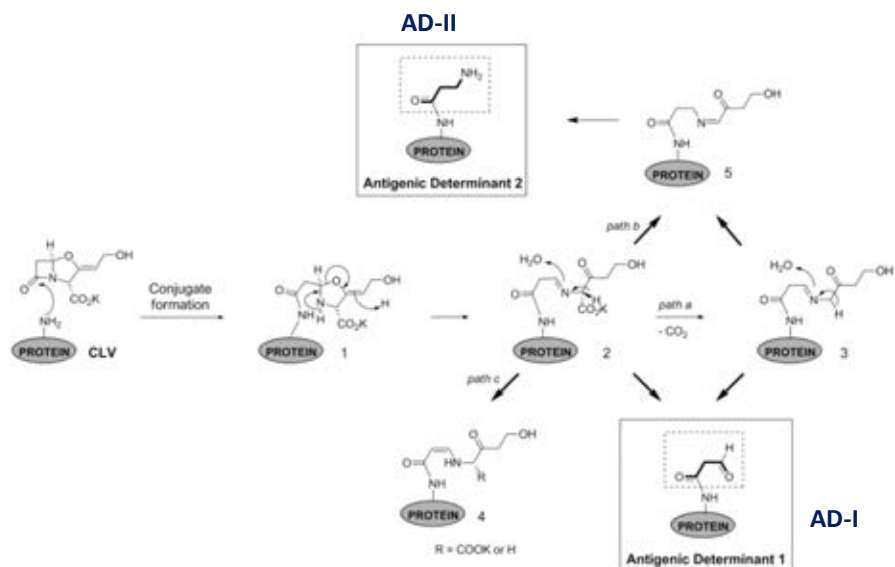


Figure 25. Degradation pathways of CLV. Obtained from Barbero *et al.* 2019.¹⁷³

From these two ADs, different analogue molecules were synthesised, with different chemical functional groups, providing them with different stability and capacity to bind to proteins. Specifically, Clav1-3 are AD-I analogue molecules, while Clav4-7 correspond to AD-II analogues (Figure 26.)

The synthetic analogues from CLV were prepared according to the methodology previously described by our group,¹⁷³ briefly:

Clav1, N-Butyl-3,3-dimethoxypropanamide, was synthesised by mixing butylamine with methyl 3,3-dimethoxypropanoate, and incubated 4 days at 60°C. After cooling, the mixture was diluted with dichloromethane and washed with hydrochloric acid (HCl). The organic layer was dried over magnesium sulphate (MgSO₄), filtered and concentrated.

Clav2, Bis(isobutylcarbonic) malonic anhydride, malonic acid was dissolved in dichloromethane (CH₂Cl₂) and cooled. The mixture was slowly mixed with N-Methyl morpholine and isobutylchloroformate, stirred and incubated for 2h at room temperature. Then, it was filtered and concentrated.

Clav3, 3,3-Diethoxypropanoic anhydride, 3,3-diethoxypropanoic acid was dissolved in CH₂Cl₂ and cooled. N-(3-Dimetilaminopropil)-N'-etilcarbodiimide (EDCI) was slowly added and incubated overnight at room temperature. The solution was washed, filtered, and concentrated.

Clav4, 3-Amino-N-butylpropanamide, N-butyl-3-(1,3-dioxoisindolin-2-yl) propanamide was mixed with hydrazine hydrate in Ethanol (EtOH) and heated at 100°C for 2h. CH₂Cl₂ was added, and then, filtered, and concentrated.

Clav5, 3-amino-propanoic acid, was purchased directly from Sigma-Aldrich (Saint Louis, USA).

Clav6, Succinimidyl 3-acetamidopropanoate, 3-acetamidopropanoic acid was diluted in CH₂Cl₂ and cooled. N-hydroxysuccinimide was slowly added and incubated overnight at room temperature. The solution was washed, filtered and concentrated.

Clav7, was commercial and purchased directly from Merck KgaA (Darmstadt, Germany).

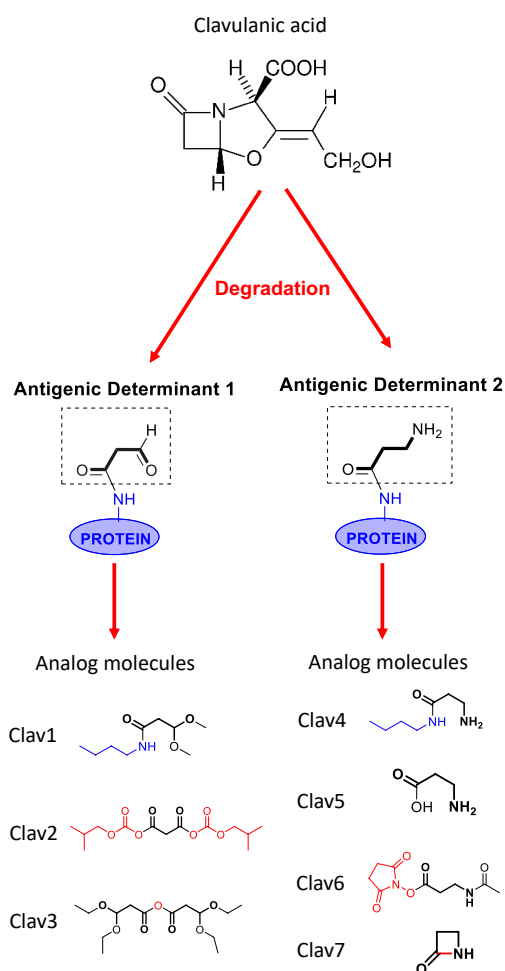


Figure 26. CLV ADs and their synthetic analogues. Black bold correspond to the AD fragment, blue to the butylamine, which mimic the conjugation to proteins, and red the functional group, important for the interaction with proteins.

SAMPLES OBTAINING

Depending on the study, between 40 and 72mL of heparinised blood was obtained by venipuncture from patients with confirmed DHRs and from healthy donors. Samples were collected by the nursing staff of the Allergy Unit from HRUM. All samples were processed immediately after collection at the research laboratory of IBIMA, from Hospital Civil (HRUM).

PERIPHERAL BLOOD MONONUCLEAR CELL ISOLATION

Peripheral Blood mononuclear cells (PBMCs) were isolated using ficoll density gradient. Ficoll has a density of 1.077g/mL, which allows separation of the granulocyte and erythrocyte fractions, with high density, from the monocyte/lymphocyte fraction, with lower density. After centrifugation without brake or acceleration, erythrocytes, neutrophils, and eosinophils sediment below the ficoll. In the upper ficoll phase, mainly lymphocytes and monocytes are found in a whitish halo. At the top of the tube is the fraction composed of plasma and platelets (Figure 27).

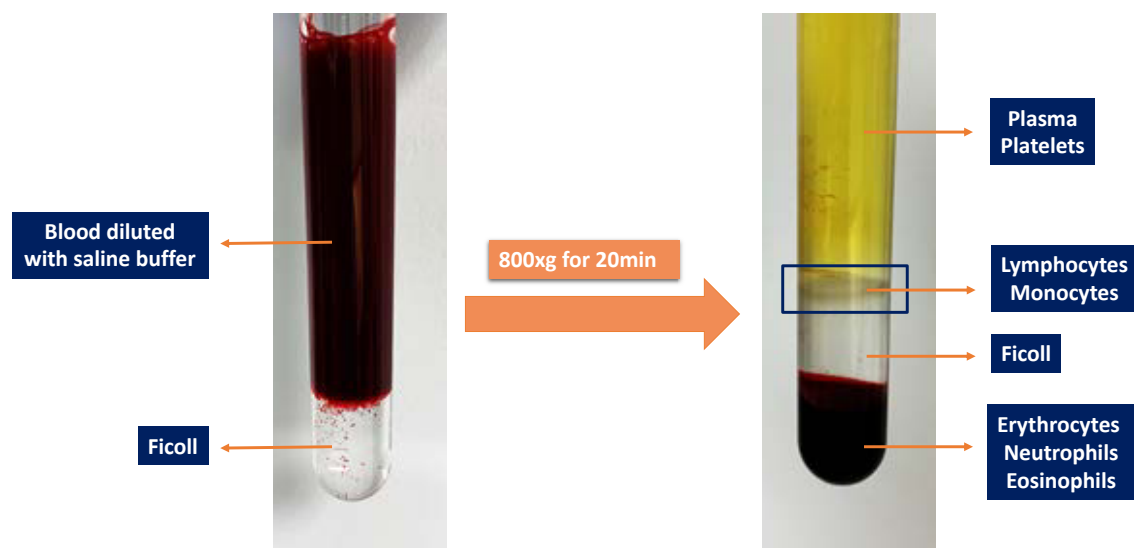


Figure 27. PBMCs isolation.

All the procedures were performed under sterile conditions, working in laminar flow cabinet, with sterile materials and reagents. Blood from each donor was diluted with an equal volume of saline solution. 3mL of ficoll was dispensed into sterile 12mL polystyrene tubes and the diluted blood was carefully dispensed on the ficoll. The samples were centrifugated at 800xg for 20min at room temperature (Figure 27). Afterwards, the plasma fraction was removed, and the PBMCs phase just below the ficoll was carefully collected with a Pasteur pipette, placed in clean 12mL tubes and washed twice with saline solution (300xg for 10min at room temperature). PBMCs were counted in a Neubauer-improved chamber and resuspended in 500 μ L of R40 medium.

CELL FREEZING AND THAWING

Isolated PBMCs were frozen in all cases, to perform the experiments in parallel between samples and days, to reduce the variability between procedures and samples. The only exception were the experiments with mDCs, where due to the low number of mDCs in peripheral blood, and after some experiments using fresh and frozen PBMCs, it was finally decided to isolate mDCs directly from fresh blood to increase the number of recovered mDCs.

7-15x10⁶ PBMCs were resuspended in 500 μ L of R40 medium and placed into sterile cryotubes, then 500 μ L of freezing medium was slowly pipetted into each cryotube, with continuous tapping. The cryotubes were placed in isopropanol freezing containers, and quickly transported to -80°C freezer for 24h. The next day, cryotubes were placed in liquid nitrogen (-196°C) until use. The samples were guarded with the guarantees of quality, traceability, and confidentiality required by LIB 14/2007, and RD1716/2011, in the Biobank of the IBIMA- HRUM.

For cellular thawing, the cryotubes were placed in a 37°C bath. Prior to complete thawing, PBMCs were removed from the bath. Then, PBMC suspensions were dispensed into 12mL tubes and cold culture medium was added dropwise up to the maximum capacity of the tube. Samples were centrifuged at 640xg for 10min at room temperature twice, to completely remove remaining DMSO.

mDCs ISOLATION

mDCs (CD1c⁺) were isolated directly from fresh PBMCs, using the CD1c (BDCA-1)⁺ dendritic cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The number of PBMCs was determined in a Neubauer-improved chamber, using trypan blue to assess cell viability. PBMCs were centrifuged at 300xg for 10min at room temperature. 1×10^8 PBMCs were resuspended in 100μL of separation buffer, 100μL CD19 μbeads, 100μL FcR blocking reagent and 100 μL of CD1c (BDCA-1)-biotin. The cells were mixed and incubated for 15min at 4°C and then washed with separation buffer and centrifuged at 300xg for 10min at 4°C. The supernatant was aspirated, and cells were resuspended in 500μL of separation buffer.

LD columns were placed in the magnetic field of the MACS separator and washed with 2mL of separation buffer. PBMCs suspension was placed on the column, and after passing completely through it, the column was washed with 1mL of separation buffer twice. The positive fraction within the column was discarded.

The unlabelled fraction was centrifuged at 300xg for 10min at 4°C, and the supernatant was aspirated and discarded. The cells were resuspended in 400μL of separation buffer and 100μL of anti-biotin μbeads, and after mixing, incubated for 15min at 4°C. After washing at 300xg for 10min at 4°C and discarding the supernatant, the cells were resuspended in 500μL of separation buffer.

The MS columns were placed in the magnetic field of the MACS separator and rinsed with 500μL of separation buffer. The cell suspension was applied onto the column. After the cell suspension had passed completely through the column, it was washed three times with 500μL of separation buffer. After the third wash, the column was removed from the MACS separator, and 1mL of separation buffer was added to the column. After pushing the plunger into the column, the mDCs were counted in a Neubauer-improved chamber, using trypan blue to assess the viability of the mDCs.

mDCs CHARACTERISATION

mDCs were characterized as $CD4^-CD8^-CD16^-CD14^-CD19^-CD1c^+$, HLA-DR⁺ cells by flow cytometry (FACSCanto II, BD Biosciences, New Jersey, USA) before (Figure 28) and after (Figure 29) isolation to ensure correct identification of the cell population, as well as to assess cell viability, and purity achieved after mDCs isolation process.

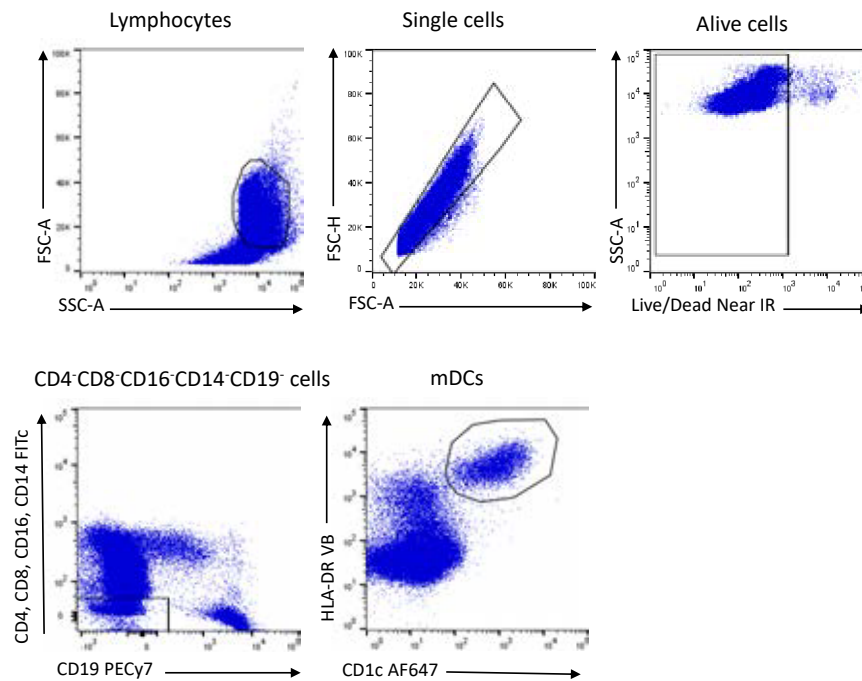


Figure 28. mDCs characterisation before purification.

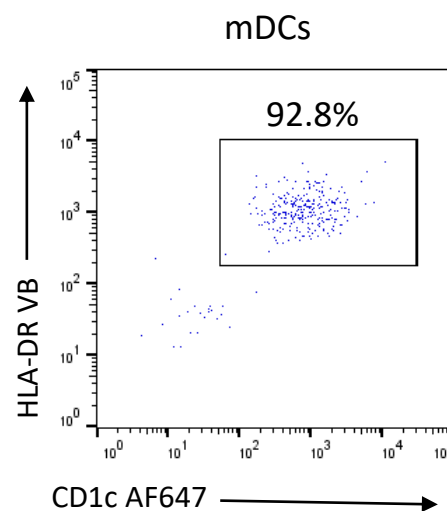


Figure 29. mDCs characterisation after purification.

MONOCYTE ISOLATION

Monocytes were isolated from frozen PBMCs by positive selection using CD14 magnetic μ beads. After thawing and centrifugation, PBMCs numbers were counted in a Neubauer-improved chamber and trypan blue. PBMCs were then centrifuged at 300xg for 10min at room temperature. 1×10^7 PBMCs were resuspended in 80 μ L separation buffer and 20 μ L of CD14 μ beads. The cells were mixed and incubated for 15min at 4°C. Then, PBMCs were washed by adding separation buffer up to the top of the 12mL tube and centrifugated at 300xg for 10min at 4°C. The supernatant was aspired and PBMCs were resuspended in 500 μ L of separation buffer.

LS columns and MACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) were used for magnetic separation. Each LS column was placed in the magnetic field of the MACS separator and rinsed with 3mL of separation buffer. 500 μ L of the cell suspension was placed into the column. After cell suspension had passed completely trough the column, it was washed by adding 3mL of separation buffer three more times. The unlabelled cell fraction (CD14⁻cells) was collected in 12mL tubes. After passing all the buffer trough the column, it was removed from the separator, and 5mL of separation buffer was added. Then, the plunger was firmly pushed into the column, and CD14⁺ cells were collected in a new 12mL tube.

Buffer containing the CD14⁻ cell fraction was centrifugated at 300xg for 10min at 4°C and frozen until their use as described previously.

MONOCYTES CHARACTERISATION

After purification, monocytes were characterised by flow cytometry. The strategy followed is showed in Figure 30. Briefly, FSC-A and SSC-A were gated to select the monocyte population. Then, duplets were discarded by plotting FSC-A vs. FSC-H and SSC-A vs. SSC-H. Dead cells were discarded, and monocytes were characterized as CD1a⁻CD16⁻HLA-DR⁺CD14⁺cells. The monocyte purity found after isolation was higher than 95%.

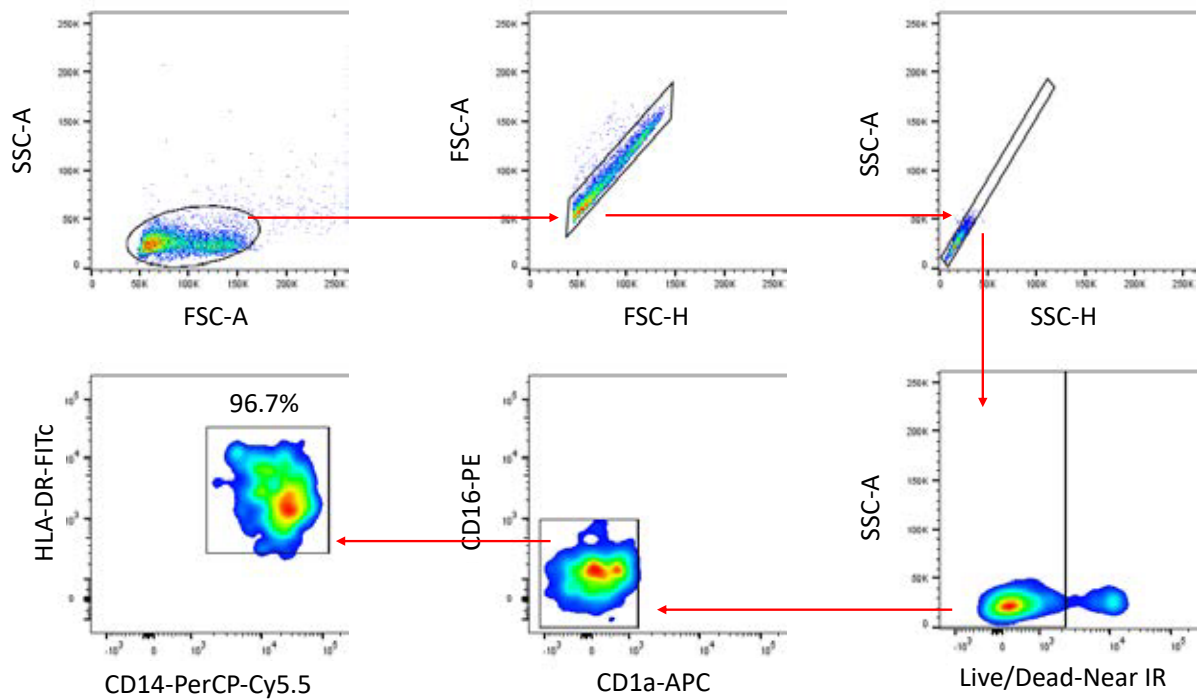


Figure 30. Monocyte characterisation.

moDCs OBTAINING

The protocol for obtaining moDCs was first described by Sallusto and Lanzavecchia in 1994.^{212,281} For this, 3×10^6 monocytes were cultured with 2mL of culture medium in a 12-well plate at 37°C in a controlled atmosphere with <5% of CO₂. The culture medium was supplemented with 200ng/mL of human recombinant GM-CSF and 100ng/mL of human recombinant IL-4. Half of the culture medium was renewed after the first 24 hours and every 48 hours thereafter. With each medium change, IL-4 and GM-CSF were also added. After 4 days of culture, a new non-adherent cell population with DC morphological characteristics can be observed by inverted microscopy (Figure 31). On the last day, immature moDCs were collected in 12mL tubes, and after centrifugation at 300xg for 10min at room temperature, moDCs were counted in an improved-Neubauer chamber.

MATERIAL AND METHODS

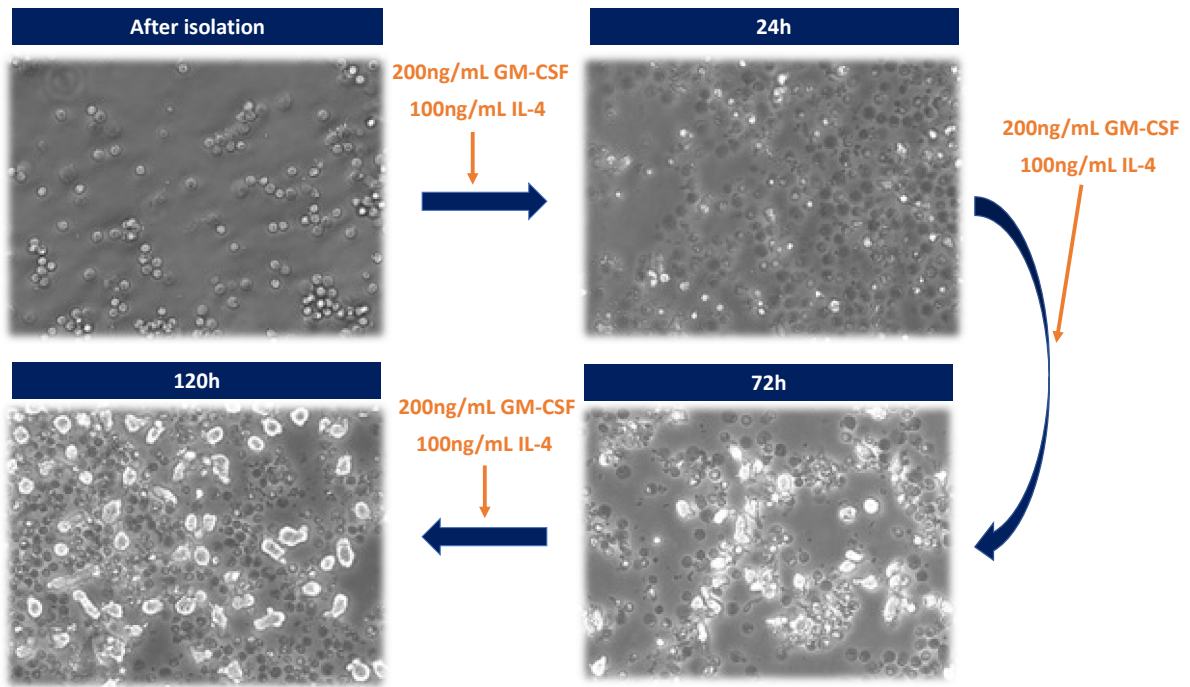


Figure 31. moDCs obtaining from monocytes. Morphological changes induced by GM-CSF and IL-4.

To test the phenotypic changes induced during monocyte culture with GM-CSF and IL-4, the CD14, HLA-DR and CD1a expression was analysed on monocytes just after the isolation process, and on moDCs after 120h of culture with GM-CSF and IL-4 (Figure 32). The expression of CD14 was much lower on moDCs than on monocytes, while monocytes did not express CD1a, but the expression of this marker highly increased on moDCs. Moreover, monocytes expressed high level of HLA-DR, although its expression was higher on moDCs.

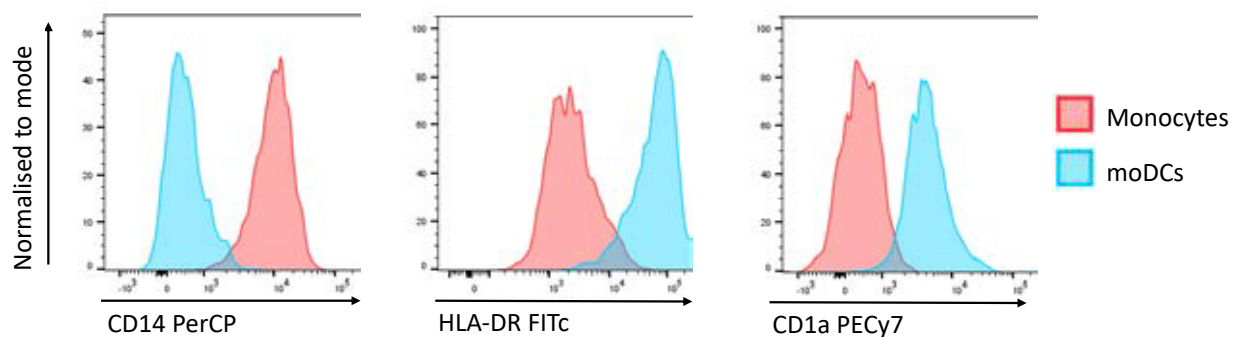


Figure 32. Phenotypic differences between monocytes (red) and moDCs (blue). MFI was expressed for each marker as normalised-to-mode-histograms.

DCs MATURATION ASSAYS

For the objectives 3 and 4, DCs (mDCs and moDCs) were cultured with the culprit drugs at different concentrations (Table 12) and/or with different CLV AD analogue molecules (Figure 26) at 0.5 and 1mM. Depending on the study, between 1.5×10^4 and 3×10^5 DCs were cultured with each drug in 96-well clear round bottom TC-treated microplates in 250 μ L of culture medium. As negative control, DCs with culture medium without stimulus was used, and as positive control, ultrapure lipopolysaccharide from *E. coli* K12 (LPS) was used at 1 μ g/mL. DCs were maintained in culture for 72h at 37°C in a controlled atmosphere with <5% of CO₂.

After 72h of culture, DCs were centrifugated at 300xg for 5min at room temperature and washed with phosphate-buffered saline (PBS). Cells were resuspended with 100 μ L of PBS and stained with monoclonal antibodies: CCR7-PerCP-CyTM5.5, CD40-PE, CD80-PECy7, CD83-APC, and CD86-FITc at 1.75-2.5 μ g/mL. Additionally, live-dead fixable near-infra red dye was added to assess DC viability after drug stimulation. After being incubated for 30min at 4°C, DCs were washed with 150 μ L of PBS and centrifuged at 300xg for 5min at 4°C.

DCs were acquired in a FACSCanto II (BD Biosciences, New Jersey, USA). Data were analysed in FlowJo (BD Biosciences, New Jersey, USA). Briefly, in a first gating step, cell size (FSC) and granularity (SSC) parameters were used to select DCs, leaving out cell debris or possible lymphocyte contamination. Then, alive cells were selected (Figure 33).

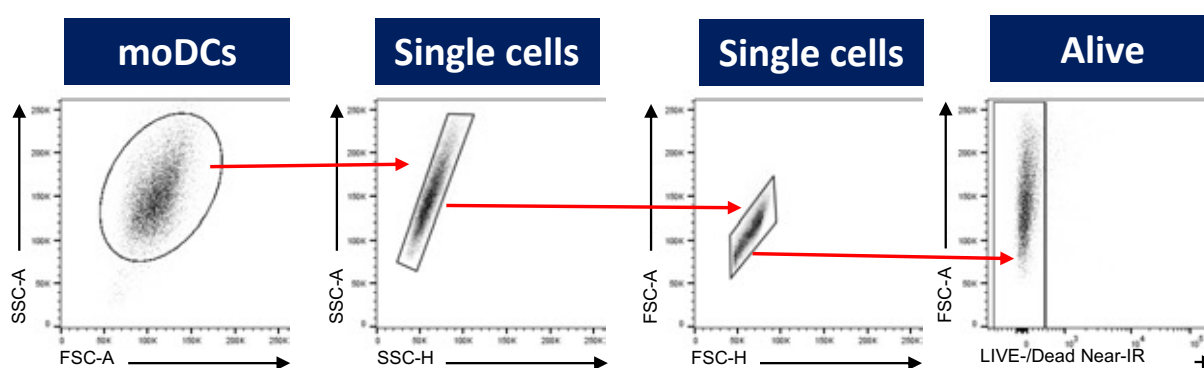


Figure 33. Flow cytometry strategy for selecting moDCs in DC maturation assays.

MATERIAL AND METHODS

The MFI as well % of expression for each marker was analysed. For this, DCs without stimulus were used to establish a cut-off of around 2.5% of expression for each marker. Figure 34 shows representative dot plots examples with moDCs without stimulus, and after culturing with LPS or drugs/ADs. Then, based on the established cut-offs, the expression of each marker was analysed for the positive control, to ensure the response to LPS, and with each drug/metabolite condition. The % of expression was always referred to the unstimulated condition, to reduce the maturation DC variability between subjects. This maturation index (MI) was calculated as follows:

$$\text{Maturation Index (MI)} = \frac{\% \text{ of marker expression on stimulated DCs}}{\% \text{ of marker expression on unstimulated DCs}}$$

Positive results were considered when MI was equal or higher than 2.

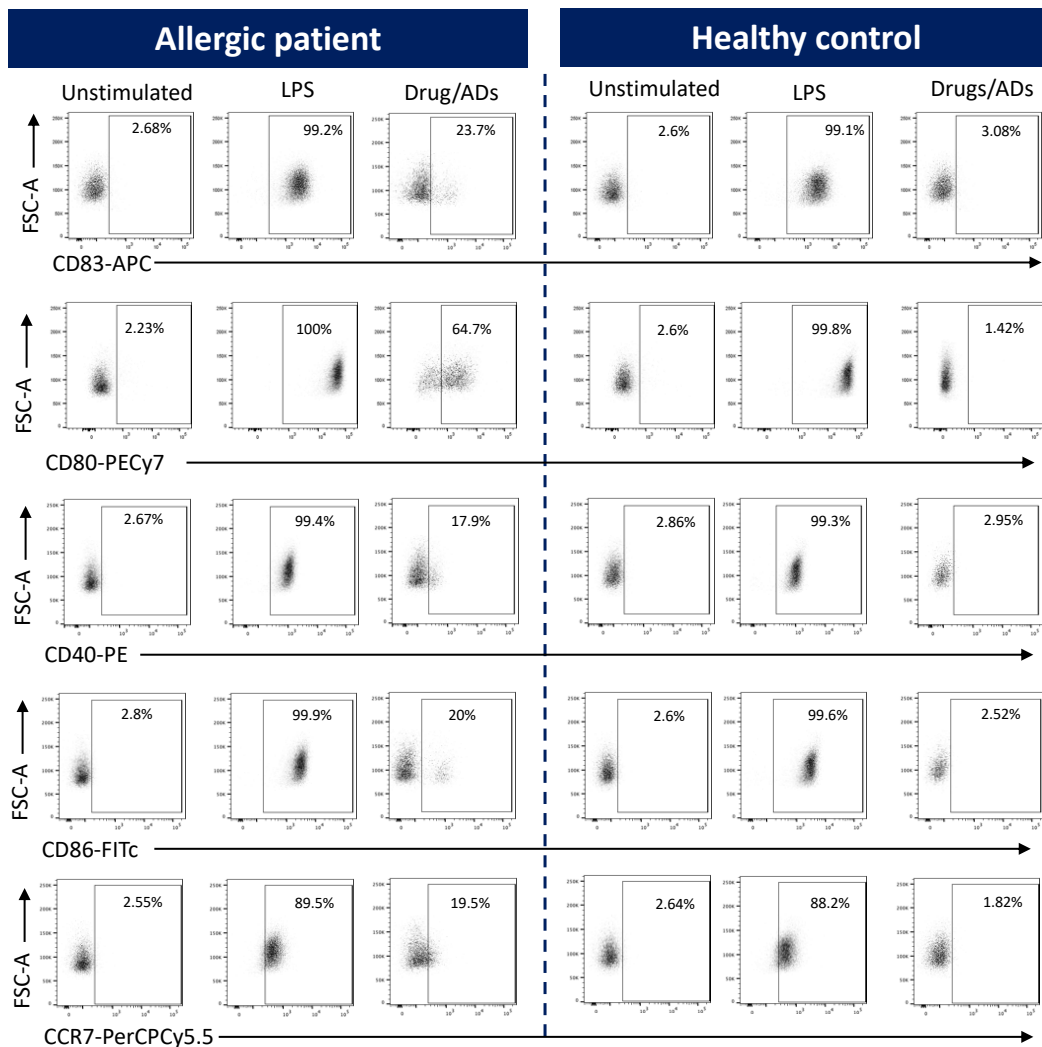


Figure 34. Representative examples of moDCs maturation assays. Dot plots and % of activation for all markers analysed on unstimulated or stimulated moDCs with LPS or drug/antigenic determinants from an allergic patient and a healthy control.

LTT CULTURES

Conventional LTT (C-LTT)

For the objectives 1, 2, and 4, 120×10^6 PBMCs were resuspended in 80 μ L of PBS and CFSE pre-reconstituted with DMSO to a final concentration of 1 μ M. PBMCs were incubated at room temperature in the dark for 10min. Then, culture medium was slowly added, and cells were centrifuged at 300xg for 10min at room temperature for 3 times. CFSE-PBMCs were counted in an improved-Neubauer chamber using trypan blue to assess cell viability.

1.5×10^5 CFSE-PBMCs were transferred to 96-well clear round bottom TC-treated microplates in 250 μ L of culture medium with the different stimuli. The cells were cultured with the drugs or CLV ADs at different concentrations (Table 12 for NIDHRs, and at 0.5 and 1mM for AX, CLV and its different synthetic analogues). CFSE-PBMCs only with culture medium was used as negative control, phytohemagglutinin (PHA) at 20 μ g/mL was used as positive control. Cells were cultured for 6/7 days at 37°C in an atmosphere with <5% of CO₂.

Drug-primed mDCs/moDCs LTT

For all the objectives, isolated mDCs or moDCs were cultured with the different stimuli for 3 days, similar to that described above for DC maturation assays. After this, DCs were washed with culture medium twice to remove the drugs present in the medium. In parallel, the remaining PBMCs after separation of monocytes or isolation of mDCs were incubated with CFSE as previous described for C-LTT. Then, DCs were co-cultured with CFSE-PBMCs in a 1:10 ratio for 6/7 days at 37°C in an atmosphere with <5% of CO₂. It is important to highlight that a condition with unstimulated lymphocytes, without DCs is necessary to establish a cut-off for spontaneous lymphocyte proliferation.

LTT FLOW CYTOMETRY ANALYSIS

After cell cultures, the proliferative response of the different cell populations was analysed by flow cytometry, in a FACSCanto II (BD Biosciences, New Jersey, USA). For the first and second objectives, related to NIDHRs, the proliferative response of CD3⁺T, CD4⁺T, CD8⁺T and NK cells was analysed. Moreover, the proliferation of subpopulations CD4⁺Th1 cells (CD3⁺CD4⁺CXCR3⁺IFN- γ ⁺), CD4⁺Th2 cells (CD3⁺CD4⁺CRTH2⁺IL-4⁺), cytotoxic NK (CD3⁻CD56⁺Perforin⁺) and inflammatory NK cells (CD3⁻CD56⁺ IFN- γ ⁺) was assessed. The selection gating strategy is shown in Figure 35.

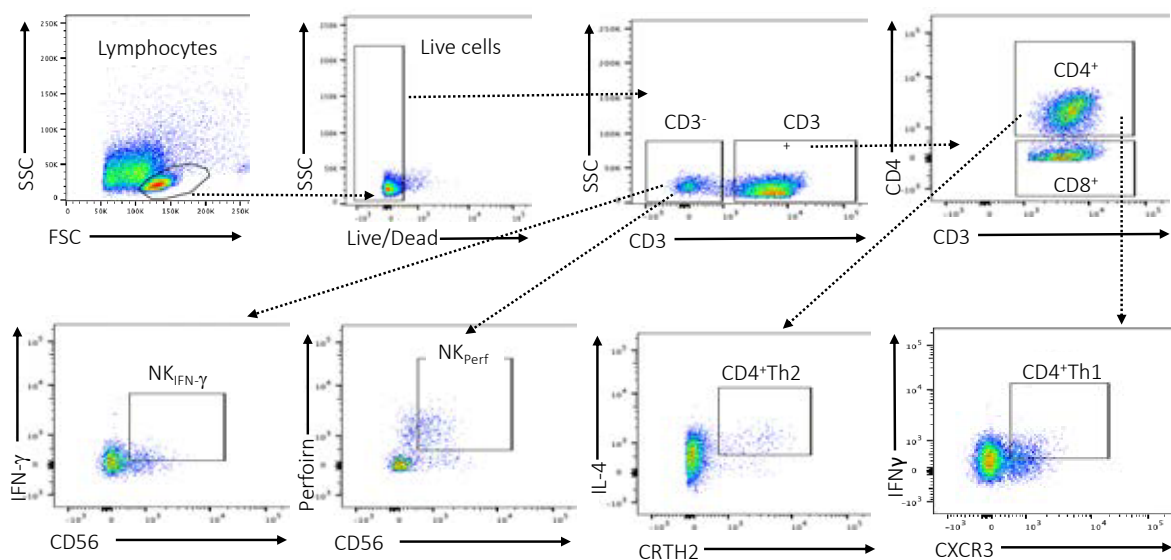


Figure 35. Flow cytometry gating strategy for evaluating NIDHRs

In relation to the third and fourth objectives, related to IDHRs, due to the high number of conditions to assess IDHRs to CLV and its ADs, as well as the small number of isolated mDCs obtained, the flow cytometry strategy was simplified as much as possible to reduce the number of cells needed to perform these studies. Therefore, only the proliferative response of CD3⁺, CD4⁺ and CD8⁺, and the subpopulations of CD4⁺Th1 (CD3⁺CD4⁺CCR5⁺) and CD4⁺Th2 (CD3⁺CD4⁺CRTH2⁺) cell was assessed (Figure 36). To ensure the correct gating strategy, isotype controls for each marker was used (Figure 37).

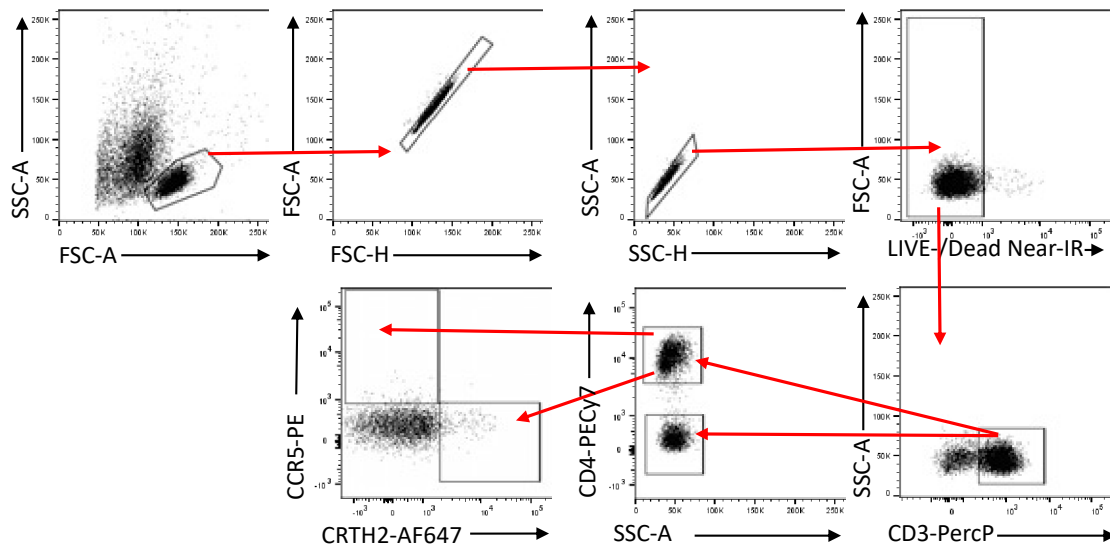


Figure 36. Flow cytometry gating strategy for evaluating IDHRs

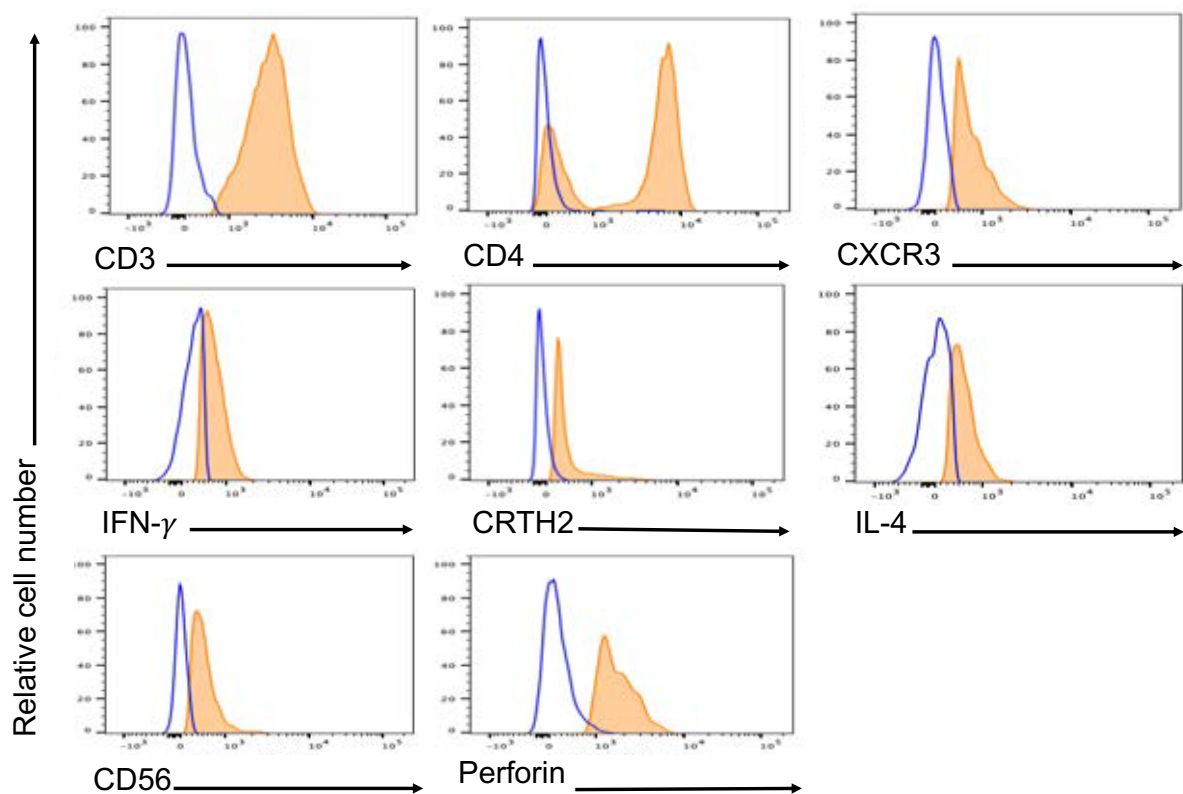


Figure 37. Isotype controls. Histograms represent the relative cell number for each marker (orange) with its respective isotype control (blue).

MATERIAL AND METHODS

The proliferative response of the different cell populations was analysed by the dilution of CFSE (expression of % CFSE_{dim} cells) (Figure 38). The unstimulated condition was used to establish a cut-off for spontaneous lymphocyte proliferation. Proliferation results were represented as proliferation index (PI), calculated as the ratio between the % of CFSE_{dim} cells in stimulated and unstimulated cells.

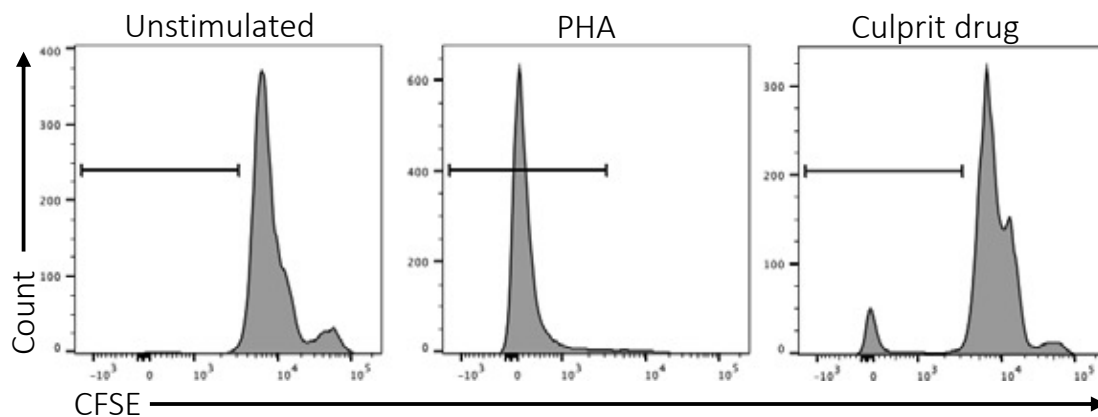


Figure 38. Representative examples of % of CFSE_{dim} cells. Histograms represent the %CFSE_{dim} cells in unstimulated cells and stimulated with PHA or the culprit drug/metabolite.

Because the inclusion of mDCs/moDCs triggers an unspecific proliferative response in lymphocyte proliferation (Figure 39), the PI for mDCs and moDCs LTT was calculated as:

$$PI = \frac{(\% \text{ CFSE}_{\text{dim}} \text{ stimulated lymphocytes} + \text{mo-DCs}) - (\% \text{ CFSE}_{\text{dim}} \text{ unstimulated lymphocytes} + \text{mo-DCs})}{\% \text{ CFSE}_{\text{dim}} \text{ unstimulated lymphocytes}}$$

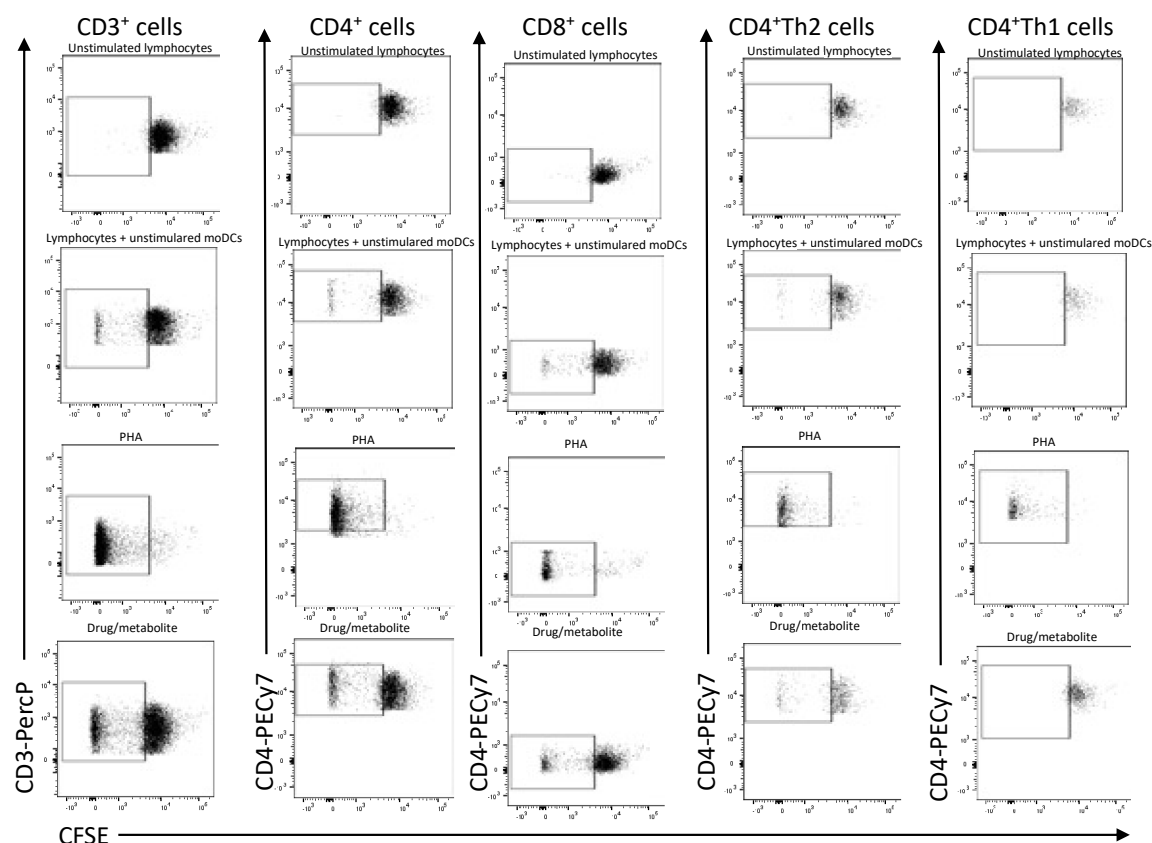


Figure 39. Representative examples of lymphocyte proliferation of different cell population. Dot plots represent the expression of CFSE in cells without stimulus, PHA and the culprit drug or metabolite.

STATISTICAL ANALYSIS

Classical statistics such as mean, median, standard deviation or standard error were calculated for continuous quantitative variables. The normality distribution of the data was assessed using Kolmogorov-Smirnov test. For pairwise comparisons of non-normally distributed quantitative data, the Mann-Whitney test was used, whereas for multiple comparisons, Kruskal-Wallis test was used. Receiver Operation Characteristic (ROC) curves were calculated for LTTs of NIDHRs to select the most appropriate cut-off that allows to differentiate between positive and negative results with the best sensitivity/specificity balance. To assess differences between qualitative variables, the Chi square (χ^2) test was performed. P-values of less than or equal to 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism v7/v8 (Dotmatics, Bishop's Stortford, UK).

CHAPTER I

Improving the sensitivity of the lymphocyte transformation test
based on flow cytometry for the evaluation on non-immediate
drug hypersensitivity reactions.

SUMMARY

Symptoms of NIDHRs appear from 1h to days or even weeks after drug administration, with the skin being the most common organ involved. Several factors make the diagnosis of these reactions difficult, such as the great **variability of clinical manifestations**, which is related to **heterogeneity in the immune mechanisms and cells involved**. Furthermore, **identification of the culprit drug or metabolites** involved in the reaction is not always easy, especially in patients using different drugs in the days or even weeks prior to the onset of symptoms. In addition, **STs have low sensitivity**, and DPT is needed in most cases to confirm tolerance or not to different drugs. Nevertheless, DPT is not possible for all drugs, not risk-free, and contraindicated for most severe reactions.

Therefore, *in vitro* tests may represent **safe alternative diagnostic** tools to assess a NIDHR. Moreover, they are useful techniques to **identify the specific drug or metabolites** responsible for triggering the reaction, but also the **clinical entity and mechanism involved**. Among them, **LTT** is the most widely **used** and **validated** *in vitro* test for evaluating NIDHRs. However, the use of radioactive reagents, the lack of standardisation, the low and variable sensitivity between studies, the technical complexity of the technique and the long time between the sample collection, and LTT results have led to LTT being considered a research tool, rather than a potential clinical technique.

In this chapter we evaluated different factors that can be used to improve LTT sensitivity. Herein, we demonstrated that the **inclusion of pre-primed moDCs as APCs** significantly **increased LTT sensitivity**. Furthermore, the analysis of the drug-specific **proliferative response of different cell population** can help not only to improve LTT sensitivity, but also to efficiently determine the concrete drug causing the reaction and better identify the specific clinical mechanism involved. Finally, we demonstrated that the use of **flow cytometry** instead of using radioactivity allows reproducible results based on different cell subpopulation in a single assay, without the need to increase the number of cells or experimental conditions required.

RESULTS

Patients and healthy controls characterisation

Samples from 37 patients with NIDHRs to different drugs confirmed by the Allergy Unit from HRUM were used in this study. The clinical characteristics of the patients and the results of the *in vivo* tests from the allergological work-up for each patient are shown in Table 15.

Most patients included in the study were females: 24 out of 37 (64.9%) and 13 were males (35.1%). The mean age was 55.2 ± 22.4 years. The most common clinical entity involved was MPE in 21 out of 37 patients (56.8%), followed by SJS/TEN in 10 (27%), AGEP in 3 (8.1%), DRESS in 2 (5.4%) and fix drug eruption (FDE) in one (2.7%). BLs are the drugs most frequently implicated in the reactions: in 14 of the 37 cases (35.8%), followed by ICM in 11 (29.7%), FQs in 4 (10.8%), xanthine oxidase inhibitors, anticonvulsants, and sulphonamides in 2 (5.4% each), and a benzodiazepine and ferrous supplement in 1 case (2.7% each). The time between drug administration and onset of symptoms was of 6.5 ± 5.5 days. The time interval between the reaction and sample collection was 160 ± 186 days.

Thirteen out of 37 patients (35%) were diagnosed by STs, 10 by IDT (27%) and 3 by PT (8.1%), 13 by DPT (35%). The remaining 11 patients (30%) were diagnosed according to the algorithm of the Spanish pharmacovigilance system (ASPS).

Samples from 21 subjects who had not reported DHRs to any drug were included in the study as the HC group.

Patients	Sex	Age	Clinical entity	Drug family	Culprit drug	Interval drug-reaction	Interval reaction-study	STs	DPT	ASPS
P-1	F	14	MPE	BL	Amoxicillin	6 days	70 days	-	+	12
P-2	M	11	MPE	BL	Amoxicillin	4 days	90 days	-	+	12
P-3	F	27	MPE	BL	Amoxicillin	7 days	130 days	-	+	12
P-4	M	12	MPE	BL	Amoxicillin	1 days	305 days	+	ND	9
P-5	F	29	MPE	BL	Amoxicillin	1 days	140 days	-	+	11
P-6	F	43	MPE	BL	Amoxicillin	2 days	730 days	-	+	11
P-7	F	46	MPE	BL	Amoxicillin	2 hours	155 days	-	+	11
P-8	F	74	MPE	BL	Amoxicillin	2 days	120 days	+	ND	11
P-9	F	34	MPE	BL	Clavulanic acid	2 hours	150 days	+	ND	8
P-10	F	82	MPE	BL	Cefixime	5 days	14 days	-	+	11
P-11	F	70	MPE	ICM	Iobitridol	10 hours	70 days	+	ND	11
P-12	F	69	MPE	ICM	Iobitridol	1 days	152 days	-	+	11
P-13	F	63	MPE	ICM	Iomeron	12 hours	30 days	+	ND	12
P-14	F	66	MPE	ICM	Iomeron	2 days	640 days	-	+	11
P-15	F	75	MPE	ICM	Iomeron	12 hours	70 days	+	ND	11
P-16	M	79	MPE	ICM	Iomeron	2 hours	710 days	-	+	11
P-17	F	77	MPE	ICM	Iomeron	12 hours	365 days	-	+	11
P-18	F	59	MPE	ICM	Iodixanol	12 hours	70 days	-	+	11
P-19	M	75	MPE	ICM	Iodixanol	10 hours	41 days	-	+	11
P-20	M	83	MPE	ICM	Iodixanol	1 days	10 days	+	ND	7
P-21	F	35	MPE	SULPH	Sulfamethoxazole	7 days	30 days	+	ND	9
P-22	M	17	SJS/TEN	BL	Amoxicillin	5 days	220 days	ND	ND	8
P-23	F	75	SJS/TEN	RCM	Iomeron	1 days	40 days	-	ND	9
P-24	F	64	SJS/TEN	SULPH	Sulfamethoxazole	2 days	180 days	ND	ND	8
P-25	M	54	SJS/TEN	QNL	Ciprofloxacin	2 days	31 days	+	ND	8
P-26	F	44	SJS/TEN	QNL	Ciprofloxacin	13 days	30 days	-	ND	8
P-27	M	50	SJS/TEN	QNL	Ciprofloxacin	4 days	200 days	ND	ND	9
P-28	F	91	SJS/TEN	QNL	Ciprofloxacin	19 days	400 days	-	ND	8
P-29	F	38	SJS/TEN	ACV	Phenobarbital	20 days	50 days	+	ND	8
P-30	F	57	SJS/TEN	ACV	Carbamazepine	20 days	150 days	+	ND	8
P-31	M	55	SJS/TEN	XOI	Allopurinol	2 days	60 days	-	ND	10
P-32	M	53	AGEP	BL	Amoxicillin-Clavulanic acid	12 days	180 days	+	ND	8
								-	ND	8
P-33	F	24	AGEP	BL	Amoxicillin-Clavulanic acid	8 hours	52 days	ND	ND	9
								ND	ND	9
P-34	M	66	AGEP	XOI	Allopurinol	3 days	86 days	-	ND	11
P-35	M	73	DRESS	BZD	Diazepam	6 days	30 days	-	ND	11
P-36	F	82	DRESS	Iron	Iron	3 days	86 days	-	ND	9
P-37	M	75	FDE	BL	Cefepime	6 days	38 days	+	ND	9

Table 15. Demographic and clinical data of patients with NIDHRs. Abbreviations: ACV, anticonvulsant; AGEP, acute generalised exanthematous pustulosis; ASPS, algorithm of the Spanish pharmacovigilance system; BL, betalactam; DRESS, drug reaction with eosinophilia and systemic symptoms; DPT, drug provocation test; F: female; FDE, fixed drug eruption; BZD, benzodiazepine; ICM, iodinated contrast media; M, male; MPE, maculopapular Exanthema; ND, not done; QNL, quinolone; ST, skin test; SJS-TEN, Steven-Johnson syndrome/toxic epidermal necrolysis; SULPH: sulphonamide; XOI: xanthine oxidase inhibitors.

Conventional LTT (C-LTT) vs. drug pre-primed moDCs LTT (dDC-LTT)

First, we evaluated and compared the results of the C-LTT and dDC-LTT assays by analysing the drug-specific proliferative response of the overall T cell population (CD3⁺T cells). The PI results for each assay, patient and drug are shown in Table 16.

Patients	Clinical entity	Culprit drug	C-LTT results	dDC-LTT results
P-1	MPE	Amoxicillin	1.32	0.58
P-2	MPE	Amoxicillin	0.81	1.11
P-3	MPE	Amoxicillin	1.65	8.81
P-4	MPE	Amoxicillin	3	5.22
P-5	MPE	Amoxicillin	1.23	1.20
P-6	MPE	Amoxicillin	0.93	1.09
P-7	MPE	Amoxicillin	2	5.57
P-8	MPE	Amoxicillin	1.32	0
P-9	MPE	Clavulanic acid	0.43	0.19
P-10	MPE	Cefixime	0.58	12.6
P-11	MPE	Ibuprofen	1.29	0
P-12	MPE	Ibuprofen	1.36	3.35
P-13	MPE	Ibuprofen	1.53	0.14
P-14	MPE	Ibuprofen	0.86	0.23
P-15	MPE	Ibuprofen	0.29	0.54
P-16	MPE	Ibuprofen	1.02	1.59
P-17	MPE	Ibuprofen	0.87	ND
P-18	MPE	Ibuprofen	3.43	10.1
P-19	MPE	Ibuprofen	ND	10.5
P-20	MPE	Ibuprofen	0.86	1.05
P-21	MPE	Sulfamethoxazole	12.41	ND
P-22	SJS/TEN	Amoxicillin	3.97	4.46
P-23	SJS/TEN	Ibuprofen	1.76	8.64
P-24	SJS/TEN	Sulfamethoxazole	6.34	13.61
P-25	SJS/TEN	Ciprofloxacin	1.21	0.93
P-26	SJS/TEN	Ciprofloxacin	1.39	10.5
P-27	SJS/TEN	Ciprofloxacin	2.87	ND
P-28	SJS/TEN	Ciprofloxacin	2.47	ND
P-29	SJS/TEN	Phenobarbital	ND	2.19
P-30	SJS/TEN	Carbamazepine	ND	1.47
P-31	SJS/TEN	Allopurinol	2.29	21.53
P-32	AGEP	Amoxicillin-Clavulanic acid	16.86 ND	10.10 2.2
P-33	AGEP	Amoxicillin-Clavulanic acid	1.24 ND	2.42 3.19
P-34	AGEP	Allopurinol	1.23	1.04
P-35	DRESS	Diazepam	1.29	5.66
P-36	DRESS	Iron	3.43	ND
P-37	FDE	Cefepime	0.96	0.77

Table 16. Proliferation Index results of CD3⁺T cells in C-LTT and dDC-LTT. Positive results are shown in bold. Abbreviations: AGEF, acute generalised exanthematous pustulosis; DRESS, drug reaction with eosinophilia and systemic symptoms; FDE, fixed drug eruption; MPE, maculopapular exanthema; SJS-TEN, Steven-Johnson syndrome/toxic epidermal necrolysis.

To compare both techniques and to establish cut-off points to differentiate between positive and negative results, based on the best balance between sensitivity and specificity, ROC curves were performed (Figure 40). To build these curves, LTT results from HC with specific drugs were included in a similar proportion to those involved in the patients' group, to reduce the possible effect of the nature of the drugs in the analysis. The area under curve (AUC) was higher for dDC-LTT (0.783) with a 95% confidence interval (CI) of 0.650-0.915 and a significant p-value (<0.0005) compared to the C-LTT with an AUC of 0.605, 95% CI of 0.472-0.737, and a p-value of 0.128.

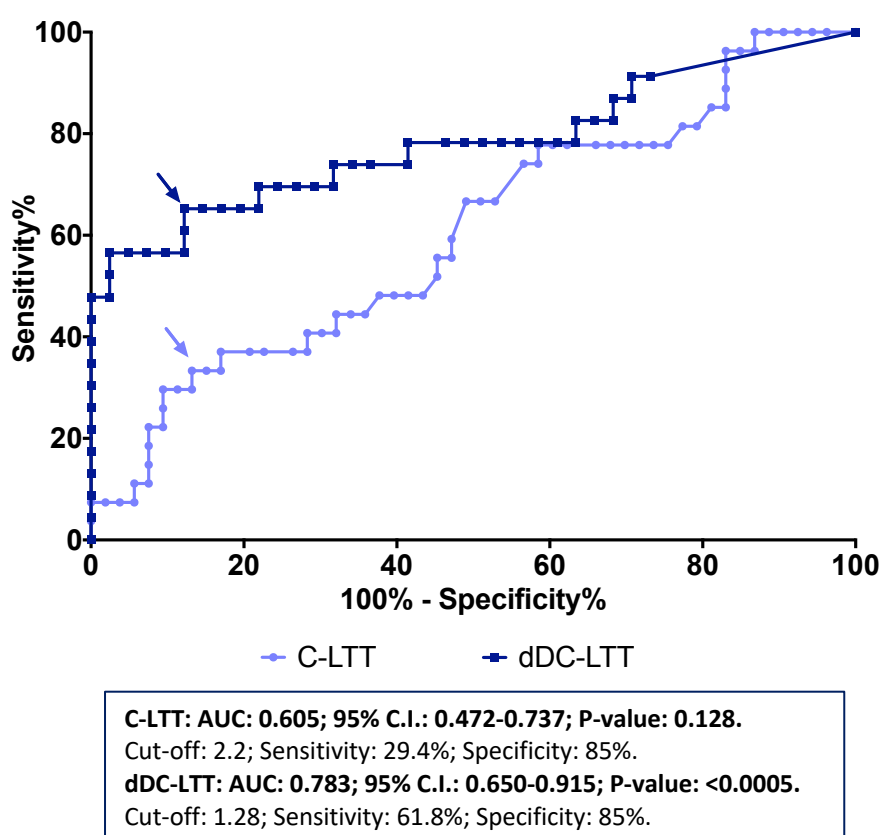


Figure 40. ROC curve analysis for the proliferative results of CD3⁺T cells in C-LTT and dDC-LTT.
Arrows represent the cut-off selected for each technique with the best balance between sensitivity and specificity.

Following ROC curve analysis, a cut-off point of 2.2 was selected for C-LTT, with a sensitivity of 29.4% and a specificity of 85%. For the dDC-LTT, a cut-off point of 1.28 was selected, with a sensitivity of 61.8% and a specificity of 85% (Figure 40). Based on these cut-off points, the contingency tables for C-LTT and dDC-LTT for positive and negative results are shown in Table 17.

General (C-LTT)			General (dDC-LTT)		
	+	-		+	-
Allergic patients	10	24	Allergic patients	21	13
Healthy controls	3	18	Healthy controls	3	18

Table 17. Contingency tables for positive and negative proliferative results of CD3⁺T cells in C-LTT and dDC-LTT.

Results indicated a significant lower percentage of positive results in AP with C-LTT (29.4%) compared to dDC-LTT (61.8%) ($p=0.026$) (Figure 41). Interestingly, the combination of C-LTT and dDC-LTT results did not improve the % of positive cases detected in AP compared to dDC-LTT alone (61.8%), although the number of positive cases in the HC group (false positive) increased slightly from 15% to 17.4% (Figure 41).

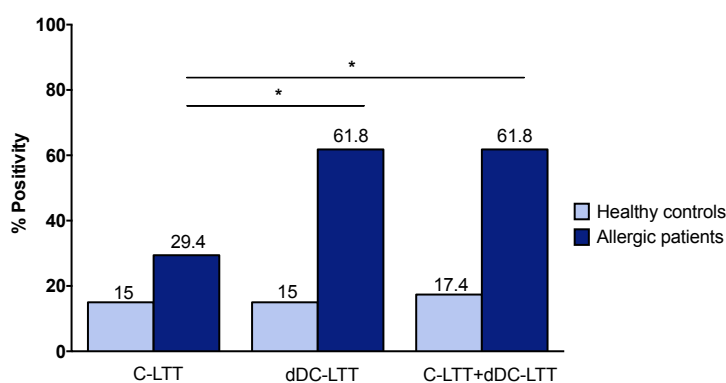


Figure 41. Positive cases detected with each LTT technique. Bars represent the % of positive cases detected in AP and HC with C-LTT and dDC-LTT, and with the combination of results from both assays. Comparisons were assessed by χ^2 test (* $p<.05$).

C-LTT vs. dDC-LTT in different clinical entities

The PI was significantly higher in patients compared to HC with dDC-LTT regardless of the clinical entity involved, while with C-LTT only significant differences were detected between SJS/TEN patients and HC (Figure 42). Among clinical entities, higher PI was only observed between SJS/TEN and MPE patients when dDC-LTT was performed (Figure 42). Notably, C-LTT with AGEP was only carried out in three samples.

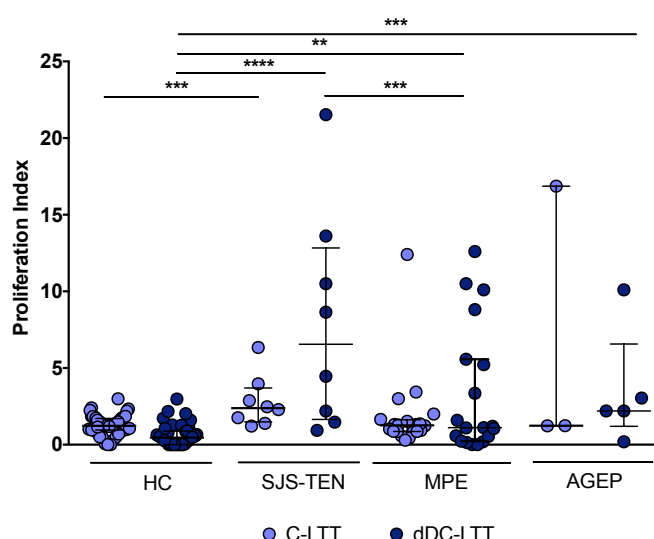


Figure 42. Proliferative response of CD3⁺T cells with C-LTT and dDC-LTT in patients with different clinical entities. Scatter plots represent proliferation index of CD3⁺T cells in healthy controls and SJS/TEN, MPE and AGEP patients with C-LTT and dDC-LTT. Comparisons were assessed by Mann-Whitney U test (** $p < .01$; *** $p < .001$; **** $p < .0001$).

Having analysed the overall sensitivity and specificity of CD3⁺T cells with both C-LTT and dDC-LTT, we next performed the same analysis, but differentiating between the three clinical entities most represented in our patients, SJS/TEN, MPE and AGEP (Figure 43). For SJS/TEN patients, the AUC was higher in dDC-LTT (0.952), 95% CI= 0.886-1.02 and p -value < 0.0001 , than in C-LTT (0.760), 95% CI=0.602-0.918, and a p -value of 0.02 (Figure 43A). Similar results were observed for AGEP, with higher AUC for dDC-LTT (0.895), 95% CI= 0.705-1.087 and a p -value of 0.006 compared to C-LTT (0.564), 95% CI=0.518-0.978 (Figure 43B). However, for MPE, similar AUC were observed in dDC-LTT (0.603), 95% CI=0.340-0.816 and C-LTT (0.564), 95% CI=0.389-0.738 (Figure 43C).

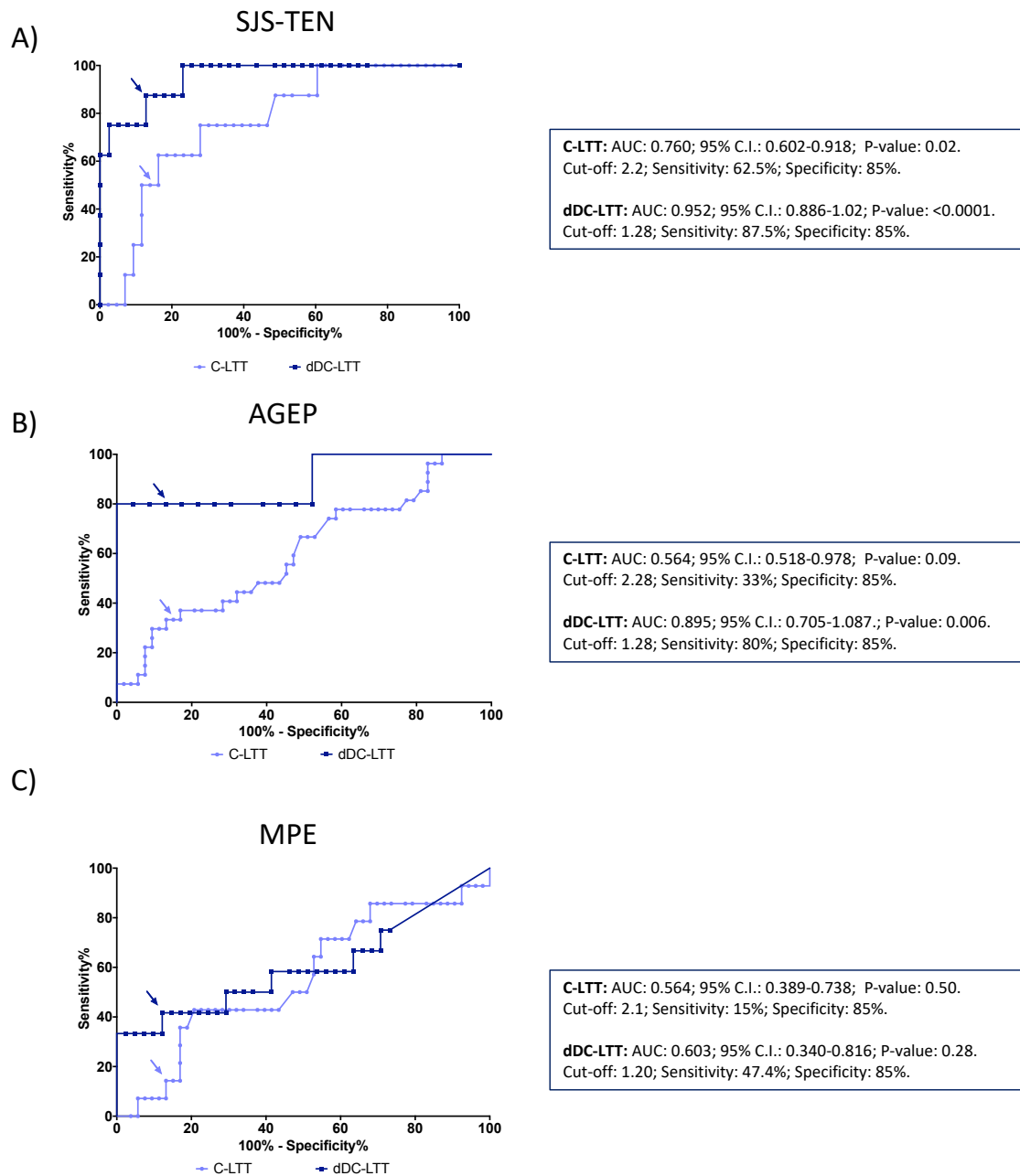


Figure 43. ROC curve analysis for the proliferative results of CD3⁺T cells in C-LTT and dDC-LTT in different clinical entities. A) SJS/TEN; B) AGEP; C) MPE. Arrows represent the cut-off selected for each technique with the best balance between sensitivity and specificity.

After performing the ROC curve analysis, the cut-off points were established to differentiate positive and negative results for each clinical entity and LTT assay. For SJS/TEN and dDC-LTT a cut-off of 1.28 (87.5% of sensitivity) and for C-LTT, 2.2 (62.5% of sensitivity) was established (Figure 42A). For AGEP, a cut-off point of 1.28 was established for dDC-LTT (80% of sensitivity) and 2.28 for C-LTT (sensitivity of 33%) (Figure 42B). For MPE with dDC-LTT, a cut-off point of 1.20 (47.4% of sensitivity) and 2.1 for C-LTT (15% of sensitivity) was settled (Figure 42C). The specificity was 85% for all clinical entities and LTT assays. Based on these cut-off points, contingency tables for positive and negative results were shown in Table 18 for each clinical entity and LTT assay.

SJS-TEN (C-LTT)			SJS-TEN (dDC-LTT)		
	+	-		+	-
Allergic patients	5	3	Allergic patients	7	1
Healthy controls	3	18	Healthy controls	3	18

MPE (C-LTT)			MPE (dDC-LTT)		
	+	-		+	-
Allergic patients	3	17	Allergic patients	9	10
Healthy controls	3	18	Healthy controls	3	18

AGEP (C-LTT)			AGEP (dDC-LTT)		
	+	-		+	-
Allergic patients	1	2	Allergic patients	4	1
Healthy controls	3	18	Healthy controls	3	18

Table 18. Contingency tables for positive and negative proliferative results of CD3⁺ cells in C-LTT and dDC-LTT by clinical entity.

The % of positive cases in AP detected by dDC-LTT was higher than that of C-LTT, independently the clinical entity involved (Figure 42). Specifically, the % of positive cases increased from 62.5% with C-LTT to 87.5% with dDC-LTT in SJS/TEN patients, from 15% to 47.4% in MPE and from 33% to 80% in AGEP patients (Figure 44). Interestingly with both C-LTT and dDC-LTT the results in MPE were lower (15% and 47.4%) compared to SJS/TEN (62.5% and 87.5%, respectively) or AGEP (33% and 80%, respectively) (Figure 44).

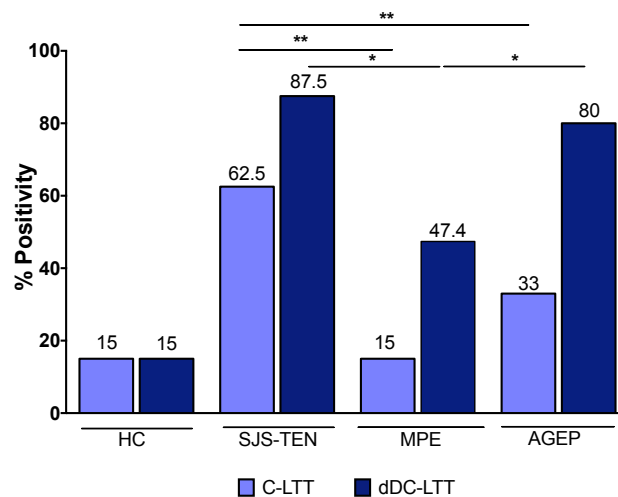


Figure 44. Positivity for CD3⁺T cells with C-LTT and dDC-LTT in healthy controls and patients with different clinical entities. Bars represent % of positivity for CD3⁺T cells in SJS/TEN, MPE, AGEP patients and healthy controls. Comparisons were assessed by X² test (*p<.05; ** p<.01).

Overall proliferative response of different cell populations

Once it has been demonstrated that dDC-LTT has higher sensitivity compared to C-LTT for assessing the proliferative response of CD3⁺T cells in the overall group of patients and in each clinical entity studied, we next focused on evaluating the proliferative response of the different cell populations, CD3⁺T, CD4⁺T, CD4⁺Th1, CD4⁺Th2, CD8⁺T, NK, NK IFN- γ ⁺, and NK PERF, analysing the results obtained only with dDC-LTT. The PI found for all cell population analysed was always higher in AP vs. HC (Figure 45). Interestingly, the overall PI observed was much higher in CD4⁺Th1 cells compared to the other cell populations analysed within the AP group (Figure 45).

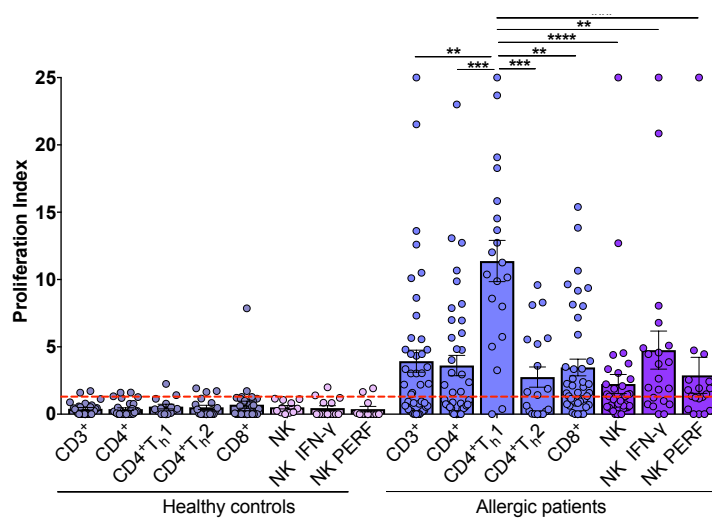


Figure 45. Proliferative response of different cell populations. Dots and bars represent proliferation index of different cell populations in the overall group of allergic patients and healthy controls. Comparisons were assessed by Kruskal-Wallis test (** p<.01; *** p<.001; **** p<.0001)

Next, we combined the proliferative results from the different cell populations to determine if the combination of their results may increase the sensitivity of the test compared with the proliferative response of CD3⁺T cells only. We observed that the combination of the proliferative response of CD3⁺T and CD4⁺Th1 cells increased the % of positivity from 61.8% to 73.9%. When CD3⁺T and NK cells proliferative results were combined, LTT sensitivity increased to 82.6%. In both cases, the combination did not reduce the specificity (Figure 46).

Moreover, the combination of the three populations, CD3⁺T, CD4⁺Th1 and NK cells increased the positivity of dDC-LTT to 87% without increasing the number of positive cases in HC (Figure 46). Interestingly, the combination of CD3⁺T and CD8⁺T cell proliferative results increased the positive cases in AP up to 91%, although a reduction in terms of specificity from 85% to 80% was also observed (Figure 46).

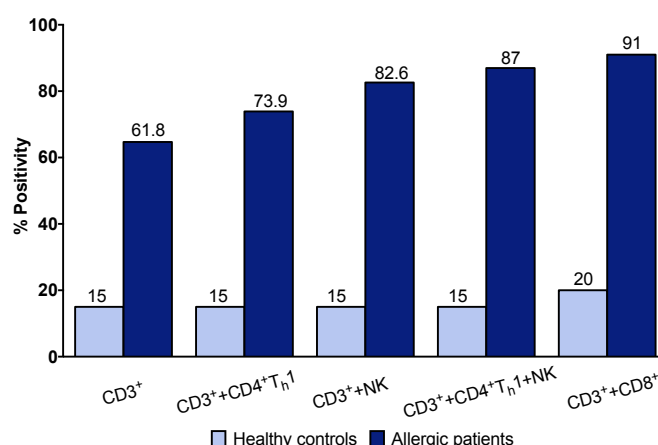


Figure 46. Combination of positive results from different cell populations. Bars represent the % of positivity for CD3⁺T cells and for combination of proliferative results of different cell populations in allergic patients and healthy controls.

Proliferative response of different cell populations and clinical entities

Next, we analysed the cell subpopulation that specifically proliferated after the involved drug stimulation depending on the clinical symptoms of the specific NIDHR. The PI was higher when assessing the proliferative response of CD4⁺Th1 cells compared to other cell populations such as NK cells, mainly inflammatory, but not cytotoxic NK cells, and CD4⁺Th2 cells (Figure 47A). Notably, the PI obtained for CD4⁺Th2 cells was significantly lower than the one obtained for other cell populations such as CD8⁺T, CD3⁺T, CD4⁺T, and CD4⁺Th1 cells (Figure 47A). Combining CD3⁺T and CD4⁺Th1 cells

proliferative results allowed the identification of all SJS/TEN patients with 85% of specificity. The same occurred when CD3⁺T and NK cells proliferative results were combined (Figure 47B). However, the combination of CD3⁺T and CD8⁺T cells proliferative results although also allowed to identify all SJS/TEN patients, the specificity was reduced from 85% to 80% (Figure 47B).

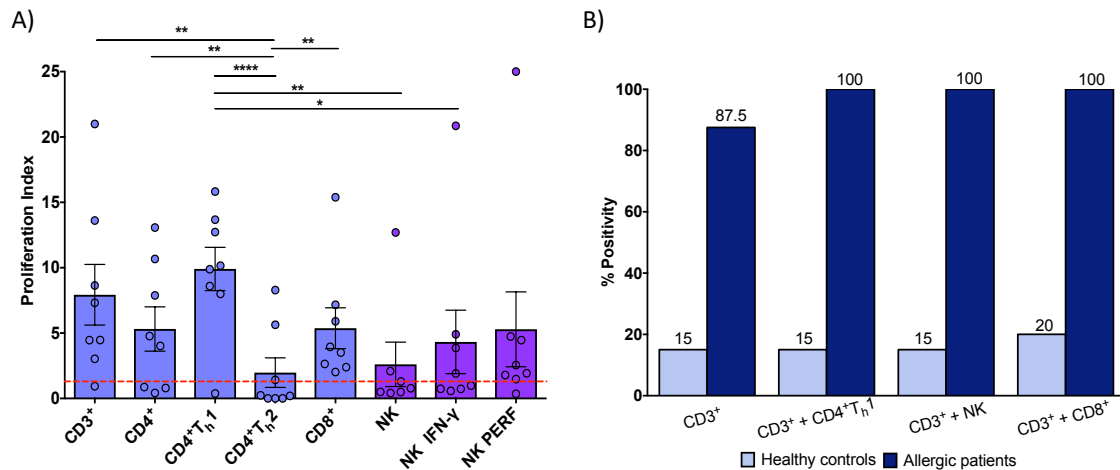


Figure 47. Proliferative response of different cell populations in SJS/TEN patients. A) Dots and bars represent the proliferation index of different cell populations in SJS/TEN patients. B) Bars represent the % of positivity combining different cell populations in patients with SJS/TEN and healthy controls. PI comparisons were assessed by Kruskal-Wallis test (* $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$).

The PI in MPE patients was higher in CD4⁺Th1 cells compared to the other cell populations analysed, except for inflammatory NK cells (NK IFN-γ) (Figure 48A).

The sensitivity of LTT increased from 47.4% (based on the proliferation of CD3⁺T cells) to 50% and to 58.3% when the proliferative results of both CD3⁺T and CD4⁺Th1 cells or CD3⁺T and NK cells were respectively combined. On the other hand, the combination of CD3⁺T and CD8⁺T cells proliferative results did not increase the % of positivity compared to the combination of CD3⁺T and NK cells (58.3%), but a reduction in specificity was observed (from 85% to 80%) (Figure 48B). Interestingly, the combination of proliferative results of CD3⁺T, CD4⁺Th1 and NK cells increased the overall detection of positive cases to 68.4% with a specificity of 85% (Figure 48B).

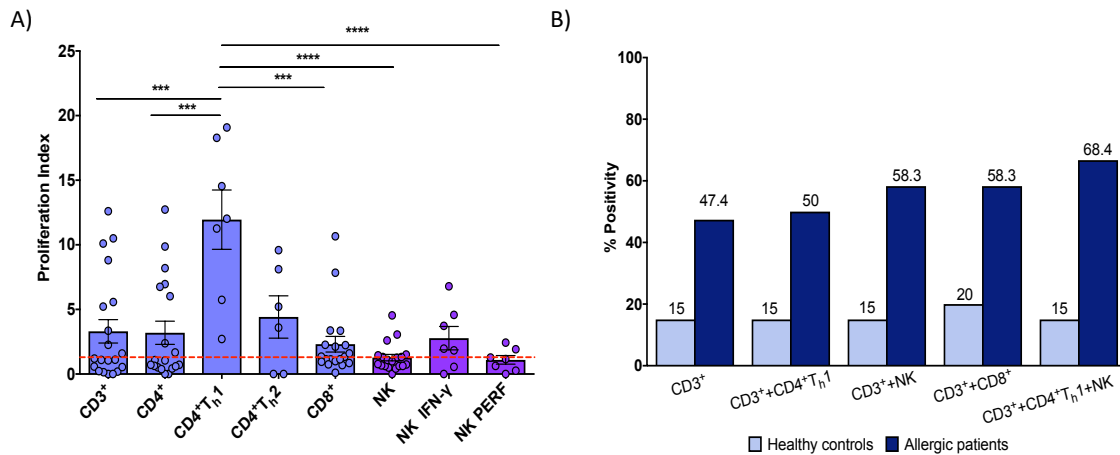


Figure 48. Proliferative response of different cell population in MPE patients. A) Dots and bars represent the proliferation index of different cell populations in MPE patients. B) Bars represent the % of positivity combining different cell populations in patients with MPE and healthy controls. PI comparisons were assessed by Kruskal-Wallis test (** $p < .001$; **** $p < .0001$).

Regarding AGEP patients, the PI of CD4⁺Th1 cells was significantly higher than of CD4⁺T and CD4⁺Th2 cells, as well as cytotoxic (NK perf), but not inflammatory (NK IFN-γ) NK cells (Figure 49A). Compared to the % of positive cases with CD3⁺T cells, the combination of the results with CD4⁺Th1 cells did not show an increase in the % of positivity. In contrast, the combination of CD3⁺T and NK cells results increased the % of detected cases to 100%, without a reduction in terms of specificity (Figure 49B). Although the combination of CD3⁺T and CD8⁺T cells proliferative results could also detect all patients with AGEP, the specificity was reduced from 85% to 80% (Figure 49B).

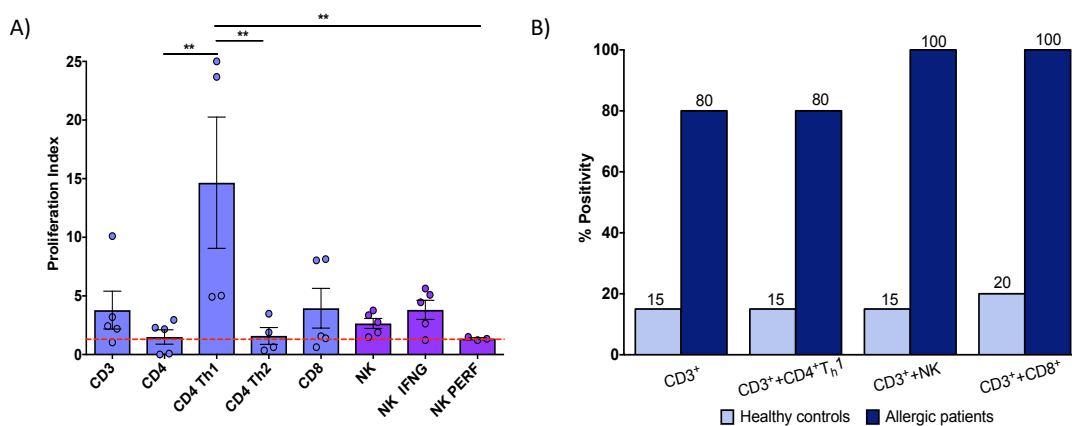


Figure 49. Proliferative response of different cell population in AGEP patients. A) Dots and bars represent the proliferation index of different cell populations in AGEP patients. B) Bars represent the % of positivity combining different cell populations in patients with AGEP and healthy controls. PI comparisons were assessed by Kruskal-Wallis test (** $p < .01$).

Finally, the % of positivity of CD4⁺T cell subpopulations (CD4⁺Th1 and CD4⁺Th2 cells) (Figure 50A), and the subpopulations of NK cells, inflammatory (NK IFN- γ) and cytotoxic (NK perf) cells (Figure 50B) were analysed by each clinical entity. Although CD4⁺Th1 cells constituted the CD4⁺T cell subpopulation with the highest % of positivity in all the clinical entities, 88%, 100%, and 100% in SJS/TEN, MPE and AGEP respectively, the % of positivity of CD4⁺Th2 cells was higher in MPE (67%), followed by AGEP (50%), and to a lesser extent, in SJS/TEN patients (37.5%) (Figure 50A). Regarding NK cell subpopulations, the % of positive cases in SJS/TEN patients was much higher in cytotoxic NK cells (87.5%) compared to the inflammatory subpopulation (50%) (Figure 50B). In contrast, the % of positive cases of cytotoxic NK cells was lower (28.6%) in patients with MPE compared to the inflammatory subpopulation (71.4%) (Figure 50B). The % of positive cases of NK cell subpopulations for AGEP patients appeared to be more balanced, although it was higher in the inflammatory NK cells (80%) than in the cytotoxic subpopulation (66%) (Figure 50B).

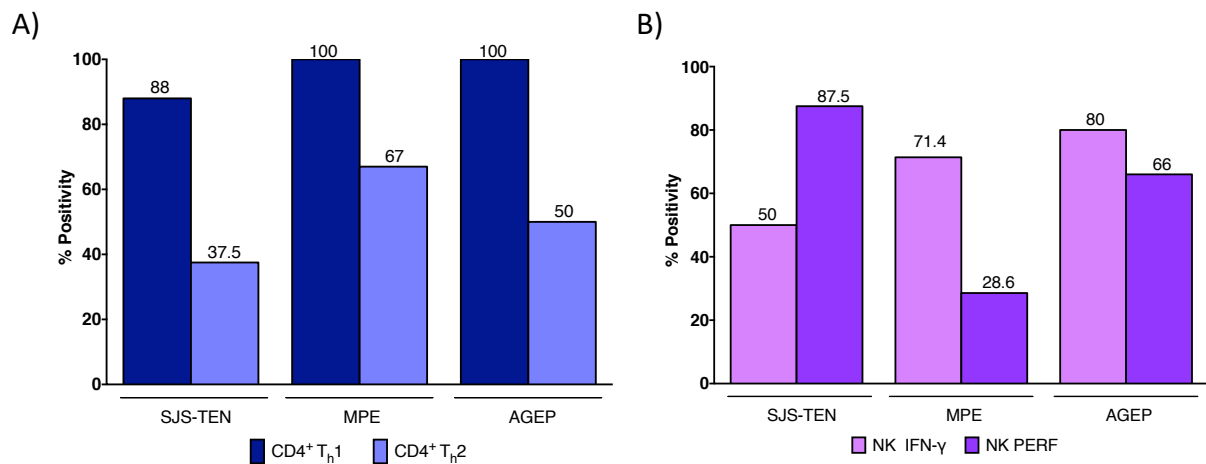


Figure 50. % of positive cases of different cell subpopulations in SJS/TEN, MPE and AGEP patients. A) Bars represent the % of positivity of CD4⁺Th1 and CD4⁺Th2 cells in the different clinical entities analysed. B) Bars represent the % of positivity of inflammatory (NK IFN- γ) and cytotoxic (NK PERF) NK cells.

DISCUSSION

Several factors may explain why LTT is nowadays considered more a research tool than a potential *in vitro* diagnostic assay for evaluating NIDHRs. One important factor is that, traditionally, the lymphocyte proliferative response has been assessed by measuring the inclusion of ^3H -thymidine in the genome of proliferative cells, determined by the increase of radioactivity. The use of these radioactive reagents makes their use in clinical routine difficult or even impracticable.^{43,129,197} Therefore, in recent decades, new techniques have been investigated to assess such a proliferative response as a consequence of drug stimulation.

Following drug stimulation of PBMCs, the release of specific cytokines has been evaluated,^{188,192,197} but the reduced number of studies, the low number of patients included, the high heterogeneity of clinical manifestations and the technical differences between laboratories make it difficult to draw precise conclusions. On the other hand, other studies have shown that the expression of some markers, such as CD69, or HLA-DR, can be associated with drug stimulation in patients with NIDHRs.^{159,193} Nevertheless, little is known about the usefulness of using flow cytometry to evaluate cell proliferation after culture with the culprit drug by the CFSE dilution assay.

The reported sensitivity of the traditional LTT, mainly based on the stimulation of monocytes or B cells as APCs is variable, ranging from 27% to 75%, depending on the study.^{36,159,187,188,192} In our study we showed that the overall sensitivity of flow cytometry C-LTT was low (29.4%), similar to previous traditional studies,^{188,196} but lower than others.^{159,186,188,253} A possible explanation for this could be that in our study, we used patients with a broad panel of clinical manifestations and drugs involved, whereas most of studies assessed the sensitivity of LTT in a specific clinical manifestation. Moreover, those studies using samples from patients with different clinical entities evaluated specific drugs, such as BLs or NMBAs, which can highly stimulate lymphocytes, thus reporting higher sensitivity.²⁸²

In a recent study carried out by our group in children with NIDHRs, we demonstrated that the sensitivity of C-LTT with CFSE for assessing NIDHRs to AX was 63.7%, while for CLV it was only 33.3%,³⁴ suggesting that the nature of the drug is also a determinant factor in LTT sensitivity, probably because of the different ways of drug-immune cells interaction or degradation.^{13,48} Altogether, C-LTT appears to have a suboptimal sensitivity for evaluating different clinical entities of NIDHR and heterogeneous drugs.

In an attempt to increase LTT sensitivity, the inclusion of professional APCs, such as moDCs, pre-stimulated with the drugs has been evaluated in some studies with promising results.^{217,272} In our study we observed a significant increase in overall LTT sensitivity from 29.4% with C-LTT to 61.8% with dDC-LTT.

Another factor that may influence the sensitivity of LTT is the specific clinical entity involved, as it has been proposed that the sensitivity of LTT is higher when evaluating mild/moderate than severe NIDHRs.^{192,266,282,283} In contrast, in our study we obtained a higher LTT sensitivity for evaluating severe NIDHRs such as SJS/TEN (62.5%) compared to mild/moderate reactions such as MPE (15%). These data are in agreement with recent studies indicating that if patients with severe reactions are well studied, the sensitivity of LTT is also high.^{14,190,191} If these observations were confirmed, they may have important implications for clinical practice, due to the very limited diagnostic tools available for patients with severe NIDHRs.

The inclusion of pre-primed moDCs increased the sensitivity of LTT compared to C-LTT in all the clinical entities studied, from 62.5% to 87.5% in SJS/TEN, from 15% to 47.4% in MPE, and from 33% to 80% in patients with AGEP. These data support the concept that moDCs can present the drugs more efficiently and amplify the immune response, especially useful for severe reactions, which has classically shown low sensitivity. Nevertheless, it cannot be completely ruled out that the nature of the drugs influences these results. For instance, in 4 patients with AGEP and SJS/TEN, the drugs involved are anticonvulsants and allopurinol, drugs clearly associated with the p-i interaction, in which the intracellular processing by APCs may not be necessary.²⁸⁴⁻²⁸⁷ Nevertheless, we observed positive results in 3 of 4 patients which these features.

In contrast to the research conducted by Yun and colleagues, who washed the APCs after stimulation,²⁸⁴ in our study we only removed the drug from the culture supernatant, so it is possible that these drugs interacted directly with HLA from moDCs in a noncovalent manner, keeping them coupled to HLA molecules, allowing interaction with TCRs, triggering lymphocyte activation without the need for internal processing by APCs. Nevertheless, further studies are needed to address this complex issue.

Several studies have shown that the analysis of the specific effector response responsible for the NIDHR may be useful to increase the sensitivity of *in vitro* assays,^{188,196,197,266,288} although since none of them has sufficient sensitivity, some authors recommend combining different assays.^{192,196,197} In addition to avoiding the use of radioactive reagents, the measurement of % of CFSE_{Dim} cells after drug stimulation allows the assessment of the proliferative response of different cell populations, including those with important implications for NIDHRs, but present at low rates.²⁸⁹

Herein, and in agreement with the general opinion, we observed that CD4⁺Th1 cells were strongly associated with NIDHRs, regardless of the clinical entity involved. In fact, the combination of CD3⁺T and CD4⁺Th1 cells proliferative results significantly increased the sensitivity of dDC-LTT, supporting the relevant involvement of this subpopulation in NIDHRs.²⁹⁰ We have also demonstrated that NK cells were also involved in the pathomechanism of NIDHRs, with inflammatory NK cells being preferentially involved in MPE, while cytotoxic NK cells were more relevant in SJS/TEN as previously described.^{88,291} Finally, we have also shown that CD4⁺Th2 cell subpopulation is relevant in MPE reactions as described by some authors.^{196,290}

Therefore, assessing the proliferative response of different cell populations by measuring the % of CFSE_{Dim} cells, combining the results, together with the inclusion of drug pre-primed moDCs as APCs significantly increased the overall LTT sensitivity to 87% (combining the proliferative results of CD3⁺T, CD4⁺Th1 and NK cells), with a specificity of 85%. Moreover, we demonstrated the involvement of specific cell subpopulations for each clinical manifestation. Therefore, the combination of proliferative results of CD3⁺T and CD4⁺Th1 or NK cells allows identification of all patients with SJS/TEN reactions; CD3⁺T and NK cells proliferative results combination allows identification of all AGEP patients; while CD3⁺T, CD4⁺Th1 and NK cells results combination increases the sensitivity of LTT to 68.4% in MPE patients, with 85% of specificity for the 3 clinical entities.

CHAPTER II

Recognition of antigenic determinants of clavulanic acid by dendritic cells and their capacity to induce of T cell proliferation in patients with immediate hypersensitivity reactions.

SUMMARY

CLV is a BL antibiotic, with low antibiotic activity, although due to its high betalactamase activity, it is frequently prescribed alongside AX. Even though it was initially considered nonimmunogenic, in recent decades, there is strong evidence regarding the capacity of CLV to trigger both IDHRs and NIDHRs.^{158,173,248,257,267,271,292-294} In fact, due to the high prescription pattern, **IDHRs** caused by **CLV** seem to **be increasing**.

Because of its **chemical instability**, diagnostic procedures to evaluate IDHRs to CLV are difficult, and current *in vitro* assays, useful and safe complementary diagnostic alternatives, have a reduced sensitivity. This **low sensitivity** can be explained by the fact that when CLV is included in the assay, it is rapidly transformed into different **degradation products**, generating a heterogeneous mixture of **metabolites** with different **capacities to interact with proteins**. Since only some of them are efficiently recognised by the immune system and are therefore responsible for triggering an IDHR, the final concentration of the concrete metabolite might be very limited, as it has been.

Consequently, in this chapter we have attempted to **identify** the **potential CLV ADs derived from CLV degradation** that are efficiently recognised by DCs, as well as to determine the **best combination** of them that can **produce the better sensitivity** of current *in vitro* assays.

For this purpose, we analysed the ability of different **CLV synthetic analogues** with **different protein binding capacity**, corresponding to **two different ADs derived from CLV degradation**, to be recognised by moDCs, as well as to determine their capacity to stimulate lymphocytes in patients with CLV-selective IDHRs compared to HC.

Herein, we demonstrated that some **specific synthetic analogues of CLV ADs** were **specifically recognised by moDCs** from AP, but not by moDCs from HC. Nevertheless, each AD was recognised in a different way, being therefore the inclusion of synthetic analogues from both ADs necessary to increase the sensitivity on moDCs maturation assays. Furthermore, pre-primed moDCs stimulated with CLV AD analogues can trigger the proliferation of lymphocytes, specifically, **CD4⁺Th2** cells, only in AP to CLV but not in HC. The combination of synthetic analogues from both ADs **increased the overall sensitivity** of **dDC-LTT** from 19% when native CLV was included to 65%, with a specificity higher than 90%.

RESULTS

Patients and healthy controls characterisation

From the subjects who were referred to the Allergy Unit of HRUM with a compatible history of an IDHR after the intake of AX-CLV during the years 2018-2020, 27 patients with confirmed CLV-selective IDHRs were included in the study. Sixteen (59.3%) were female and 11 (40.7%) were male. The age of the patients was 52.07 ± 14.6 years. The most frequent clinical entity was ANA in 22 of 27 cases (81.48%) followed by URT with or without ANG in 3 (11.11%), and AS in 2 (7.41%).

The severity of each reaction was graded according to the Ring-Messner classification for anaphylactoid reactions.²⁹⁵ Twelve of 27 patients had a severity type I reaction (44.44%), 7 (25.94%) a grade II reaction, 6 (22.22%) of grade III reaction, and two (7.4%) a reaction of grade IV. The time interval between reaction and sample collection was $696 \pm 10,638$ days. The mean interval between drug intake and symptom onset was 33.96 ± 27.85 min. All reactions occurred after the intake of AX-CLV. Twenty patients suffered one episode (74.07%) and 7 patients reported two episodes (25.93%).

All patients included in the study were confirmed as CLV-IDHRs selective patients by positive ST to CLV, and negative ST to AX and BP (BP-OL and MDM). Moreover, tolerance to AX was confirmed in all patients by negative DPT. Demographic and clinical data from the patients included in the study can be consulted in Table 19.

A group of 13 sex- and age-matched subjects with no reported hypersensitivity reactions to any drugs were included in the study as HC group. Eight of 13 were female (61.54%) and 5 were male (38.46%). The mean age of the HC group was 37.26 ± 11.97 years.

Patients	Sex	Age	Reaction	Severity grade	Time Interval (days) reaction /study	Time interval (minutes) drug administration/onset of symptoms	Drug	Episodes	ST			DPT
									CLV	AX	BP-OL/MDM	AX
P-1	F	42	Anaphylaxis	II	60	60	AX-CLV	1	+	-	-	-
P-2	F	33	Anaphylaxis	III	2920	30	AX-CLV	2	+	-	-	-
P-3	F	66	Anaphylaxis	I	3650	30	AX-CLV	1	+	-	-	-
P-4	M	66	Urticaria/Angioedema	I	30	60	AX-CLV	1	+	-	-	-
P-5	M	69	Anaphylaxis	I	3650	60	AX-CLV	1	+	-	-	-
P-6	F	47	Anaphylaxis	II	60	15	AX-CLV	2	+	-	-	-
P-7	F	34	Anaphylaxis	III	185	60	AX-CLV	1	+	-	-	-
P-8	F	42	Urticaria	I	730	60	AX-CLV	1	+	-	-	-
P-9	F	66	Anaphylaxis	I	167	5	AX-CLV	1	+	-	-	-
P-10	F	37	Anaphylaxis	I	210	10	AX-CLV	2	+	-	-	-
P-11	M	66	Anaphylaxis	II	30	60	AX-CLV	2	+	-	-	-
P-12	M	44	Anaphylaxis	III	130	30	AX-CLV	1	+	-	-	-
P-13	M	40	Anaphylaxis	III	31	30	AX-CLV	1	+	-	-	-
P-14	M	33	Anaphylaxis	I	240	120	AX-CLV	1	+	-	-	-
P-15	F	28	Anaphylaxis	III	570	15	AX-CLV	1	+	-	-	-
P-16	M	31	Urticaria/ Angioedema	I	1825	10	AX-CLV	1	+	-	-	-
P-17	F	33	Anaphylactic Shock	IV	365	40	AX-CLV	2	+	-	-	-
P-18	M	62	Anaphylaxis	II	1765	45	AX-CLV	1	+	-	-	-
P-19	F	48	Anaphylaxis	I	153	15	AX-CLV	1	+	-	-	-
P-20	M	56	Anaphylaxis	II	730	10	AX-CLV	1	+	-	-	-
P-21	F	39	Anaphylaxis	III	134	5	AX-CLV	1	+	-	-	-
P-22	M	58	Angioedema	I	35	72	AX-CLV	1	+	-	-	-
P-23	F	66	Anaphylaxis	II	55	5	AX-CLV	2	+	-	-	-
P-24	M	71	Anaphylactic Shock	IV	30	5	AX-CLV	1	+	-	-	-
P-25	F	71	Anaphylaxis	I	30	30	AX-CLV	1	+	-	-	-
P-26	F	41	Anaphylaxis	I	212	30	AX-CLV	1	+	-	-	-
P-27	F	60	Anaphylaxis	II	1460	5	AX-CLV	2	+	-	-	-

Table 19. Demographic and clinical data of patients with CLV-selective IDHRs. Abbreviations: AX, amoxicillin; BP-OL, benzylpenicilloyl octa-L-lysine; CLV, clavulanic acid; DPT, drug provocation test; F, female; M, male; MDM, minor determinants mixture; ND, not done; SPT, skin prick test.

moDCs maturation in response to CLV and its AD synthetic analogues

Based on our previous experience, the most suitable concentrations of AX and CLV for inclusion in moDC maturation assays and dDC-LTT ranged from 250 μ g/mL to 25 μ g/mL.^{34,186,199,201,202,217,296} These concentrations correspond to about 1mM and 0.1mM. To assess that the inclusion of synthetic analogues of CLV ADs did not affect the viability of moDCs, we cultured moDCs from CLV-selective AP (Figure 51), and from HC (Figure 52) for 3 days with LPS at 1 μ g/mL, CLV, and the different synthetic AD analogues from CLV at 1mM and 0.5mM. The % of cells expressing low (alive cells) and high (dead cells) live/dead near IR dye was analysed for each condition.

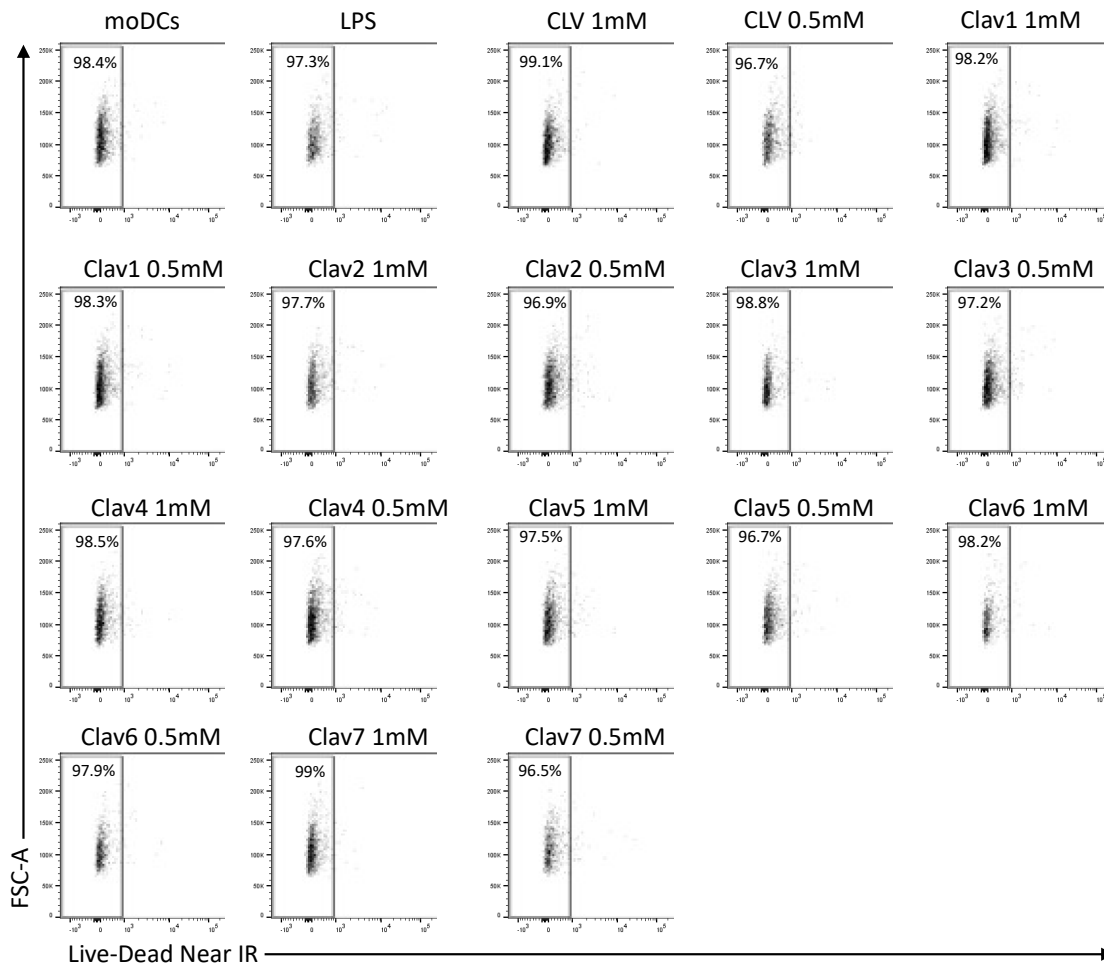


Figure 51. Viability of moDCs from CLV-allergic patients with different stimuli. Dot plots represent the alive/dead moDCs after stimulation with LPS at 1 μ g/mL, CLV at 1 and 0.5mM, and Clav1-7 at 1mM and 0.5mM for 72h.

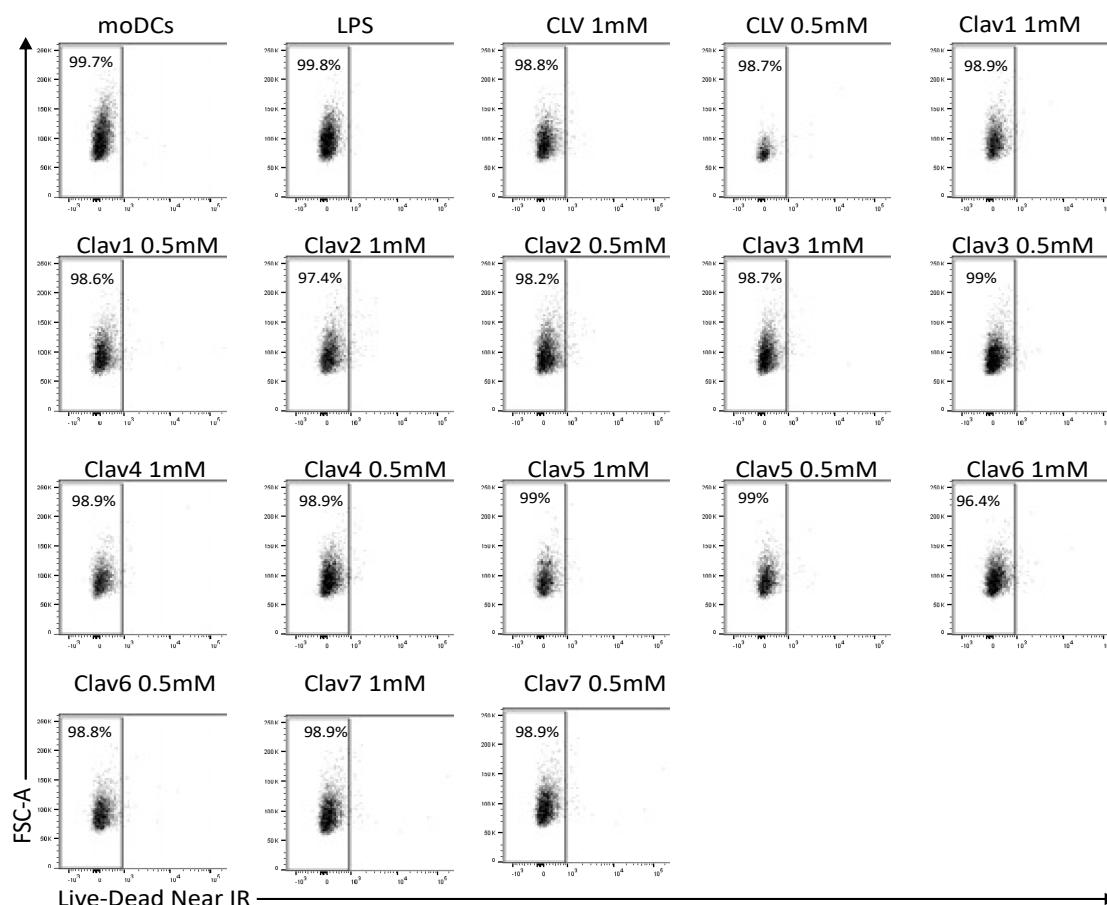


Figure 52. Viability of moDCs from healthy controls with different stimuli. Dot plots represent the alive and dead moDCs after stimulation with LPS at $1\mu\text{g}/\text{mL}$, CLV at 1 and 0.5mM, and Clav1-7 at 1mM and 0.5mM for 72h.

For both CLV-AP (Figure 51) and HC (Figure 52) moDCs viability was above 95%, regardless of the stimulus used. Therefore, CLV and Clav1-7 were included at 1mM and 0.5mM in the *in vitro* assays. Due to the large number of conditions, to facilitate the reader's interpretation of the graphics, only the highest concentration (1mM) will be represented, because the results with this concentration were better.

The MI was higher for CCR7 on moDCs from CLV-AP after stimulation with Clav1 and Clav2 from AD-I and Clav6 from AD-II (Figure 53A), although only Clav2 showed significant differences compared to the MI obtained after native CLV stimulated moDCs. Interestingly, higher MI of CD40, CD80, and CD83 was observed in CLV-AP compared to HC, irrespective of the synthetic analogue included (Figure 53B-D). Compared to the MI obtained with native CLV in AP, differences were only observed after stimulation with Clav2 and Clav7 for CD40 (Figure 53B), and after stimulation with all synthetic analogues except Clav3 for CD80 (Figure 53C).

The MI of moDCs from CLV-AP for CD86 was higher after stimulation with all synthetic AD analogues from CLV, except Clav3, Clav4, and Clav5 compared to HC (Figure 53E). Only Clav6 reported a higher MI than stimulation with native CLV (Figure 53E) in CLV-AP. Interestingly, native CLV only triggered higher MI of CD83 and CD86 (Figure 53D, E), but not of CCR7, CD40, or CD80 in CLV-AP compared to HC (Figure 53A-C).

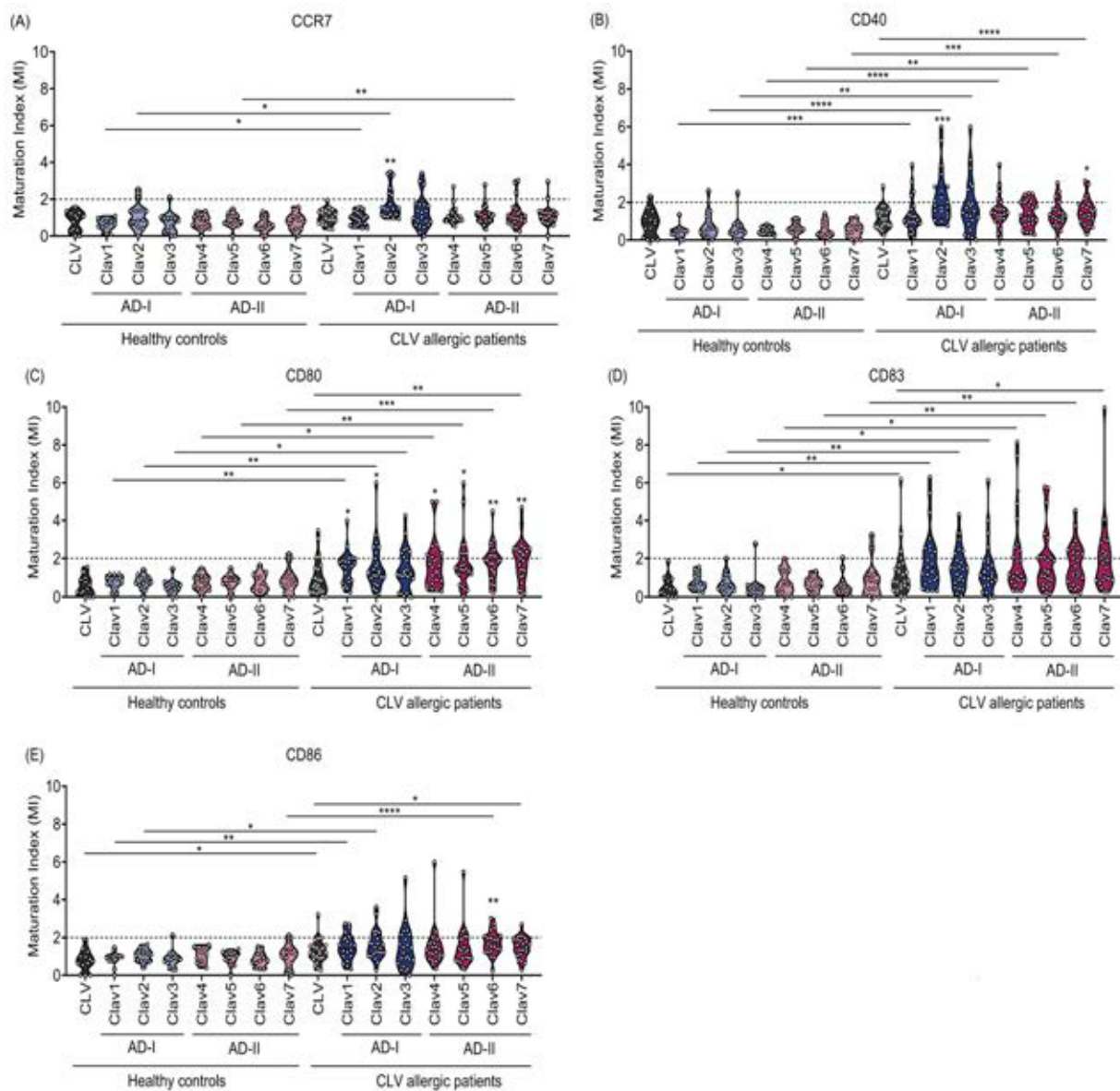


Figure 53. moDCs maturation induced by CLV and its synthetic AD analogues. Violin plots represent maturation index of moDCs from CLV allergic patients and healthy controls. A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86. MI comparisons were assessed by Mann-Whitney U test (* p<.05; ** p<.01; *** p<.001; **** p<.0001).

The % of positive cases was higher for CCR7 in CLV-AP moDCs after stimulation with Clav2 (29%), followed by Clav3 and Clav6 (14% both) (Figure 54A). For CD40, the highest % of positivity was also observed for Clav2 (43%), followed by Clav5 (33%) (Figure 54B). On the other hand, for CD80, the highest % of positivity was obtained for CLV-AP moDCs stimulated with Clav7 (48%), followed by Clav4-7 (33%) and Clav3 (29%) (Figure 54C). For CD83 in CLV-AP, the highest % of positivity was obtained for Clav5-7 (43%) followed by Clav1 (38%) (Figure 54D). Finally, for CD86, the highest positivity values were found for Clav2 and Clav6 (29%), followed by Clav3 and Clav5 (25%) (Figure 54E).

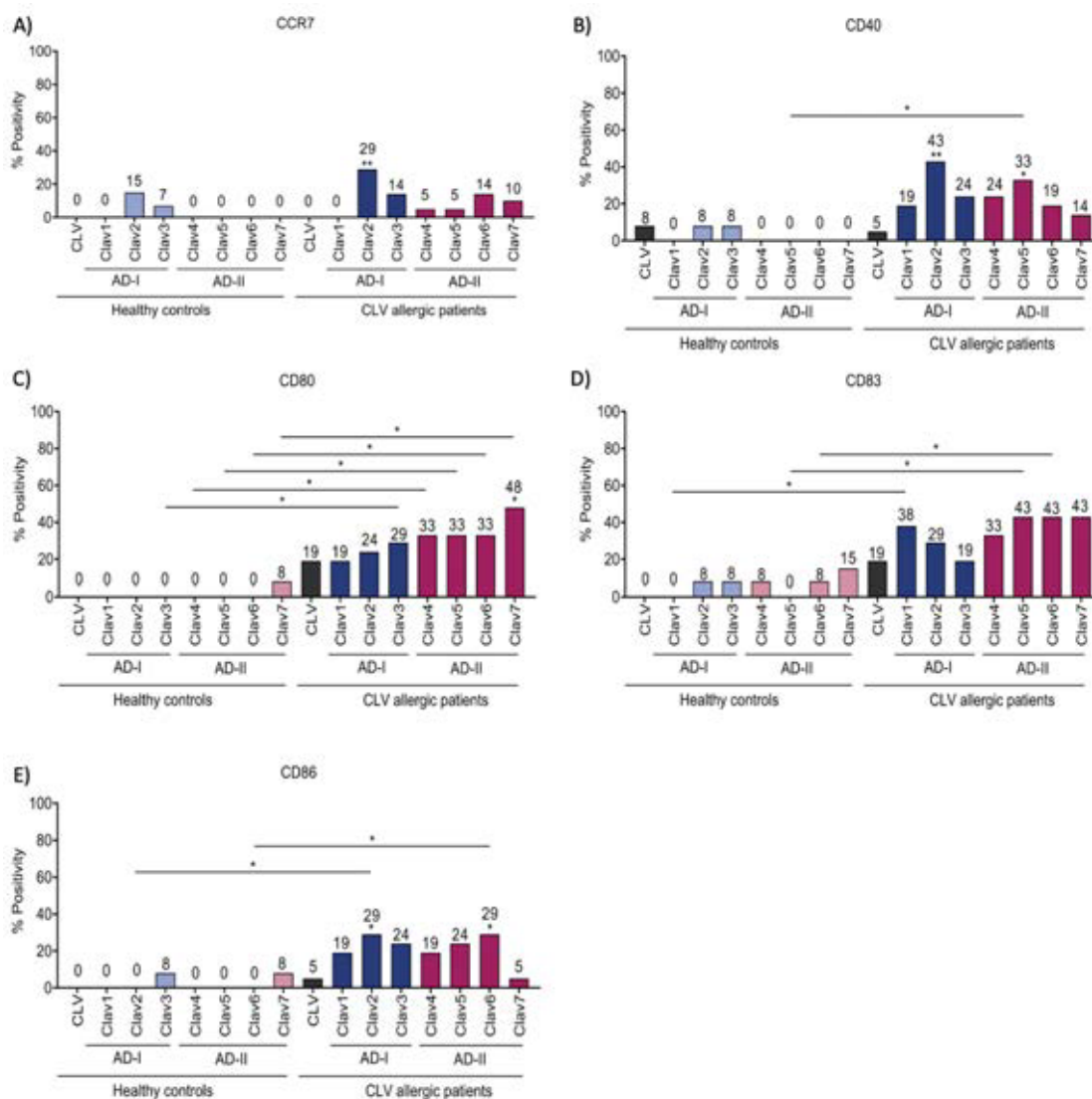


Figure 54. Positivity of moDCs maturation assays induced by CLV and its synthetic AD analogues. Bars represent % of positive cases for each maturation marker from CLV allergic patients and healthy controls in moDCs maturation assays. A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86. Positivity comparisons were assessed by χ^2 test (* $p < .05$; ** $p < .01$).

The % of positive cases in moDCs from HC was lower than 10% independently of the synthetic analogue included, or the marker analysed (Figure 54A-E), except for CCR7 and Clav2 (15%) (Figure 54A). Interestingly, native CLV reported extremely low values of positive cases for CCR7 (0%), CD40 and CD86 (5% each) and CD80 and CD83 (19% each). No positive cases were detected in HC, regardless of the synthetic analogue included or the marker studied (Figure 54A, C-E), except for CD40 (8%) (Figure 54B).

Since differences exist in terms of the synthetic analogue of CLV AD included and the activation marker studied, we then combined the results of maturation assays with synthetic analogues from each AD. Subsequently, we combined the results with all the synthetic analogues from both ADs together. Finally, we assessed whether the inclusion of native CLV has an added value in addition to the inclusion of CLV ADs in terms of % of positivity (Figure 55A-E).

A higher % of positivity was detected in CLV-AP moDCs with the combination of synthetic analogues from AD-I for CCR7 (38%) and CD40 (62%) compared to the combination of AD-II synthetic analogues, 14% and 38% for CCR7 and CD40, respectively (Figure 55A, B).

In contrast, the % of positivity was higher when synthetic analogues from AD-II were combined (62%, 52% and 52%) compared to the combination of synthetic analogues from AD-I (43%, 43%, and 48%) for CD80, CD83, and CD86 respectively (Figure 55C-E).

Interestingly, combining MI results of the synthetic analogues from both CLV ADs, I and II, significantly increased the positivity in CCR7 to 48%, CD40 to 76%, CD80 to 67%, CD83 to 57%, and CD86 to 62% (Figure 55A-E). The number of positive cases in HC group after AD combination was less than 25% for all markers analysed.

The inclusion of results with native CLV had not an added value to the combination of synthetic analogues from both ADs, regardless of any of the markers analysed, except for CD40, where the number of positive cases increased from 76% with the combination of results from both ADs to 86% when native CLV was added, although the number of positive cases in the HC group also increased from 23% to 31%.

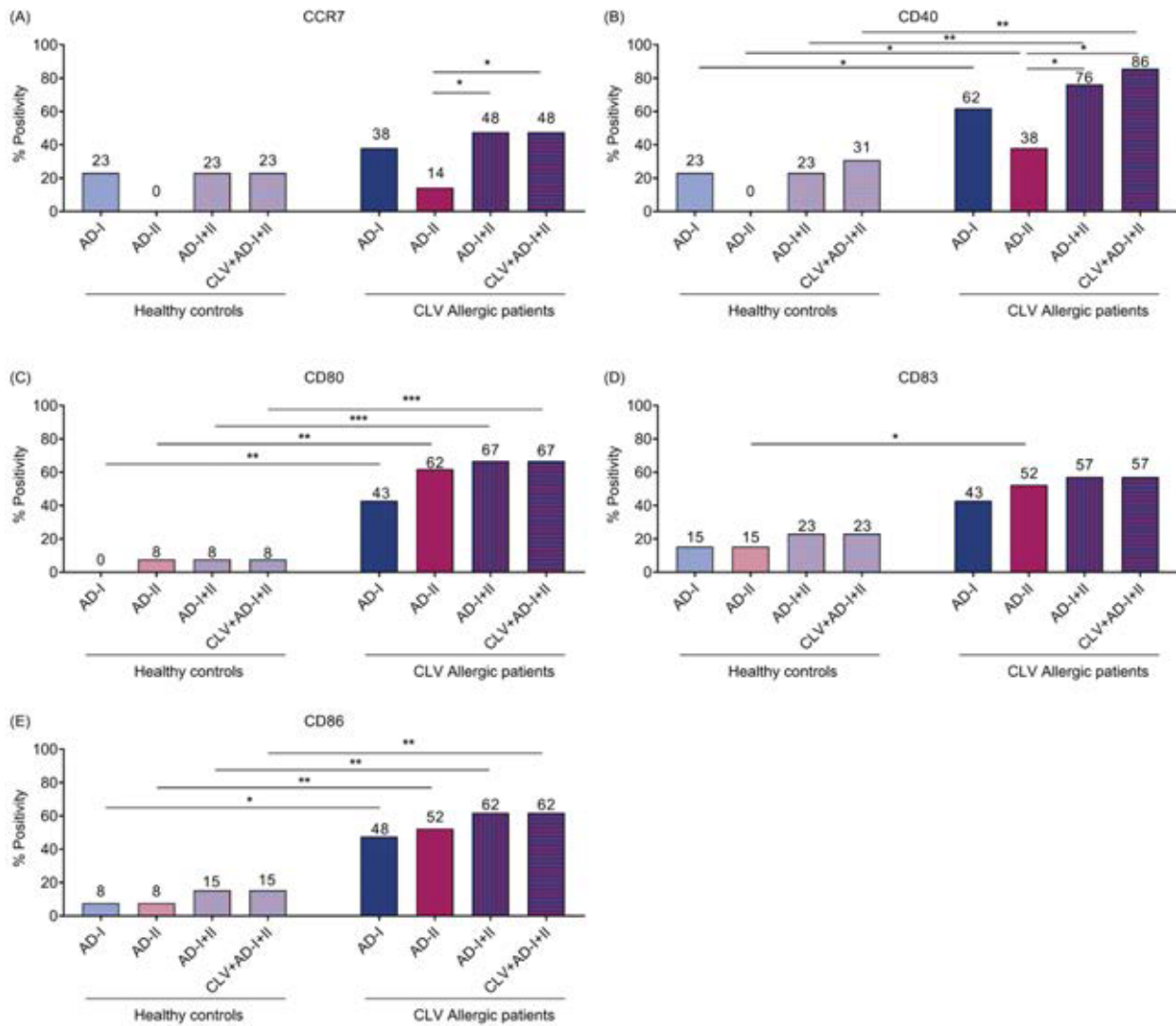


Figure 55. Combination of positive results of moDCs maturation assays. Bars represent % of positive cases with the combination of synthetic analogues for each maturation marker from CLV allergic patients and healthy controls. A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86.

Positivity comparisons were assessed by χ^2 test (* $p < .05$; ** $p < .01$; *** $p < .001$).

Since a different recognition pattern seems to depend on the nature of the specific CLV AD involved, from the total number of positive cases, we assessed the specific recognition of each AD (Figure 56). Interestingly, the majority of positive cases for CCR7 and CD40, exclusively recognised synthetic analogues from AD-I, 70% for CCR7 (Figure 56A) and 52% for CD40 (Figure 56B). In addition, 20% and 24% of positive cases for CCR7 and CD40 respectively specifically recognised AD-II synthetic analogues, while a small number of cases (10% for CCR7 and 24% for CD40) simultaneously recognised synthetic analogues from both ADs (Figure 56A, B).

On the other hand, for CD80, CD83, and CD86, most positive cases simultaneously recognised synthetic analogues from both ADs (57%, 67%, and 62% for CD80, CD83, and CD86 respectively) (Figure 56C-E). Conversely, 36%, 25%, and 23% of positive cases for CD80, CD83, and CD86 exclusively recognised AD-II synthetic analogues, while to a lesser extent, 7%, 8% and 15% only recognised synthetic analogues from AD-I for CD80, CD83 and CD86 respectively (Figure 56C-E).

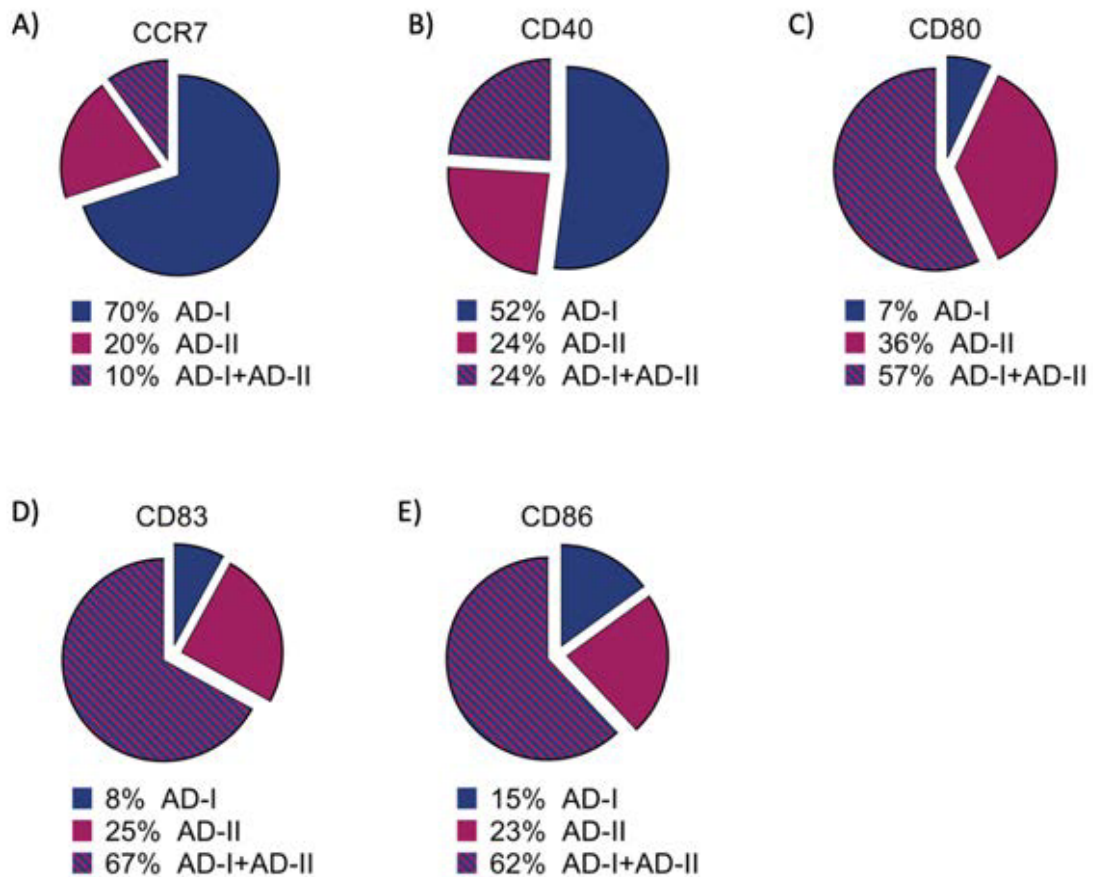


Figure 56. Specific recognition of each AD from maturation positive cases. Parts of whole represent the % of recognition of each AD from moDC maturation assay positive cases for A) CCR7; B) CD40; C) CD80; D) CD83 and E) CD86.

T cell proliferation in response to CLV and its AD synthetic analogues

The synthetic analogues of CLV ADs, only induced higher PI of CD3⁺T cell after moDCs stimulation with Clav4, and Clav5 (Figure 57A) in CLV-AP compared to HC, while the stimulation with Clav1, Clav2, Clav4 and Clav5 induced higher proliferation of CD4⁺T cells (Figure 57B) in CLV-AP compared to HC. No higher PI was observed in CLV-AP vs. HC when CD8⁺T and CD4⁺Th1 cell proliferation was analysed, independently the synthetic analogue included (Figure 57C, D). Interestingly, the highest differences between CLV-AP and HC were observed when the proliferative response of CD4⁺Th2 cells was analysed, mainly after moDCs stimulation with Clav2 and Clav4-7 (Figure 57E). Notably, native CLV did not increased the PI in CLV-AP compared to HC independently of the proliferation of cell population studied (Figure 57A-E).

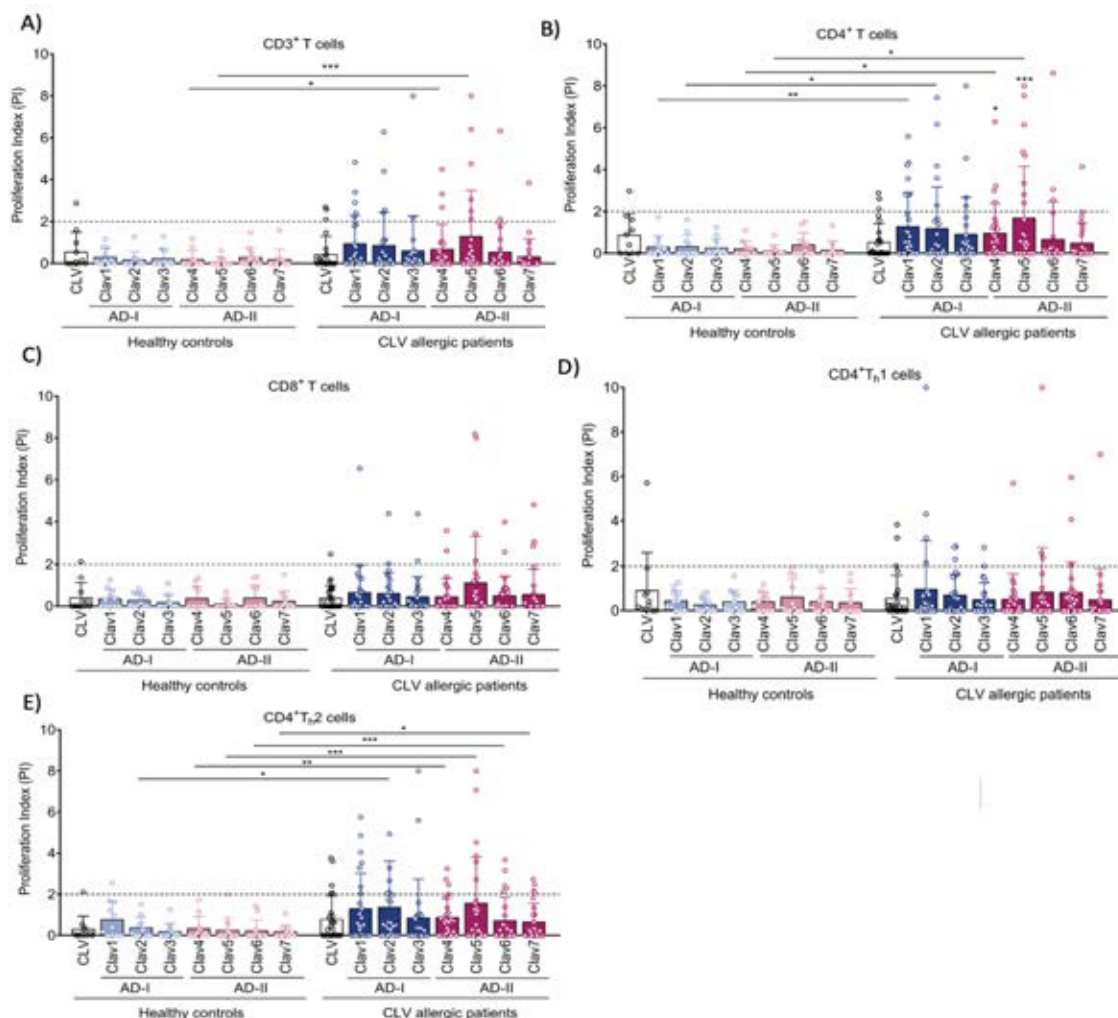


Figure 57. Lymphocyte proliferation induced by CLV and its synthetic AD analogues. Bars and plots represent proliferation index from CLV allergic patients and healthy controls in different lymphocyte populations. A) CD3⁺T cells; B) CD4⁺T cells; C) CD8⁺T cells; D) CD4⁺Th1 cells; E) CD4⁺Th2 cells. PI comparisons were assessed by Mann-Whitney U test (* $p < .05$; ** $p < .01$; *** $p < .001$).

Regarding the % of the CD4⁺T cells subpopulations, CD4⁺Th1 cells were slightly increased in HC compared to CLV-AP (Figure 58A), while CD4⁺Th2 cells appeared to be increased in CLV-AP (Figure 58B). Significant differences were confirmed between CLD-AP and HC when CD4⁺Th2/CD4⁺Th1 cells ratio was calculated (Figure 58C). Significant differences were found between CLV-AP vs. HC regarding the CD4⁺Th2/CD4⁺Th1 cells ratio. However, no differences were observed within CLV-AP or HC group after stimulation with CLV or synthetic analogues of CLV ADs (Figure 58).

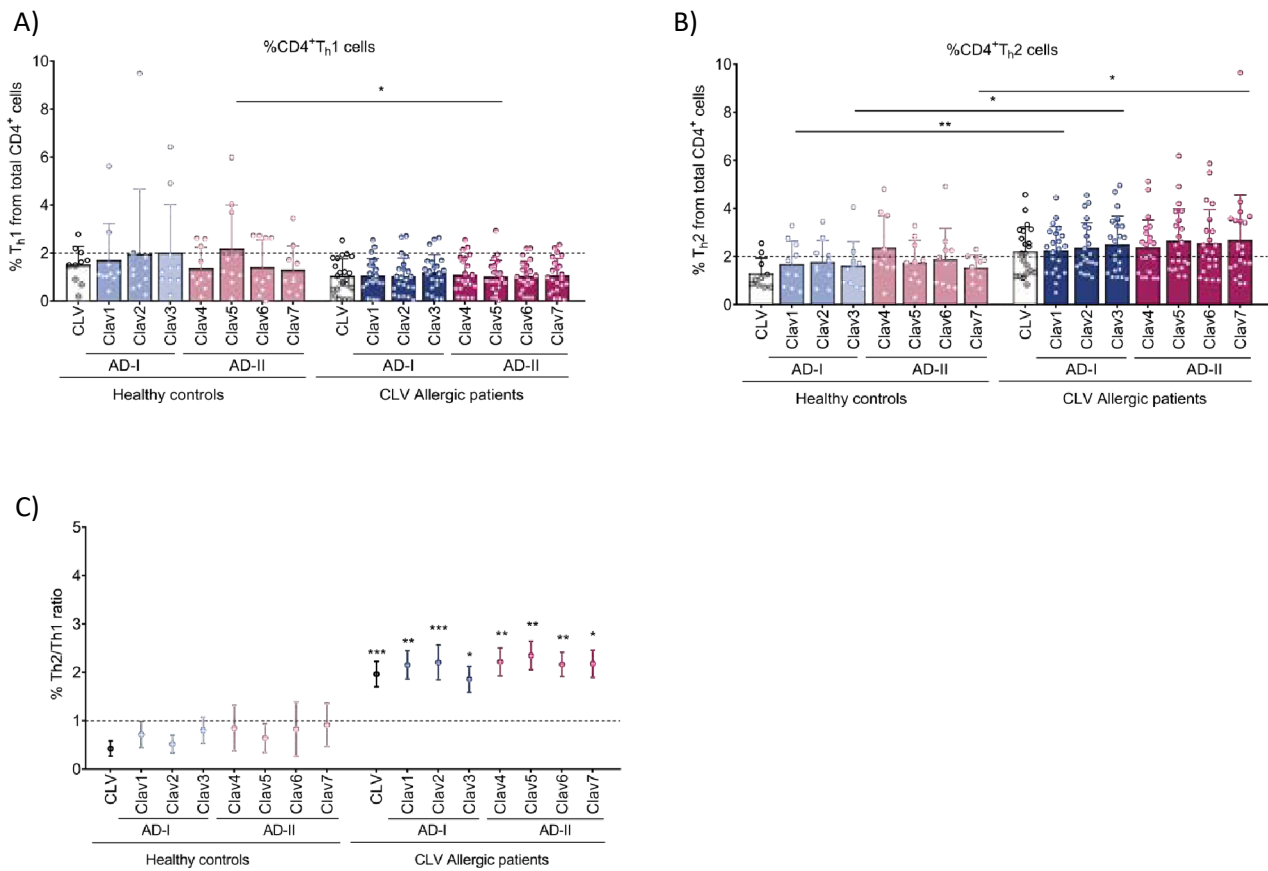


Figure 58. Frequency of CD4⁺T cell subpopulations in CLV-allergic patients and healthy controls. Bars and dots represent % of A) CD4⁺Th1 cells; B) CD4⁺Th2 cells after stimulation with CLV and its synthetic AD analogues in CLV allergic patients and healthy controls. C) Dots represent the ratio between CD4⁺Th2 and CD4⁺Th1 cells after stimulation with CLV and its synthetic AD analogues in CLV-allergic patients and healthy controls. Comparisons were assessed by Mann-Whitney U test (* p<.05; ** p<.01; *** p<.001).

Since significant proliferative differences were only observed for CD3⁺T, CD4⁺T, and especially CD4⁺Th2 cells from CLV-AP compared to HC after stimulation with synthetic ADs analogues from CLV (Figure 59), for the subsequent analysis, we only

focused on these cell populations, as the results for CD8⁺T and CD4⁺Th1 cell results seem not to be as relevant in IDHRs to CLV.

In CD3⁺T cells, the highest % of proliferative positive cases was detected after moDCs stimulation from CLV-AP with Clav5 (23%), followed by Clav1 (19%) and Clav2 (15%) (Figure 59A). Similarly, in CD4⁺T cells, the highest % of positive cases was obtained after moDCs stimulation from CLV-AP with Clav5 (31%), followed by Clav1 (27%) and Clav2 (19%) (Figure 59B). In CD4⁺Th2 cells, the highest % of positivity was found after CLV-AP moDCs stimulation with Clav1 (27%), followed by Clav3, Clav5 and Clav6 (23% each). The number of positive cases in HC was 0%, independently the CLV AD synthetic analogue included, and cell population analysed (Figure 59), except Clav1 for CD4⁺Th2 cells (Figure 59C). The % of positive cases was lower than 20% when native CLV was included in LTT experiments, reporting 9% of positivity in HC, regardless the cell population studied.

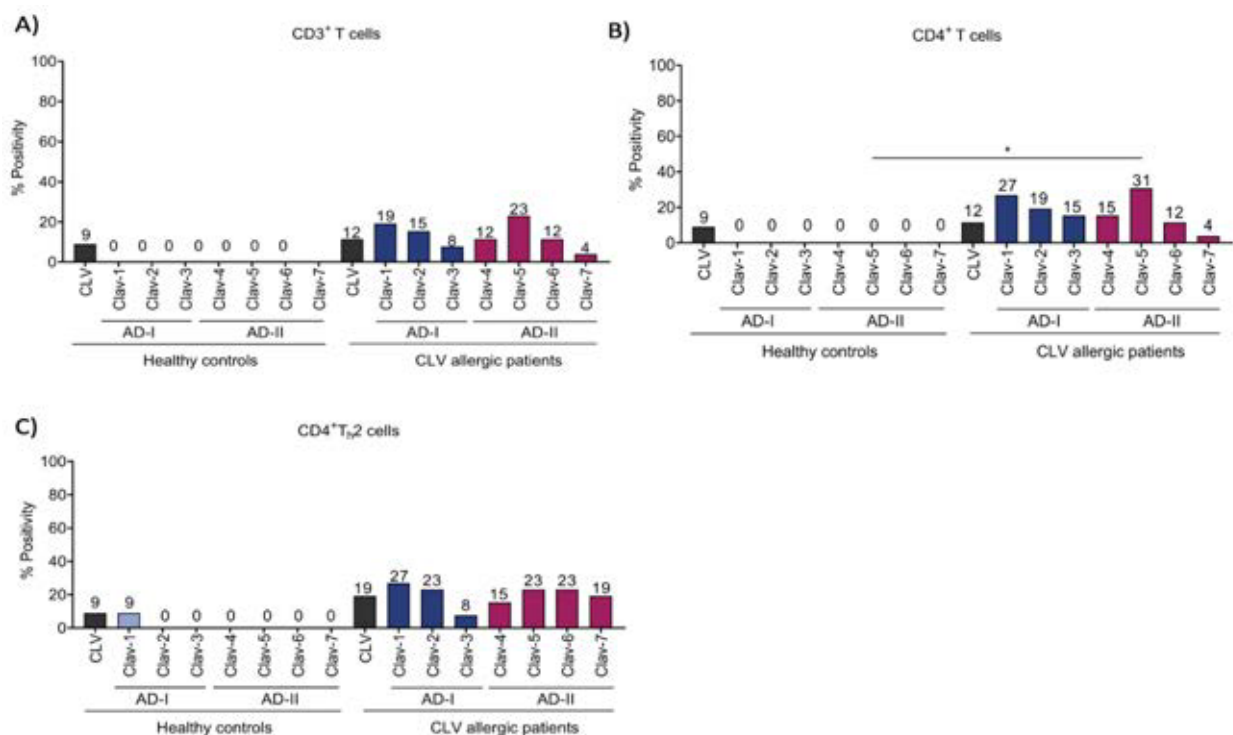


Figure 59. Positivity of lymphocyte proliferation assays induced by CLV and its synthetic AD analogues. Bars represent % of positive cases of proliferative response for each cell population analysed in CLV allergic patients and healthy controls. A) CD3⁺T cells; B) CD4⁺T cells; C) CD4⁺Th2 cells. Positivity comparisons were assessed by X² test (* p<.05).

The combination of proliferative results of synthetic analogues from each AD increased the % of positivity to 35% and 42% in CD3⁺T and CD4⁺T cells respectively (Figure 60A, B). When the proliferative response of CD4⁺Th2 cells was evaluated, the combination of results from synthetic analogues of CLV AD-I increased the % of positivity to 42%, while those from AD-II increased to 54% (Figure 60C). The combination of results from synthetic analogues from both CLV ADs together increased the positivity to 46%, 50% and 65% when evaluating the proliferative response of CD3⁺T, CD4⁺T, and CD4⁺Th2 cells respectively (Figure 60). The % of positivity was lower than 10% independently the cell subpopulation or synthetic analogue combination used in HC (Figure 60). The inclusion of results from native CLV did not increase the % of positivity compared to the combination of AD-I and AD-II, although it increased the % of positive cases in HC when assessing CD4⁺Th2 cells (from 9 to 18%) (Figure 60C).

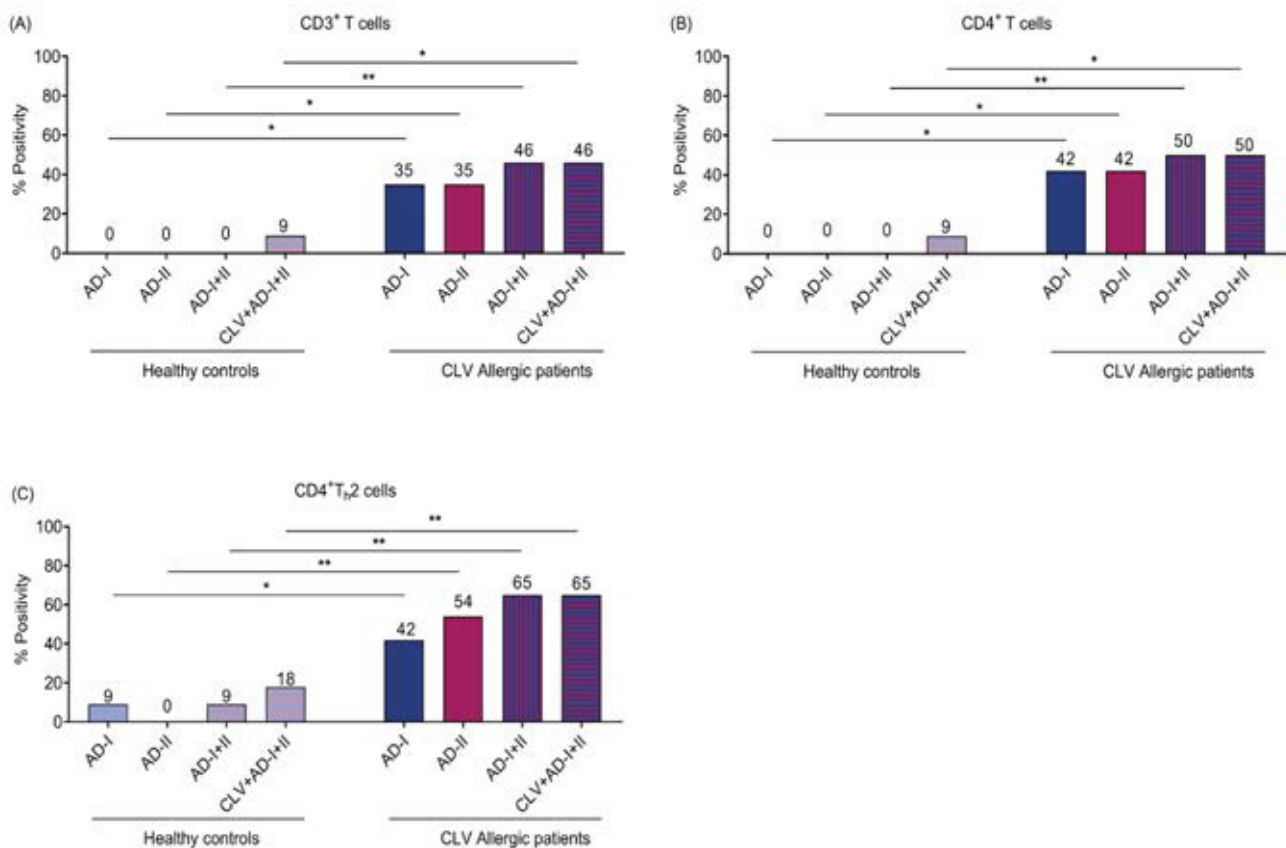


Figure 60. Combination of results from lymphocyte proliferative assays. Bars represent % of positive cases after the combination of proliferative lymphocyte results with synthetic analogues from each CLV ADs for different cell populations from CLV allergic patients and healthy controls. A) CD3⁺Tcells; B) CD4⁺Tcells; C) CD4⁺Th₂ cells. Positivity comparisons were assessed by X² test (* p < .05; ** p < .01).

From the lymphocyte proliferation positive cases, 50% simultaneously recognised synthetic analogues from AD-I and AD-II, while 25% specifically recognised synthetic analogues from AD-I or AD-II when assessing CD3⁺T cell proliferation (Figure 61A). A similar pattern of recognition was observed for CD4⁺T cells, where 70% of positive cases simultaneously recognised synthetic analogues from both ADs, while 15% specifically recognised those from AD-I or AD-II (Figure 61B). From the positive cases of CD4⁺Th2 cells, 47% simultaneously recognised synthetic analogues from both ADs, whereas 35% specifically recognised those from AD-II and 18% those from AD-I (Figure 61C).

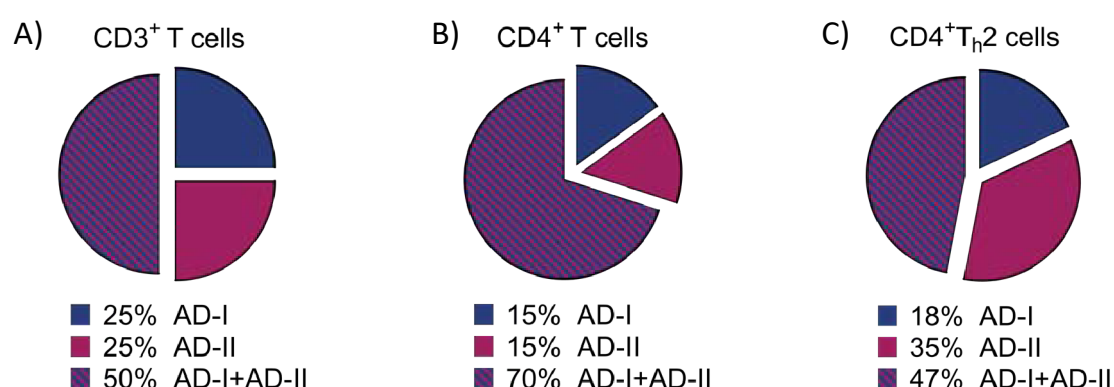


Figure 61. Specific recognition of each AD from the positive proliferative results. Part of whole represent the % of recognition of each AD from lymphocyte proliferative assay positive cases for A) CD3⁺Tcells; B) CD4⁺T cells; C) CD4⁺Th2 cells.

Combinations of results from the cell populations most importantly involved in the study (CD3⁺T, CD4⁺T and CD4⁺Th2 cells) with synthetic analogues from both ADs and native CLV did not increase the % of positive cases compared to the evaluation of synthetic analogues from both CLV ADs when only the proliferative response of CD4⁺Th2 cells was assessed (Figure 62A).

Finally, we evaluated which concrete analogues from AD CLV have a positive influence on the results of the lymphocyte proliferative assay. The inclusion of all synthetic analogues, except Clav4 and Clav5, improved the sensitivity of CD4⁺Th2 cell proliferative results, with a contribution in terms of positivity from highest to lowest: Clav1>Clav6>Clav2>Clav3>Clav7 (Figure 62B).

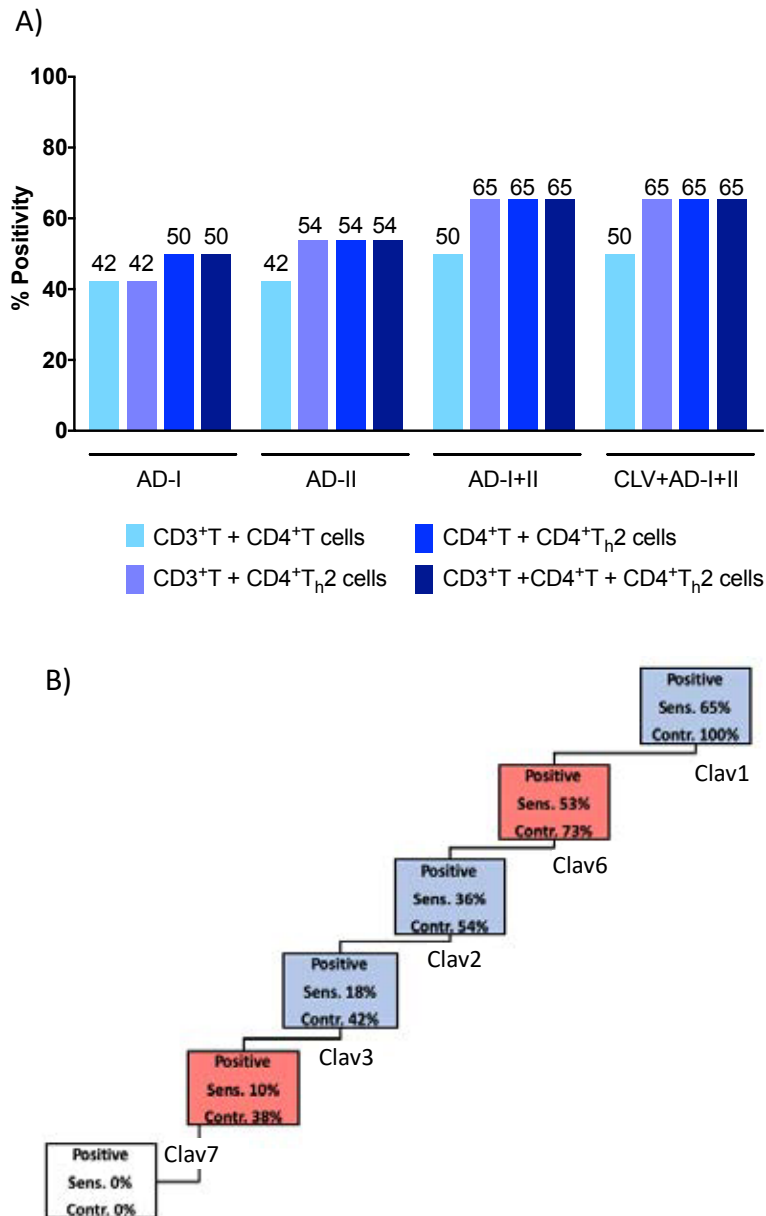


Figure 62. Combination of proliferative results from different cell populations and contribution of each synthetic analogue to lymphocyte proliferation. A) Bars represent the combination of proliferative results from different cell populations and synthetic analogues in CLV allergic patients. B) Diagram represents the contribution of each concrete synthetic analogue from CLV AD-I (blue) and AD-II (red) in the proliferative response of CD4⁺Th2 cells from CLV allergic patients

DISCUSSION

IDHRs to CLV have increased in recent decades due to its high prescription alongside AX, mainly because of its betalactamase activity.^{267,293,297} Nevertheless, their diagnosis is a complex process because STs have low sensitivity and DPT cannot be performed with CLV itself. Therefore, an accurate diagnosis of an IDHR after AX-CLV intake has to be confirmed in most cases by the good tolerance of AX by DPT, a procedure that is not risk-free, and is contraindicated for most severe reactions.^{34,128,269,294,298}

Because of these limitations of *in vivo* test, the use of *in vitro* tests may represent useful and safe alternatives for CLV IDHRs clinical routine. Nevertheless, the CLV-sIgE detection is not possible,²⁵⁶ and the sensitivity of BAT or HRT is limited.^{267,299} One possible explanation for the low sensitivity reported in these tests could be that CLV was highly degraded in solution, with only some of the CLV metabolites having ability to interact with proteins and thus responsible for triggering an IDHR. However, as the native CLV is directly included in the tests, the final concentration of each degradative metabolite may be low enough to trigger the immune response *in vitro*.

The inclusion of metabolites instead of the native drug has been evaluated in different studies with promising results.^{91,95,300-303} Nevertheless, the identification of the CLV degradative metabolites is very difficult,^{173,252} mainly because CLV degradation occurs very rapidly, between 5-10 times faster than in other BLs,^{237,238} and its degradative metabolites can be detected within minutes.²⁵¹ In addition, the reaction has been reported to be self-accelerated, because the degradative intermediates also act as strong catalysts.^{236,252}

In a previous study, we have demonstrated that synthetic analogues from one AD derived from CLV degradation (AD-I) can trigger the basophils degranulation in patients with IDHRs to CLV, but not in HC, increasing the BAT sensitivity. Interestingly, the metabolites inducing basophil activation were those with capacity to react to proteins¹⁷³ Nevertheless, the complete CLV recognition process has not been fully elucidated. Therefore, we now try to determine the recognition of these synthetic analogues using moDCs from CLV-AP and HC, and to analyse their maturation status, as well as their capacity to trigger T cell proliferation, as it has been used to evaluate different drugs previously.^{91,199,217}

In this study, we have shown that some of the synthetic analogues from both ADs are efficiently and specifically recognised by moDCs from CLV-AP. Moreover, the maturation moDCs response following stimulation with the synthetic analogues was higher than stimulation with native CLV, as the latter only triggered the upregulation of CD83 and CD86, whereas the former triggered the moDC expression of CD40, CD80, CD83 and, although to a lesser extent, of CCR7 and CD86. Interestingly, we observed that synthetic analogues have differential behaviours inducing the expression of different maturation markers on moDCs, with AD-I preferentially promoting the expression of CCR7 and CD40, whereas those from AD-II were associated with the expression of CD80, CD83, and CD86. These data may suggest that both ADs were efficiently recognised by moDCs from CLV-AP, but in different ways, so that the inclusion of both ADs might be necessary to amplify the immunological response and increase the sensitivity moDCs maturation assays.

The proliferative response of lymphocytes after stimulation with BLs has been evaluated previously in NIDHRs.^{36,43,266,282,304} Nevertheless, only one recent study carried out by our group in a paediatric cohort has separately assessed the lymphocyte proliferative response to AX or CLV.³⁴ In the study carried out by Prieto *et al.* the lymphocyte stimulation with CLV reported a sensitivity of 33.3%.³⁴ In the present study, we obtained a sensitivity of 19% when native CLV was included. However, in the study of Prieto *et al.* children with NIDHRs were evaluated, whereas in this study we evaluated adults with IDHRs. Furthermore, in this study we stimulated moDCs with CLV, and as it has been suggested, drugs are not potent DC maturation inducers. Therefore, there is a possibility that only a semimature DC state is achieved, not allowing subsequent full stimulation of autologous lymphocytes.^{217,305,306} Moreover, the overall low sensitivity found in both studies with CLV may be explained by the not inclusion of the specific metabolites responsible for triggering the DHR.

In this regard, when synthetic analogues from both AD-I and AD-II with different reactivity were included, the sensitivity of lymphocyte proliferative assays increased to 65% with a specificity higher than 90%, similar to other studies with different drugs.^{34,186,301}

Other factors such as the severity of the reaction,^{14,190,191} or the time elapsed between reaction and sample collection could also have an impact in the sensitivity of proliferative assays.^{43,174} Because of this, and due to the low number of patients included in the study, these factors cannot be completely ruled out.

Eighty-two % of patients with positive CD4⁺Th2 proliferative response, main cell subpopulation involved in IDHRs, recognised synthetic analogues from AD-II, while only 65% recognised those from AD-I. Because synthetic AD-II analogues preferentially triggered the overexpression of CD80, CD83, and CD86, rather than CCR7 or CD40, these maturation markers seem to be more relevant during the presentation process. Nevertheless, the fact that 18% specifically recognised synthetic analogues from AD-I, while 25% specifically recognised those from AD-II, might make the inclusion of synthetic analogues from both ADs is necessary. These differences between ADs and synthetic analogues could be explained by their different ability to modify proteins, as synthetic analogues can interact with different proteins, with different density and distribution, and due to this heterogeneity, the inclusion of different synthetic analogues is important for drug recognition.^{239,307,308}

Herein we have demonstrated that synthetic analogues from AD-I were recognised by moDCs from CLV-AP, with the ability to trigger mainly the proliferation of CD4⁺Th2 cells. Nevertheless, we have also shown that synthetic analogues from AD-II were also efficiently recognised. This may suggest that the aldehydic functionality of the structure is an important requirement for recognition by moDCs from CLV-AP. In addition, their ability to interact with proteins is also important, due to Clav4 and Clav5 did not trigger T cell response, because although they have the unaltered amine functionality, they are not activated to interact with proteins.

CHAPTER III

Involvement of autologous myeloid dendritic cells in the assessment of immediate hypersensitivity reactions to amoxicillin and clavulanic acid.

SUMMARY

Having confirmed in the previous chapters that moDCs improved the sensitivity of *in vitro* assays in both IDHRs and NIDHRs, we next asked whether other types of DC could play a more important role for drug recognition and the consequent activation of autologous lymphocytes.

Therefore, in this study we evaluated the usefulness of analysing **maturation, migration, and activation markers on moDCs and mDCs** in response to AX and CLV stimulation in selective AP with IDHRs to AX or CLV and in HC. Moreover, we analysed their capacity to **present** the drugs and **stimulate the proliferation of different lymphocyte populations**, comparing to the results with the traditional assay, consisting of the direct drug stimulation of PBMCs (C-LTT).

Herein, we demonstrated that **mDCs** have a **greater capacity** to upregulate the expression of **CCR7, CD40, CD80, CD83, and CD86** in response to the specific drug responsible for triggering the IDHR compared to moDCs. Additionally, mDCs have a greater ability to **promote lymphocyte proliferation**, mainly of **CD4⁺Th2 cells**, in response to the concrete drug, compared to using moDCs or C-LTT, although important **differences** were found depending on whether the **responsible drug** was AX or CLV.

RESULTS

Patients and healthy controls characterisation

From patients who attended to the Allergy Unit of HRUM with a compatible history of IDHRs to BLs between 2018 and 2019, 28 patients with a confirmed diagnosis of IDHR to AX or CLV were selected and included in the study. 14 of them (50%) (group A) were selective to AX, while 14 (50%) (group B) were selective to CLV. Demographic characteristics and clinical data of APs are shown in Table 20. The majority of the patients, 18 out of 28, were female (64%), while 10 were male (36%). The mean age was 45.39 ± 13.98 years. Most reactions were ANA in 20/28 patients (71%), followed by URT/ANG in 8/28 (29%).

The severity of the reactions was graded according to the Brown severity classification.³⁰⁹ Ten of the 28 reactions were grade I reactions (35.7%), 10/28 were grade II reactions (35.7%) and 8/28 were reactions of grade III (28.6%). The mean time interval between drugs intake and onset of symptoms was 34.64 ± 22.28 min, and the time interval between reaction and sample collection was 4.68 ± 4.04 years. Twenty out of 28 patients (71%) reported only one episode, whereas 8/28 (29%) reported two. Twenty of 28 patients (71%) reported an IDHR after the intake of AX-CLV, and 8/28 (29%) after the intake of AX alone.

All patients from group A were diagnosed by ST positive to AX, and negative to CLV, benzylpenicilloyl octa-lysine and benzylpenicilloate. DPT with PV was also performed to confirm good tolerance to other BLs. The only exception was the patient A9 who was diagnosed by DPT positive to AX after ST negative to AX, CLV, benzylpenicilloyl octa-lysine and benzylpenicilloate.

All patients from group B were diagnosed by positive ST to CLV, and negative to benzylpenicilloyl octa-lysine and benzylpenicilloate. The selectivity of CLV allergic patients was confirmed by negative DPT to AX.

Patient	Sex	Age	Reaction	Severity	Drug-Reaction	Reaction-study	Episodes	Drug	ST			DPT	
									AX	CLV	Bp*	PV	AX
A1	F	40	ANA	III	30	4	1	AX-CLV	+	-	-	-	n.d.
A2	F	19	ANA	II	10	3	1	AX-CLV	+	-	-	-	n.d.
A3	F	59	ANA	III	15	2	1	AX-CLV	+	-	-	-	n.d.
A4	F	33	ANA	III	10	4	2	AX-CLV	+	-	-	-	n.d.
A5	F	58	ANA	II	10	4	1	AX	+	-	-	-	n.d.
A6	M	49	ANA	II	10	5	1	AX	+	-	-	-	n.d.
A7	M	499	ANA	III	30	1	1	AX-CLV	+	-	-	-	n.d.
A8	M	69	ANA	II	60	13	1	AX	+	-	-	-	n.d.
A9	M	40	URT/ANG	I	60	13	1	AX	-	-	-	-	+
A10	F	64	URT/ANG	I	60	2	2	AX-CLV	+	-	-	-	n.d.
A11	F	51	ANA	III	30	5	1	AX	+	-	-	-	n.d.
A12	F	29	URT/ANG	I	60	9	2	AX	+	-	-	-	n.d.
A13	F	25	URT/ANG	I	60	1	2	AX	+	-	-	-	n.d.
A14	M	59	ANA	I	10	1	1	AX-CLV	+	-	-	-	n.d.
B1	F	42	ANA	II	60	3	1	AX-CLV	-	+	-	n.d.	-
B2	F	33	ANA	II	30	11	2	AX-CLV	-	+	-	n.d.	-
B3	F	66	ANA	I	30	13	1	AX-CLV	-	+	-	n.d.	-
B4	M	66	URT/ANG	I	60	2	1	AX-CLV	-	+	-	n.d.	-
B5	F	47	ANA	II	15	2	2	AX-CLV	-	+	-	n.d.	-
B6	F	34	ANA	II	60	1	1	AX-CLV	-	+	-	n.d.	-
B7	M	30	AN	I	30	3	1	AX-CLV	-	+	-	n.d.	-
B8	F	52	ANA	III	5	2	1	AX-CLV	-	+	-	n.d.	-
B9	F	42	URT/ANG	I	60	4	1	AX-CLV	-	+	-	n.d.	-
B10	F	29	ANA	I	5	2	1	AX-CLV	-	+	-	n.d.	-
B11	F	37	ANA	I	10	2	2	AX-CLV	-	+	-	n.d.	-
B12	M	65	ANA	II	60	1	2	AX-CLV	-	+	-	n.d.	-
B13	M	44	ANA	III	60	11	1	AX-CLV	-	+	-	n.d.	-
B14	M	40	ANA	III	30	7	1	AX-CLV	-	+	-	n.d.	-

Table 20. Demographic and clinical data from patients with selective immediate hypersensitivity reactions to amoxicillin (A1-14) or clavulanic acid (B1-14). Abbreviations: F, female; M, male; ANA, anaphylaxis; URT/ANG, urticaria/angioedema; AX, amoxicillin; CLV, clavulanic acid; unk, unknown; SPT, skin prick test; DPT, drug provocation test; PV, penicillin V; n.d., not done. *ST to BP was assessed by using bencilpenicilloyl octa-lysine and benzylpenicilloate.

Migration, activation, and maturation marker expression on different DC populations.

First, we determined the expression of different migration, activation, and maturation markers on unstimulated mDCs and moDCs from AP and HC, to determine whether differences exist. In unstimulated conditions, data shown an increased expression of CCR7 in mDCs compared to moDCs from both AP and HC (Figure 63A). In contrast, higher CD40, CD80, and CD86 expression was observed on unstimulated moDCs compared to unstimulated mDCs from both AP and HC (Figure 63B-D). No differences were observed regarding the expression of CD83 between unstimulated mDCs and moDCs (Figure 63E). To note, no differences were detected within each DC population between AP and HC (Figure 63) independently the marker analysed.

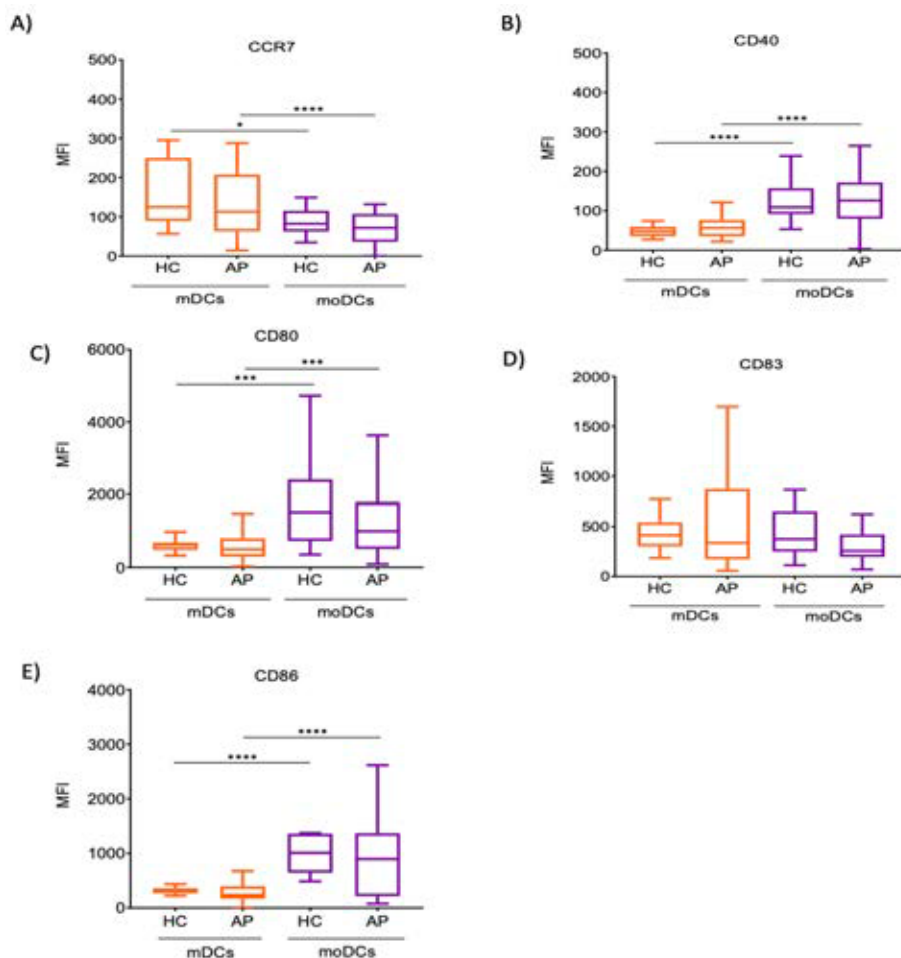


Figure 63. Expression of markers on unstimulated DCs. Box-Plot represent Mean Fluorescence Intensity (MFI) of migration, activation and maturation markers on mDCs (orange) and moDCs (purple) from healthy controls (HC) and allergic patients (AP). Comparisons were assessed by Mann–Whitney test (* $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$).

Drug-stimulated mDC and moDCs maturation assays

Stimulation with AX increased the expression of CCR7, CD40, CD80, and CD83 in AX-AP compared to HC with both concentrations of AX, and of CD86 with 0.5mM of AX (Figure 64). Conversely, only high expression of CCR7, CD83, and CD86 was observed when moDCs from AX-AP were stimulated with the culprit drug (Figure 64A, D, E). No differences were observed between AX-AP and HC in terms of CD40 and CD80 expression (Figure 64B, C).

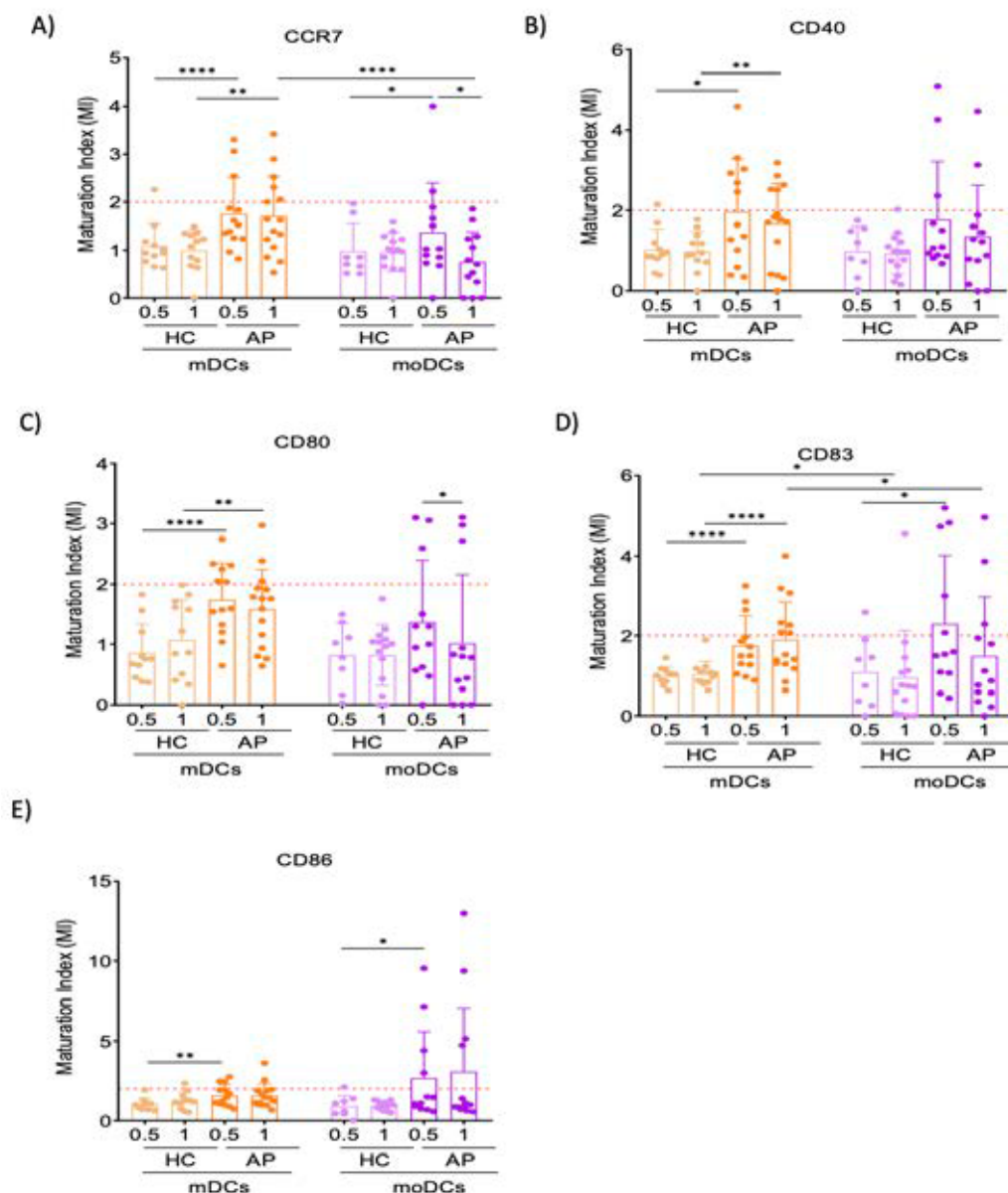


Figure 64. mDCs and moDCs maturation assays in amoxicillin allergic patients and healthy controls. Bars and dots represent maturation index (MI) values of mDCs (orange) and moDCs (purple) from healthy controls (HC) and amoxicillin (AX) allergic patients (AP) stimulated with AX at 0.5 and 1mM. A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86. Comparisons were assessed by Mann–Whitney test (*p < .05; **p < .01; ***p < .001; ****p < .0001).

Stimulation of mDCs in CLV-AP with the culprit drug increased the expression of CCR7, CD80, and CD86 at the concentration of 0.5mM and 1mM (Figure 65A, C, E), and of CD83 at 0.5mM (Figure 65D) compared to HC. No significant differences were observed for CD40 expression on mDCs between CLV-AP and HC (Figure 65B). Stimulation of moDCs from CLV-AP did not increase the expression of any of the markers studied (Figure 65A-C, E), except for CD83 at 1mM from CLV-AP compared to HC.

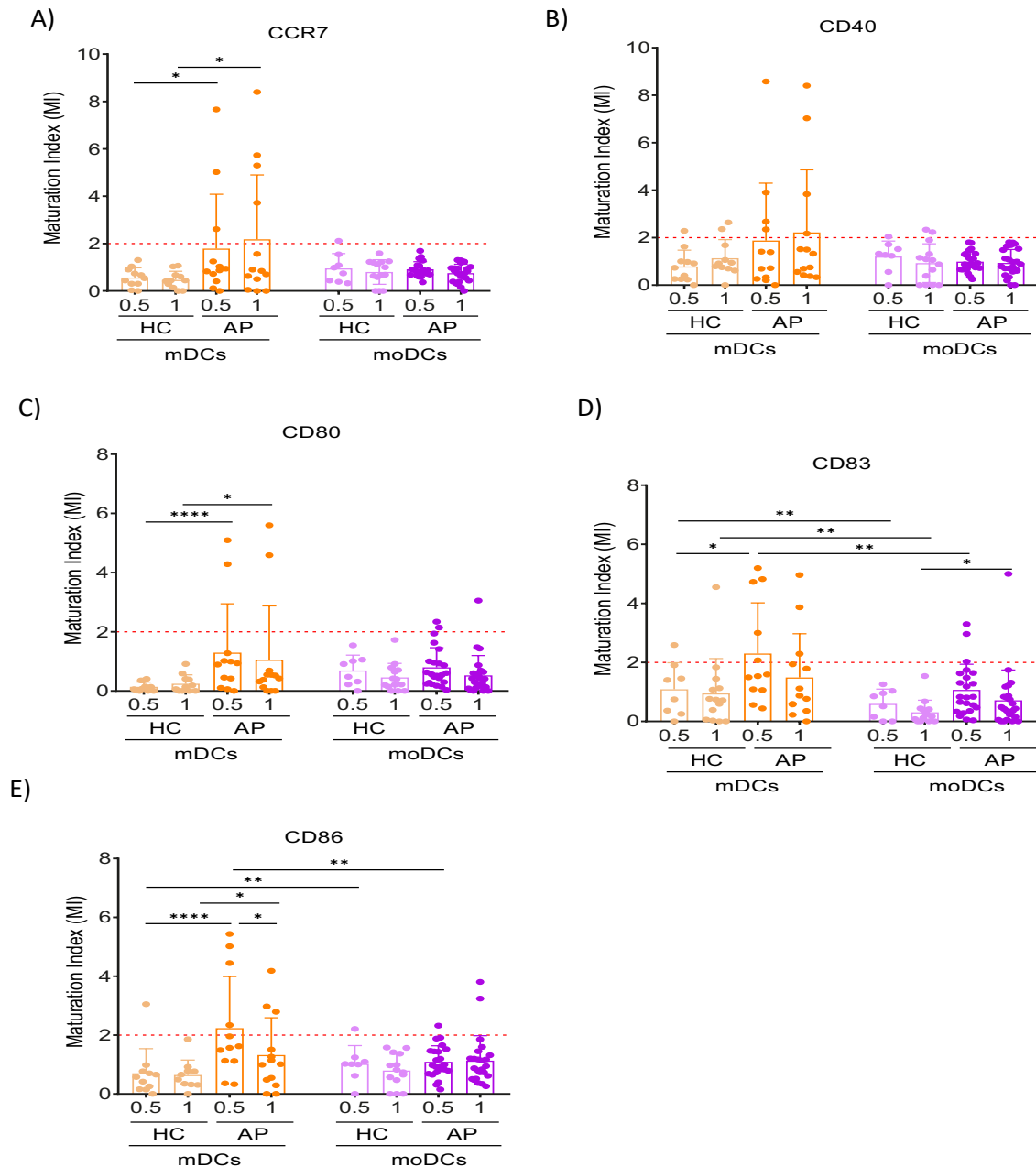


Figure 65. mDCs and moDCs maturation assays in clavulanic acid allergic patients and healthy controls. Bars and dots represent maturation index (MI) values of mDCs (orange) and moDCs (purple) from healthy controls (HC) and clavulanic acid (CLV) allergic patients (AP), stimulated with CLV at 0.5 and 1mM. A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86. Comparisons were assessed by Mann–Whitney test (* $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$).

In terms of the maturation assays positivity for each marker and DC population, higher positivity was observed when mDCs from AX-AP were stimulated with the culprit drug (47% for CCR7, 47% for CD40, 60% for CD80, and 47% for CD83) compared to the stimulation of moDCs (15% for CCR7, 31% for CD40, 31% for CD80, and 39% for CD83) (Figure 66A-D). The only exception was for CD86, whose % of positivity was similar in both DC populations, mDCs (27%) and moDCs (31%) (Figure 66E). In HC, the % of positivity was lower than 10% irrespective of the DC population and marker analysed (Figure 66A-E), except in mDCs for CD86 (17%) (Figure 66E).

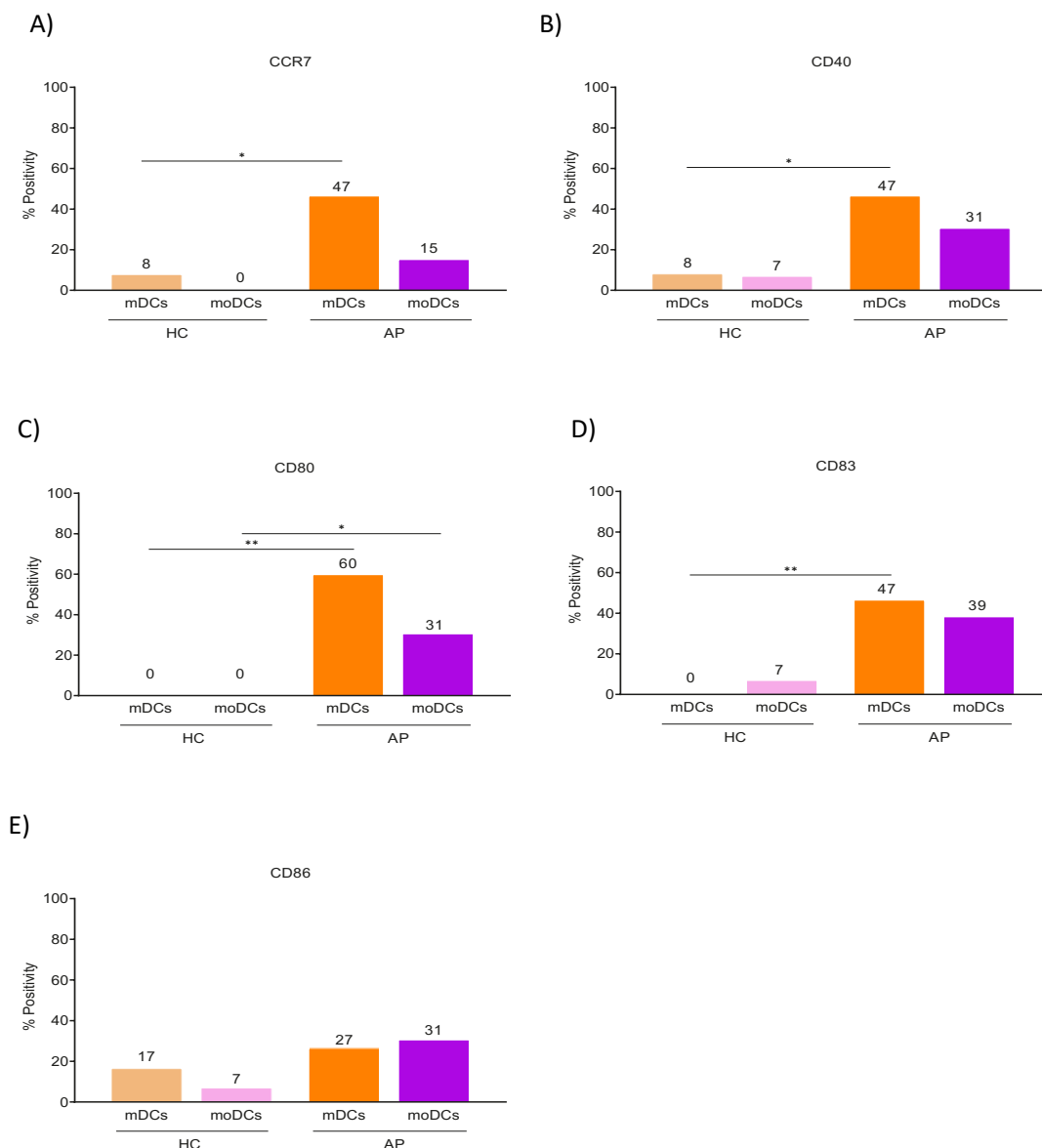


Figure 66. Positivity of mDCs and moDCs maturation assays in amoxicillin allergic patients and healthy controls. Bars represent % of positivity mDCs (orange) and moDCs (purple) from healthy controls (HC) and amoxicillin (AX) allergic patients (AP) stimulates with AX. A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86. Comparisons were assessed by χ^2 test (* p < .05; ** p < .01).

A similar trend was observed in CLV-AP, in the assays using the culprit drug, CLV, mDCs reported a higher % of positivity for all markers (43% for CCR7, 50% for CD40, 21% for CD80, and 36% for CD86), compared to stimulation of moDCs (0% for CCR7 and CD40, 8% for CD80, and 13% for CD86) (Figure 67A-C, E), with the exception again of CD83, with similar % values in mDCs (14%) and moDCs (17%) (Figure 67D). The % of positive cases in mDCs and moDCs from HC stimulated with CLV, showed values below 10% for CCR7, CD80, and CD83 (Figure 67A, H, I), although higher % was observed in mDCs and moDCs for CD40 (25% and 21% respectively) (Figure 67B), and in mDCs for CD86 (17%) (Figure 67E).

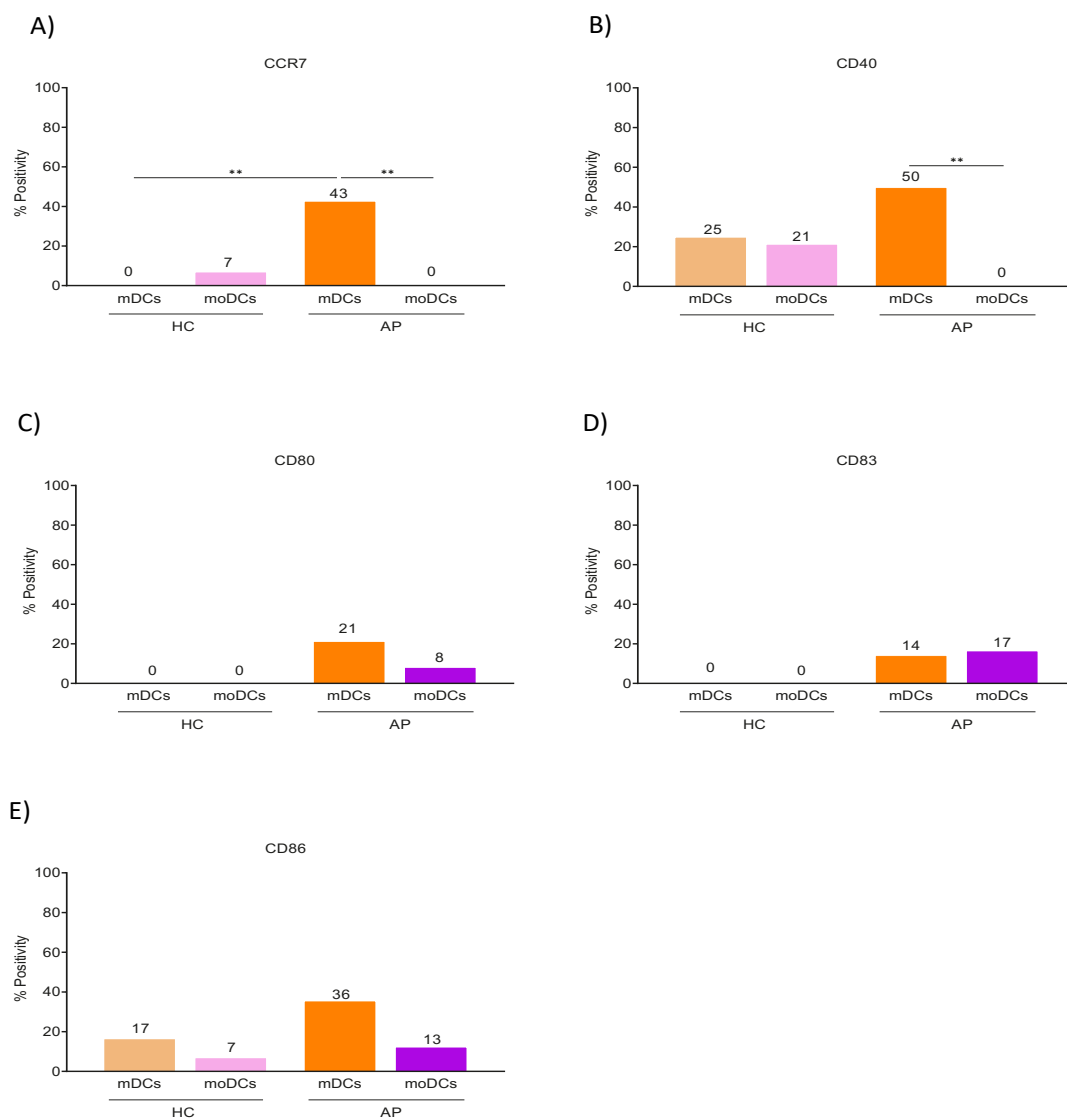


Figure 67. Positivity of mDCs and moDCs maturation assays in clavulanic acid allergic patients and healthy controls. Bars represent % of positivity of mDCs (orange) and moDCs (purple) from healthy controls (HC) and clavulanic acid (CLV) allergic patients (AP) stimulated with CLV. A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86. Comparisons were assessed by χ^2 test (** $p < .01$).

Evaluation of drug specificity of mDC and moDCs maturation assays

Next, we evaluated the specificity of the maturation assay to the drug involved in the reaction. For this, we analysed the response of mDCs and moDCs from AX-AP or CLV-AP with the non-culprit drug, CLV or AX respectively. to assess their ability to differentiate between the two drugs. Data showed that the overall MI found was low for all the markers and DC population analysed (Figure 68), with only significant differences between mDCs and moDCs with CLV at 1mM for CD40 (Figure 68B) and CD80 (Figure 68C).

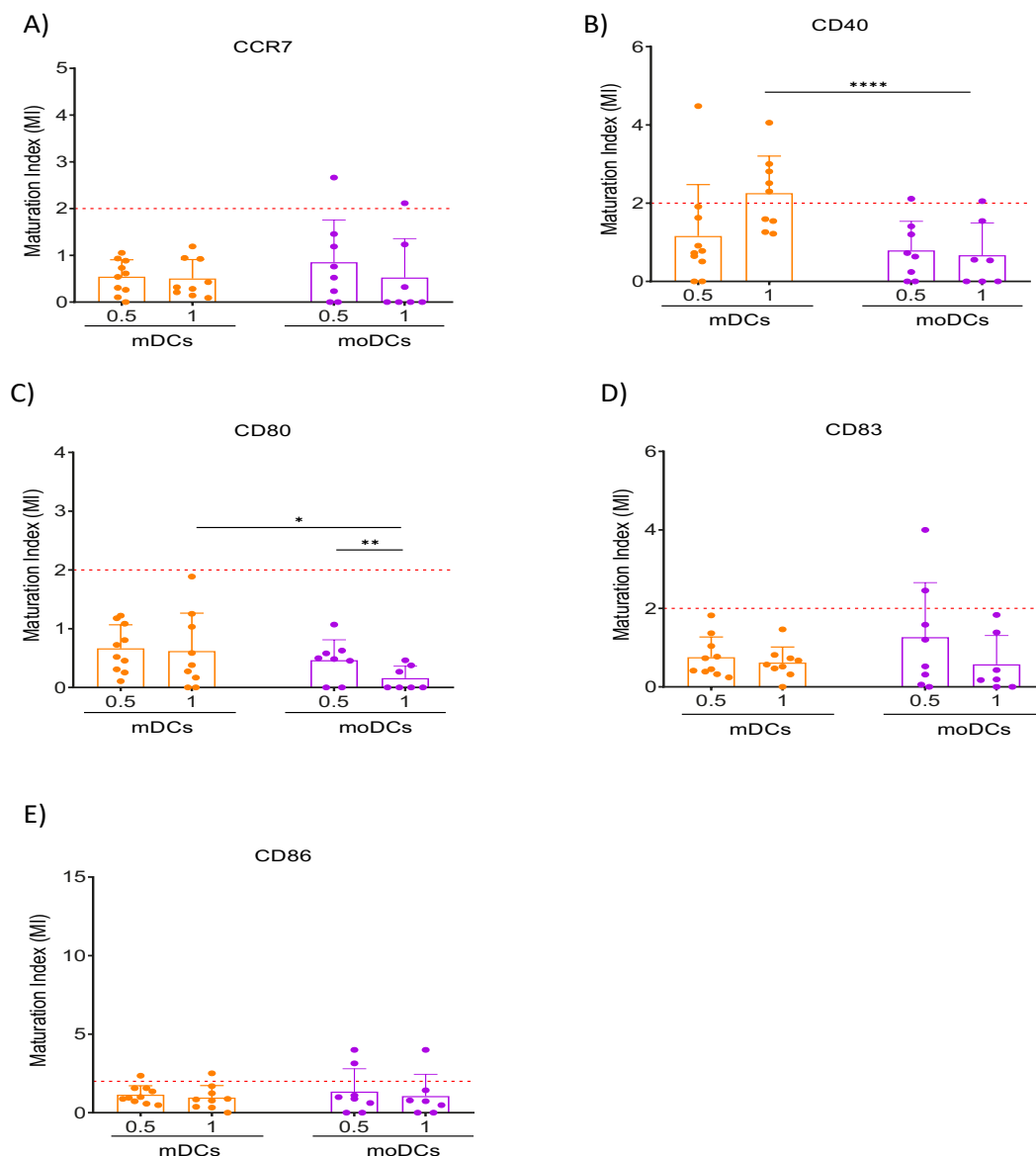


Figure 68. mDCs and moDCs maturation assays in amoxicillin allergic patients stimulated with the non-culprit drug. Bars and dots represent maturation index (MI) values of mDCs (orange) and moDCs (purple) from amoxicillin (AX) allergic patients (AP) stimulated with clavulanic acid (CLV) at 0.5 and 1mM. A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86. Comparisons were assessed by Mann–Whitney test (*p < .05; **p < .01; ****p < .0001).

Similarly, mDCs and moDCs from CLV-AP stimulated with AX reported an overall low MI (Figure 69), with only significant differences between mDCs and moDCs with AX at 0.5mM for CD40 (Figure 69B) and CD83 (Figure 69D).

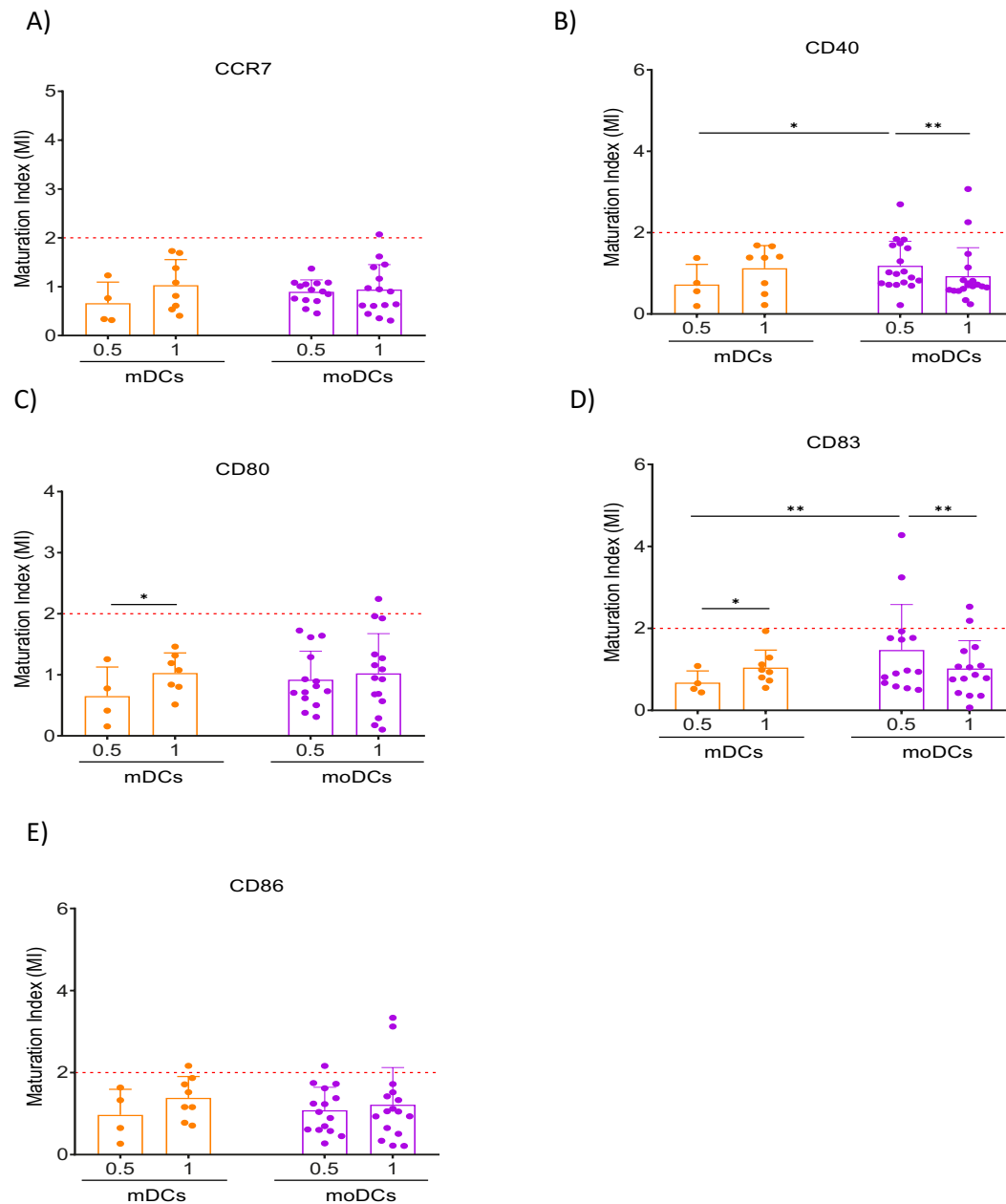


Figure 69. mDCs and moDCs maturation assays in clavulanic acid allergic patients stimulated with the non-culprit drug. Bars and dots represent maturation index (MI) values of mDCs (orange) and moDCs (purple) from clavulanic acid (CLV) allergic patients (AP) stimulated with amoxicillin (AX) at 0.5 and 1mM. A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86. Comparisons were assessed by Mann–Whitney test (*p < .05; **p < .01; ***p < .0001).

Regarding the % of positivity in AX-AP stimulated with CLV, no positive results were obtained in mDCs for CCR7 (Figure 70A), CD80 (Figure 70C), and CD83 (Figure 70D). Nevertheless, 46% of positivity was observed for CD40 (Figure 69B) and 18% for CD86 in mDCs (Figure 70E). The % of positivity obtained after stimulation of moDCs from AX-AP with CLV was higher than that obtained for mDCs (10% with CCR7, 20% with CD83 and CD86) (Figure 70A, D, E), with the exception of CD40 (10%) (Figure 70B) and CD80 (0%) (Figure 70C).

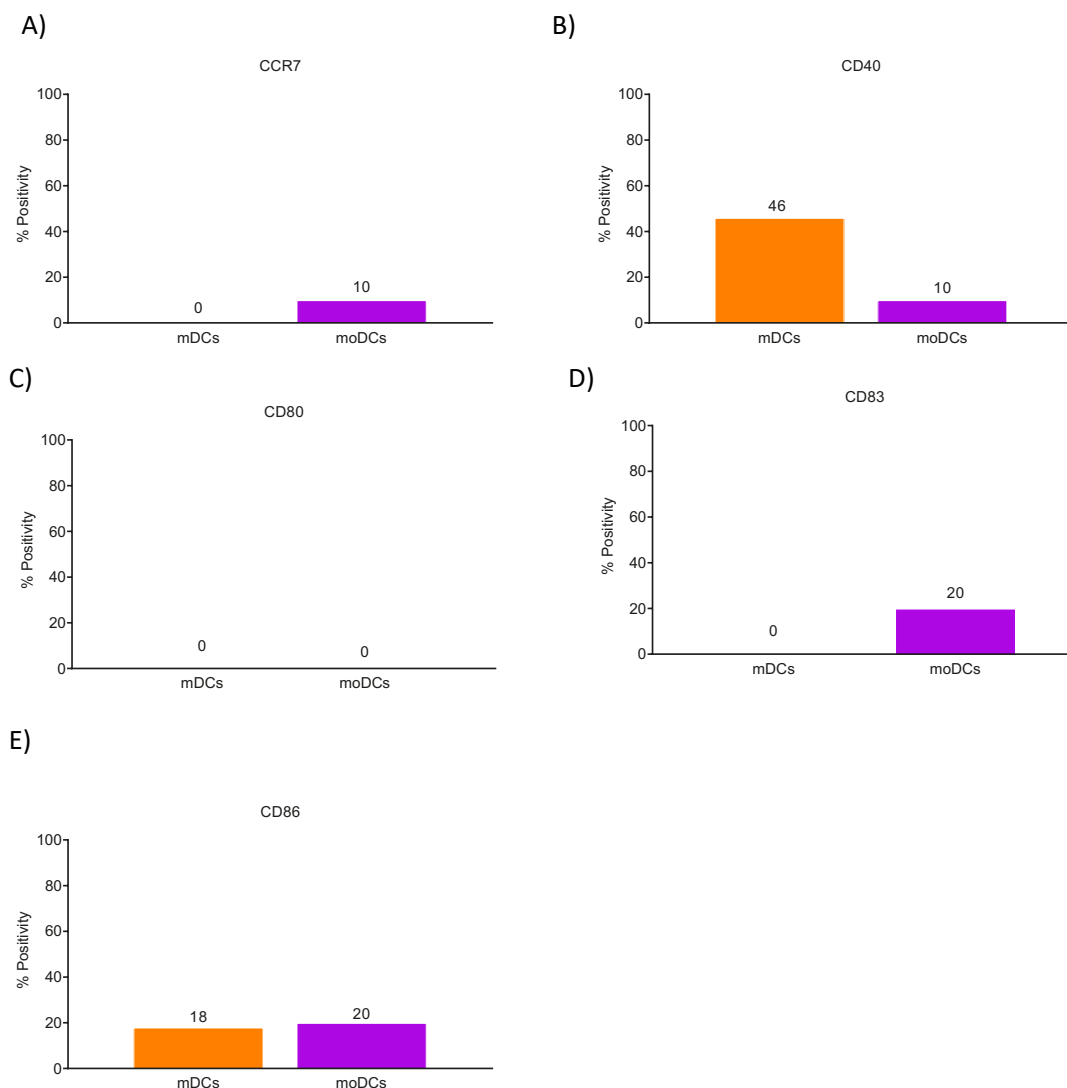


Figure 70. Positivity of mDCs and moDCs maturation assays in amoxicillin allergic patients stimulated with the non-culprit drug. Bars represent % of positivity of mDCs (orange) and moDCs (purple) from amoxicillin (AX) allergic patients (AP) stimulated with clavulanic acid (CLV). A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86. Comparisons were assessed by χ^2 test.

In mDCs from CLV-AP stimulated with AX, no positive cases were detected for any of the analysed markers (Figure 71A-D), except for CD86 (13%) (Figure 71E). The positivity for moDCs from AX-AP stimulated with CLV, although with overall low positivity, was observed at higher values compared to mDCs, 5% for CCR7, 11% for CD40, 11% for CD80, 21% for CD83, and 26% for CD86 (Figure 71).

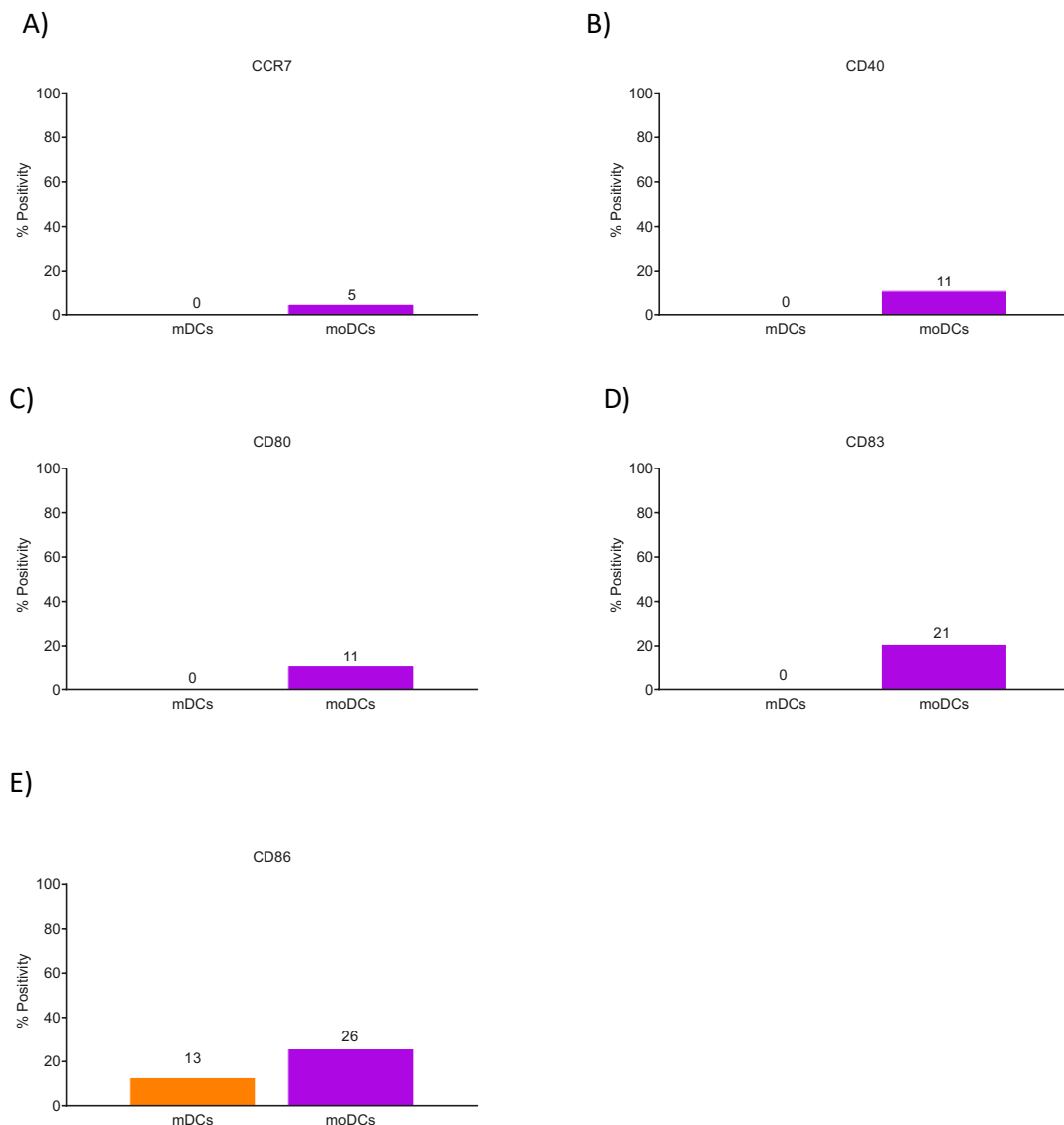


Figure 71. Positivity of mDCs and moDCs maturation assays in clavulanic acid allergic patients stimulated with the non-culprit drug. Bars represent % of positivity of mDCs (orange) and moDCs (purple) from clavulanic acid (CLV) allergic patients (AP) stimulated with amoxicillin (AX). A) CCR7; B) CD40; C) CD80; D) CD83; E) CD86. Comparisons were assessed by χ^2 test.

Drug-stimulated mDC and moDCs capacity to activate T cell populations

LTT with or without pre-primed mDCs and moDCs were carried out. Data demonstrated that higher PI was observed when mDCs from AX-AP were stimulated with the culprit drug, AX at 1mM and the proliferative response of CD3⁺T, CD4⁺T, and CD4⁺Th2 cells was assessed compared to HC (Figure 72A, B, E), but not when the proliferative response of CD8⁺T or CD4⁺Th1 cells was analysed (Figure 72C, D). In the case of AX-stimulated moDCs only significantly higher proliferative response of CD4⁺Th2 cells was observed in AX-AP compared to HC (Figure 72E). C-LTT, consisting of direct PBMCs stimulation with the culprit drug increased the proliferative response of all T cell populations (Figure 72A-C, E), except for CD4⁺Th1 cells (Figure 72D) compared to HC.

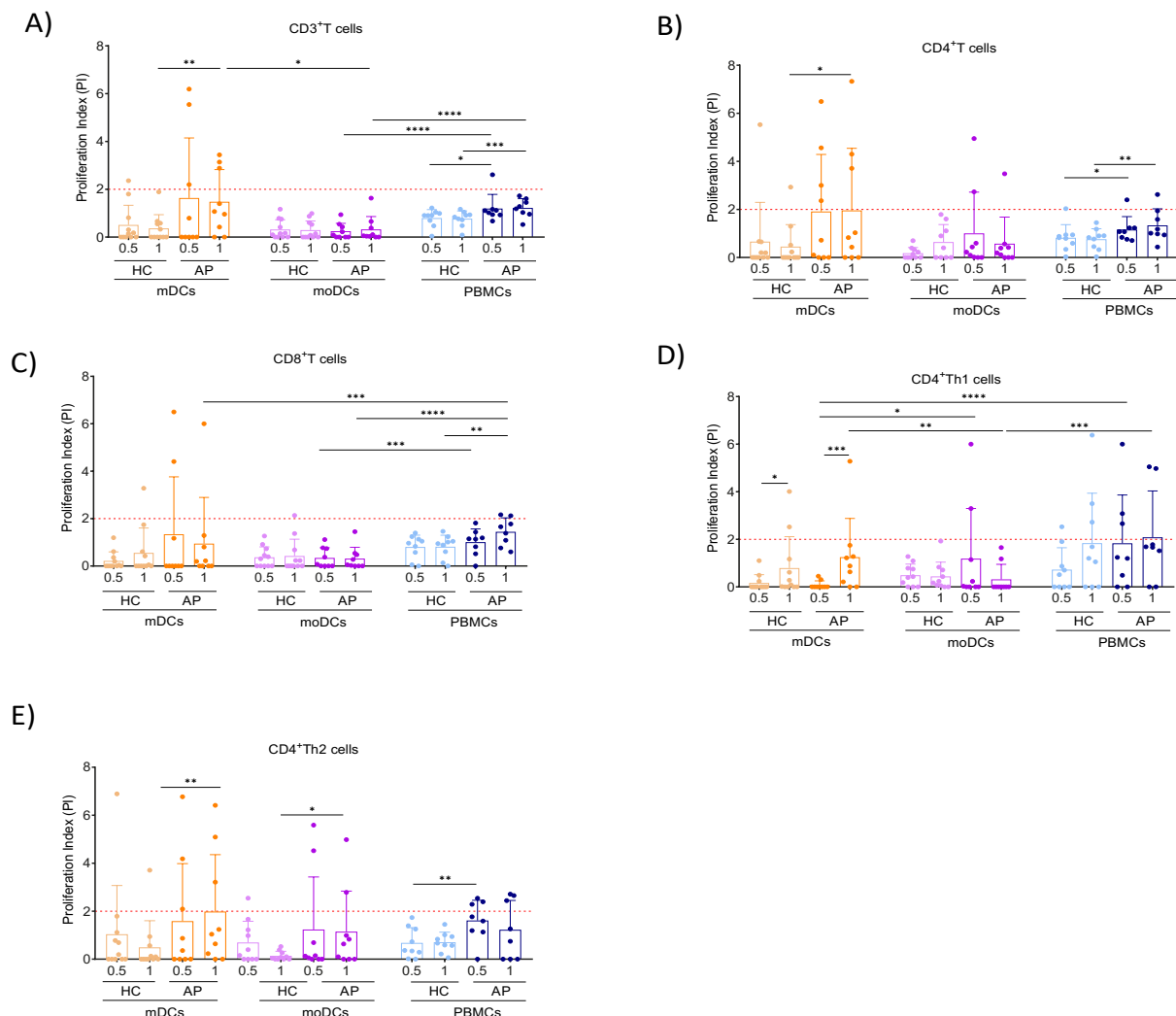


Figure 72. Proliferation Index of different T cell populations in amoxicillin allergic patients. Bars and plots represent proliferation Index (PI) of A) CD3⁺T, B) CD4⁺T, C) CD8⁺T, D) CD4⁺Th1, and E) CD4⁺Th2 cells with amoxicillin (AX) pre-primed-mDCs (orange), -moDCs (purple) or direct PBMCs stimulation (blue), in healthy controls (HC) and AX-allergic patients (AP). Comparisons were calculated by Mann–Whitney test (*p < .01; **p < .01; ***p < .001, ****p < .0001).

Similar to AX-AP, when CLV-pre-primed mDCs were included as APCs, higher PI was observed in CD3⁺T, CD4⁺T, and CD4⁺Th2 cell from CLV-AP compared to HC (Figure 73A, B, E), but not when the proliferative response of CD8⁺T or CD4⁺Th1 cells was assessed (Figure 73C, D). On the other hand, no proliferative differences were observed between CLV-AP and HC with the inclusion of CLV-pre-primed moDCs or with the conventional LTT (Figure 73).

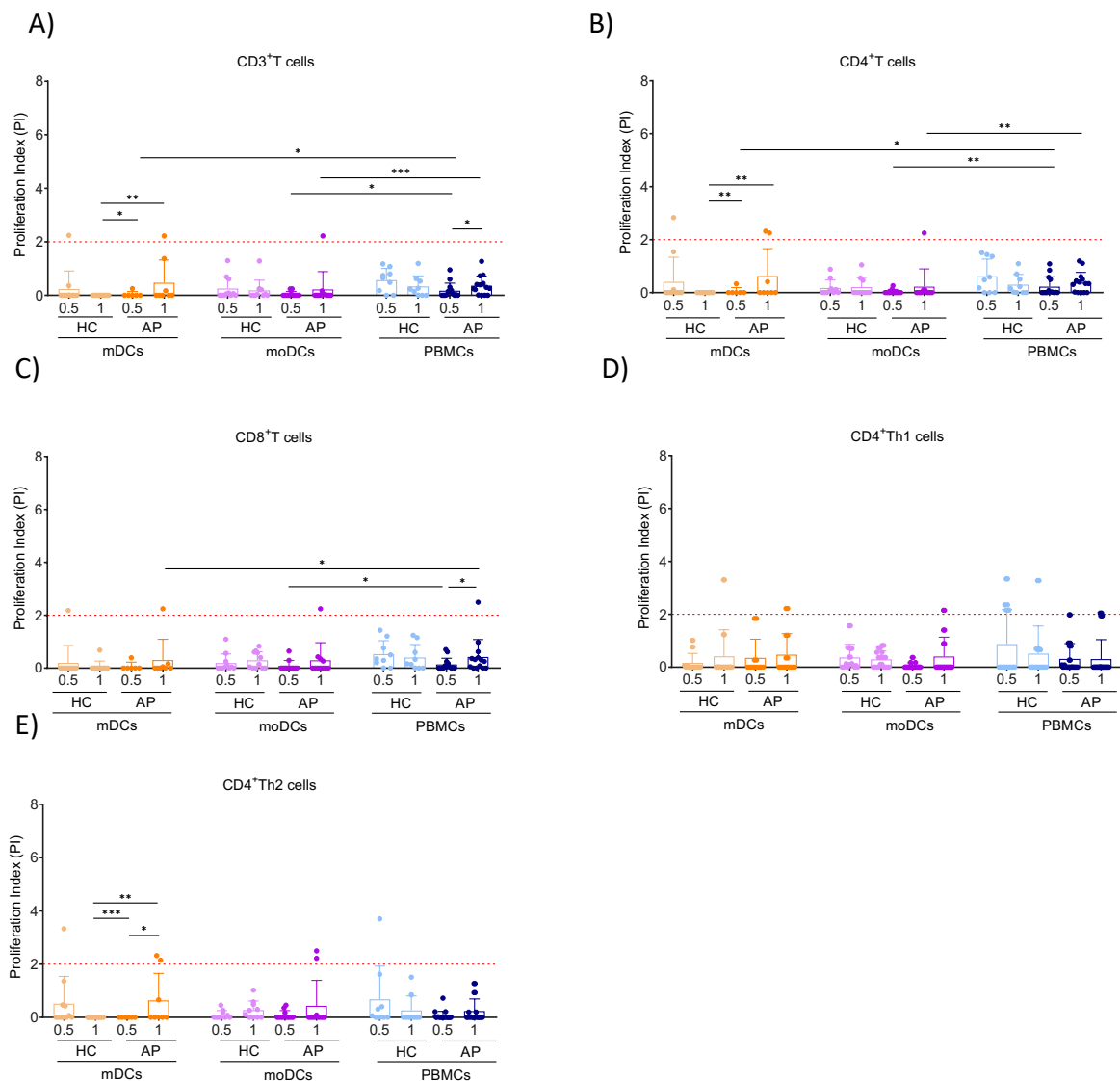


Figure 73. Proliferation Index of different T cell populations in clavulanic acid allergic patients. Bars and plots represent proliferation Index (PI) of A) CD3⁺T, B) CD4⁺T, C) CD8⁺T, D) CD4⁺Th1, and E) CD4⁺Th2 cells with clavulanic acid (CLV) pre-primed-mDCs (orange), -moDCs (purple) or direct PBMCs stimulation (blue), in healthy controls (HC) and CLV-allergic patients (AP). Comparisons were calculated by Mann–Whitney test (*p < .01; **p < .01; ***p < .001).

Then, we analysed the results in terms of LTT positivity. The highest % of positivity was obtained when AX-pre-primed mDCs were included in the assay and the proliferation of CD3⁺T, CD4⁺T, CD4⁺Th1, and CD4⁺Th2 cells was assessed (67% each) (Figure 74A, B, D, E), while it was only 22% for CD8⁺T cells (Figure 74B). No positive cases were detected with the inclusion of AX-pre-primed moDCs when the proliferation of CD3⁺T or CD8⁺T cells was assessed (Figure 74A, C), while the positivity for CD4⁺T cells was only of 33% (Figure 74B), and of 22% for CD4⁺Th1 and CD4⁺Th2 cells (Figure 74D, E). In the C-LTT, the best % of positivity was found for CD4⁺Th2 cells (50%), followed by CD4⁺Th1 and CD8⁺T cells (25% each), and CD3⁺T and CD4⁺T cells (13% each) (Figure 74). The % of positive cases was lower than 15%, independently the LTT assay performed, or the proliferative T cell response analysed in HC.

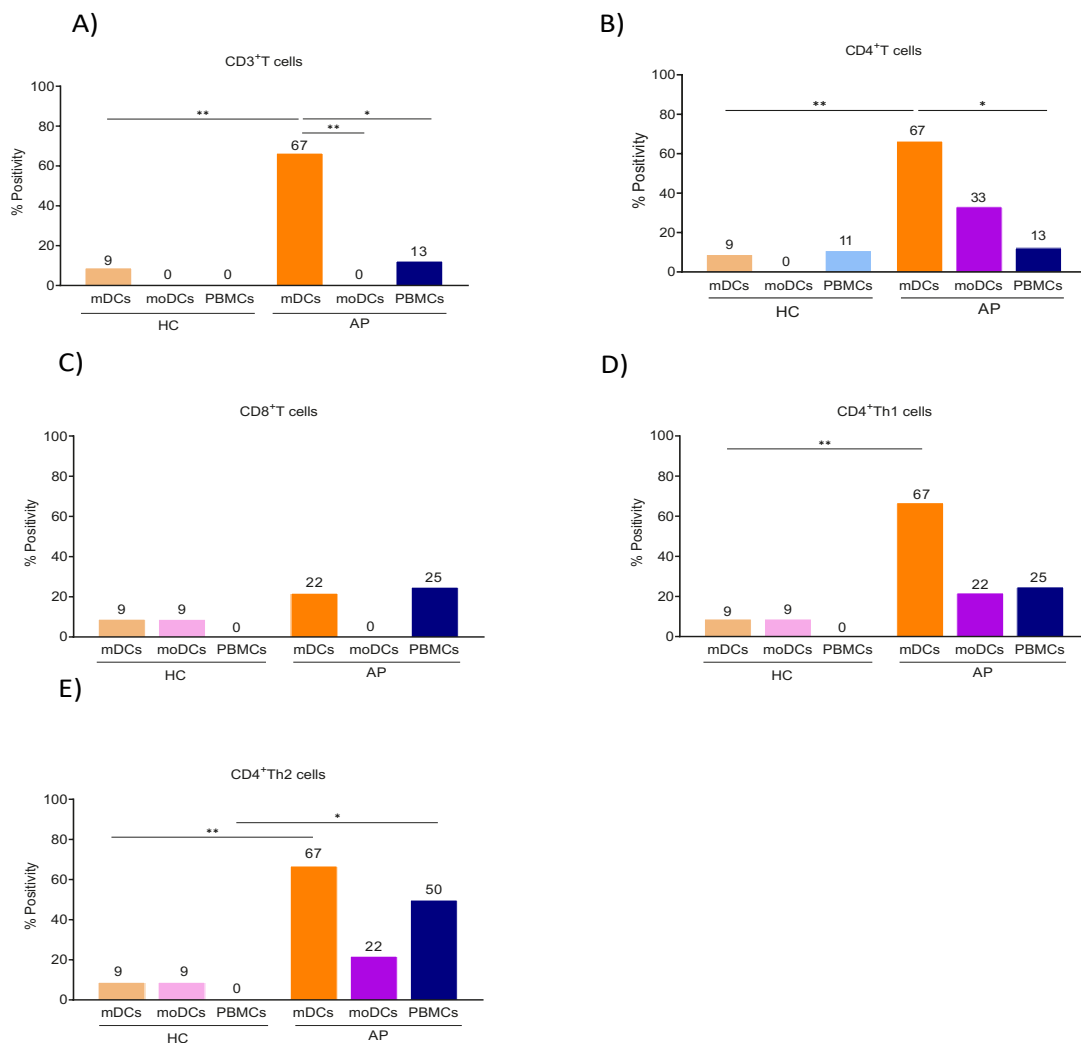


Figure 74. Positivity of lymphocyte proliferative assays. Bars represent the % of positive cases for A) CD3⁺T, B) CD4⁺T, C) CD8⁺T, D) CD4⁺Th1, and E) CD4⁺Th2 with amoxicillin (AX) pre-primed mDCs (orange), moDCs (purple) or direct PBMCs stimulation (blue) from healthy controls (HC) and AX allergic patients (AP). Comparisons were calculated by X² test (*p < .01; **p < .01).

The % of positive cases from CLV-AP stimulated with the culprit drug, CLV was lower than those obtained with AX-AP, although the trend was similar, the highest % of positivity was obtained for mDCs in CD3⁺T, CD4⁺T, and CD4⁺Th2 cells (25% each) (Figure 75A, B, E), followed by CD8⁺T and CD4⁺Th1 cells (13% each) (Figure 75E). The inclusion of CLV-pre-primed moDCs reported very low % of positivity, being the highest values (20%) when assessing the proliferative response of CD4⁺Th2 cells (Figure 75E). The direct PBMCs stimulation with CLV reported the lowest values of positivity, being only up to 7% in CD8⁺T and CD4⁺Th1 cells (Figure 75C, D), while no positive cases were detected with CD3⁺T, CD4⁺T, and CD4⁺Th2 cells (Figure 75A, B, E). Notably, the % of positive cases in HC were lower than 10%, regardless of the proliferative T cell population analysed and the LTT assay performed (Figure 75).

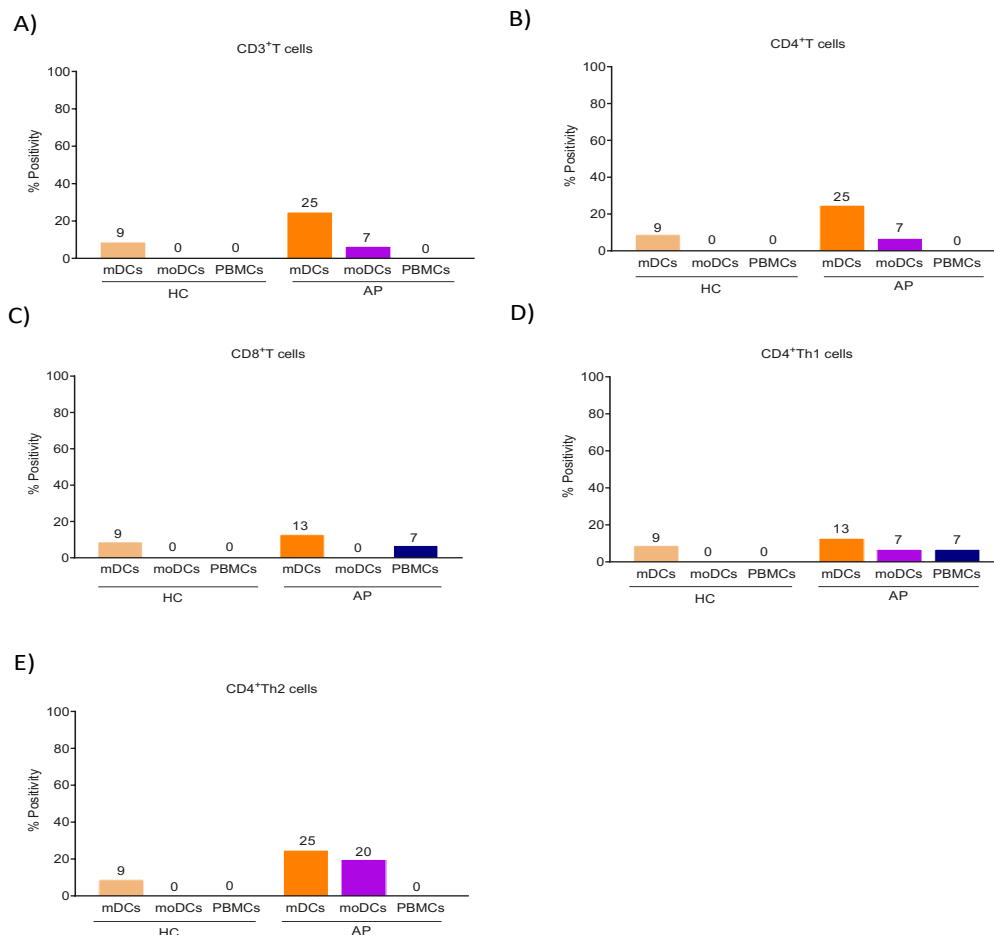


Figure 75. Positivity of lymphocyte proliferative assays. Bars represent the % of positive cases for A) CD3⁺T, B) CD4⁺T, C) CD8⁺T, D) CD4⁺Th1, and E) CD4⁺Th2 with clavulanic acid (CLV) pre-primed mDCs (orange), moDCs (purple) or direct PBMCs stimulation (blue) from healthy controls (HC) and CLV allergic patients (AP). Comparisons were calculated by X² test (*p < .01; **p < .01).

Finally, to assess the specificity of the in vitro methods, we evaluated the lymphocyte proliferation of the LTT cultures, by stimulating mDCs, moDCs or PBMCs with the non-culprit drug. This time we focused only on the proliferative response of CD4⁺Th2 cells, as these seem to be the most relevant cell population involved in IDHRs to BLs. The PI obtained in cells from AX-AP stimulated with CLV reported general low values, irrespective the LTT assay performed, although it was higher with direct stimulation of PBMCs or stimulation of moDCs compared to the ones with mDCs (Figure 76). Similarly, stimulation of PBMCs from CLV-AP with AX showed higher proliferation than that obtained after the inclusion of AX-pre-primed mDCs and moDCs (Figure 76B). In terms of % of positivity, no positive cases were detected in AX-AP stimulated with CLV when mDCs was used as APCs, although 11% of positive cases were detected with CLV-pre-primed moDCs, and 38% after direct PBMCs stimulation with CLV (Figure 76C). Similarly, no positive cases were observed with AX-pre-primed mDCs or moDCs in CLV-AP, while 21% of positive cases were detected with AX stimulation of PBMCs (Figure 76D).

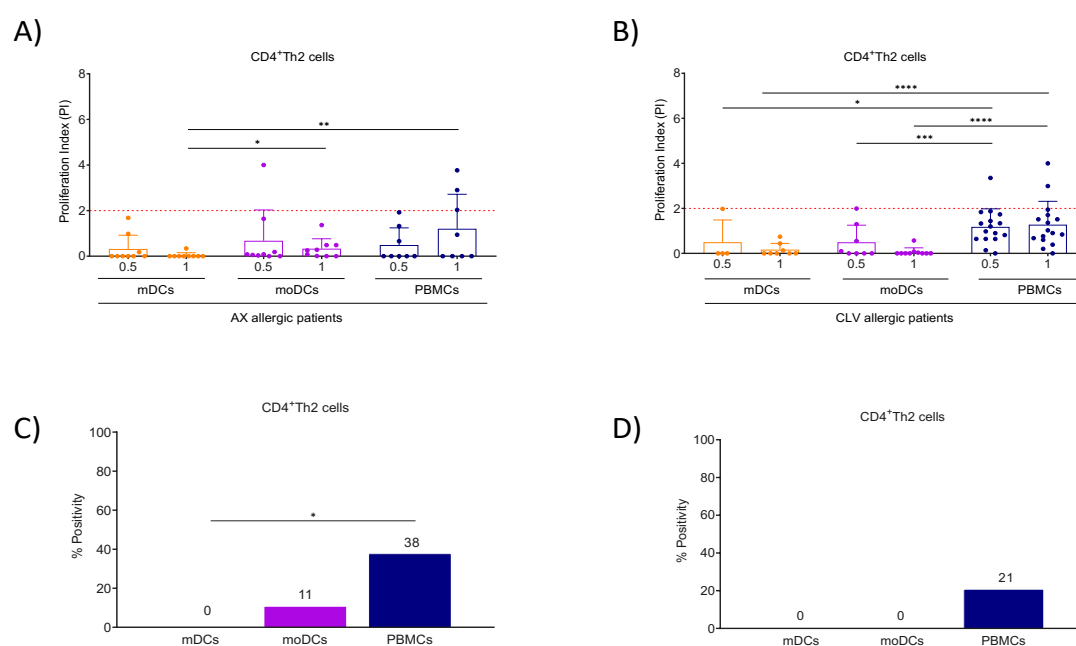


Figure 76. Proliferation Index and positivity of CD4⁺Th2 cells. (A, B) Bars, plots represent proliferation index (PI) of CD4⁺Th2 cells with pre-primed mDCs (orange), moDCs (purple) or direct PBMCs drug stimulation (blue). (A) Cells from amoxicillin (AX) allergic patients (AP) stimulated with clavulanic acid (CLV); (B) cells from CLV AP stimulated with AX. Comparisons were calculated by Mann–Whitney test (* $p < .01$; ** $p < .01$; **** $p < .001$, **** $p < .0001$). (C, D) Bars represent the % of positive cases with pre-primed mDCs (orange), moDCs (purple) or direct PBMCs stimulation (blue). (C) Cells from AX AP stimulated with CLV; (B) cells from CLV AP stimulated with AX. Comparisons were calculated by χ^2 test (* $p < .01$).

DISCUSSION

The analysis of migration, activation, and maturation markers on DCs after the stimulation with drugs may represent useful complementary alternatives in the diagnosis of IDHRs, in combination with current *in vivo* test, with limited sensitivity or even dangerous for patients,^{275,310} mainly for those with more severe reactions, and also in combination with other *in vitro* assays, because they do not have an optimal sensitivity for assessing IDHRs to BLs.^{34,160,267,272,282,283,311-313}

Although the true “sentinels” against internal and external antigens in blood and tissues are mDCs,³¹⁴ most studies used moDCs because they can be easily transformed *in vitro* from blood circulating monocytes and in high numbers.³¹⁵ Despite the fact that moDCs are present in nature, they only develop under inflammatory conditions, and after resolution, they disappear.³¹⁵⁻³¹⁷ Due to the different nature of mDCs and moDCs, their specific response to drugs may also be different, especially when IDHRs were evaluated, because of their differential contribution in immediate reactions.

In this study, we observed that, at steady state, mDCs expressed a higher level of CCR7 compared to moDCs, while the latter population expressed higher levels of costimulatory molecules such as CD40, CD80, and CD86. These observations may suggest that although both DC populations have high phagocytic and antigen processing activity,^{201,318} immature moDCs were more activated at baseline, with a lower predisposition to migrate to LNs, contrary to what was observed for mDCs.^{319,320} Accordingly, our results showed that, after drug stimulation, mDCs from AP expressed much higher levels of CCR7 than moDCs, suggesting the high capacity of the formers to migrate efficiently to LNs, mimicking the pathomechanism of DCs during acute phase of IDHRs.

Several studies have shown that DC maturation markers, such as CD40, CD80, CD83, and CD86 were highly expressed on drug-stimulated moDCs from AP with NIDHRs.^{199,217} Related to this, Sanchez-Quintero and colleagues reported a 45.8% of positivity for CD86 expression on AX-stimulated moDCs from AX-AP with NIDHRs.²⁰² In our study, we obtained a positivity of 31% for the same marker. The lower expression found in the present study could be explained by the different involvement of moDCs in NIDHRs or IDHRs. Consequently, the expression of these markers was much higher expressed after AX stimulation of mDCs from AX-AP (47%-60%) compared to moDCs.

Similarly, mDCs from CLV-AP after the stimulation with the culprit drug induced higher expression of maturation markers compared to HC. Nevertheless, the proliferation index and % of positivity were much lower in CLV- than in AX-AP. This could be explained by the higher degradation rate of CLV compared to AX.^{173,237-239} Because of its degradation, different CLV metabolites can be generated, with different capacities to interact with proteins and to form drug-carrier conjugates, and thus with different abilities to be recognised by the immune system,¹⁷³ as we demonstrated in the previous chapter. Furthermore, results with mDCs showed better results than moDCs or the conventional LTT. Therefore, we hypothesised that mDCs are more sensitive and ready-to-act than moDCs in patients with IDHRs to BLs and may require a lower concentration of these CLV reactive metabolites to be activated than other APCs such as moDCs, monocytes or B cells.

LTT has been mainly used to evaluate NIDHRs with variable sensitivity between studies, mostly due to different factors such as the nature of the drug, the immune mechanism involved, the severity of the reaction, the clinical entities, the time interval between reaction and sample collection, or the technique used to assess lymphocyte proliferation.^{43,73,159,186,187,190,191,272} In a recent study, Mori and colleagues reported a sensitivity of 52% with a specificity of 92% in a group of children with NIDHRs to AX or AX/CLV.³⁶ Similar sensitivity was found by Prieto *et al.* (52.9%) with important differences between AX (63.6%) and CLV (33.3%),³⁴ suggesting the importance of the nature of the drug in LTT, similar to our observations in this study.

To our knowledge, few studies have been conducted before to evaluate the usefulness of LTT in IDHRs. In a study carried out by Luque and colleagues in patients with IDHRs to BLs, they found a LTT sensitivity of 64.5%,¹⁸⁶ higher than in our study. The differences between studies may be explained by the different methodology used and the different clinical entities involved, since in Luque's study, more than 30% of the patients were allergic to BP and none to CLV, being most of them cross-reactors.

In our study, the highest sensitivity of LTT was obtained when assessing the proliferative response of CD4⁺Th2, similar to what was observed in our study with IDHRs to CLV and its metabolites.³²¹ In contrast, for NIDHR we have shown that the combination of different cell populations involved in the reaction improved the sensitivity of LTT.²⁹⁶ This highlights the great heterogeneity of NIDHRs compared to

IDHRs, as well as the importance of analysing the proliferative response of the specific cell populations involved in each concrete immune mechanism.^{296,321}

It has been demonstrated that the use of pre-primed moDCs trigger the proliferation of autologous T cells in response to drugs in patients with NIDHRs to AX, with greater efficacy than other APCs such as monocytes or B cells,²¹⁷ similar to other drugs such as heparins.²⁷² Nevertheless, the sensitivity of LTT is still not optimal for drugs, and concretely to AX and CLV, probably because they are not strong stimulators compared to proteins or other allergens. Because of their weak stimulation capacity, it is possible that drug stimulated DCs only achieved a semimature status, characterised by the rapid expression of activation and maturation markers, but weak cytokine release.^{217,305,322} This may explain why, although higher expression of costimulatory molecules was observed on moDCs after drug stimulation, they have not full capacity to stimulate and activate autologous lymphocytes.

In our study, only about 20% of LTT sensitivity was observed after the inclusion of pre-primed moDCs, lower than in other studies with NIDHRs, probably because the different mechanism involved in IDHRs and NIDHRs, as well as the different involvement of moDCs in these two DHRs. However, the inclusion of pre-primed mDCs increased the sensitivity of LTT to 67% with AX, in line with other studies that demonstrated that mDCs have a strong ability to trigger T cell proliferation and activation.³²² Although the use of CLV-pre-primed mDCs also reported the best % of sensitivity compared to the inclusion of pre-primed moDCs or direct drug stimulation of PBMCs, its sensitivity was much lower (25%), probably because of the instability of CLV structure and the generation of heterogeneous degradative metabolites.^{173,236-239,251,256,271,321}

CONCLUSIONS

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- ◆ The use of drug pre-primed mDCs as APCs significantly increases the sensitivity of LTT for evaluating NIDHRs to different drugs and clinical entities.
- ◆ The analysis of the % CFSE_{Dim} cells after drug stimulation by flow cytometry allows to determine the proliferative response of different cell subpopulations in a reliable and safe way and in a single test. The combination of proliferative results of the effector cell populations involved in each immune mechanism significantly increases the sensitivity of LTT keeping a high specificity.
- ◆ CLV has low capacity to stimulate mDCs from selective CLV-AP with IDHRs and to trigger the proliferation of autologous lymphocytes, probably due to its high degradation rate in solution. This might explain the different sensitivity results obtained for AX and CLV, regardless the APC included or LTT technique used.
- ◆ Some synthetic analogues (AD-I and AD-II) from CLV degradation interact efficiently and specifically with mDCs from selective CLV-AP with IDHRs, inducing their maturation with a different recognition pattern.
- ◆ The inclusion of some synthetic analogues from both CLV AD-I and AD-II: Clav1, Clav2, Clav3, Clav6, and Clav7 are necessary to increase the sensitivity of LTT.
- ◆ The highest LTT sensitivity in patients with IDHRs to BLs is obtained when CD4⁺Th2 cell proliferative response is assessed.
- ◆ Following drug stimulation, mDCs from BL-AP with IDHRs express higher levels of migration, activation, and maturation markers compared to mDCs. Their inclusion as APCs significantly increases the sensitivity of LTT compared to the inclusion of pre-primed mDCs or C-LTT.

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ANNEX I. PUBLISHED ARTICLES

.

ORIGINAL ARTICLE

Dendritic cells inclusion and cell-subset assessment improve flow-cytometry-based proliferation test in non-immediate drug hypersensitivity reactions

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Abstract

Background: Lymphocyte transformation test (LTT) has been widely used to evaluate non-immediate drug hypersensitivity reactions (NIDHRs). However, the lack of standardization and the low sensitivity have limited its routine diagnostic use. The drug presentation by dendritic cells (DCs) and the assessment of proliferation on effector cells have shown promising results. Flow-cytometry-based methods can help apply these improvements. We aimed to assess the added value of using drug-primed-DCs and the determination of the proliferative response of different lymphocyte subpopulations in NIDHRs.

Methods: Patients with confirmed NIDHR were evaluated by both conventional (C-LTT) and with drug-primed-DCs LTT (dDC-LTT) analysing the proliferative response in T cells and other effector cell subpopulations by using the fluorescent molecule, carboxyfluorescein diacetate succinimidyl ester (CFSE).

Results: The C-LTT showed a significantly lower sensitivity (29.4%) compared with dDC-LTT (61.8%), which was confirmed analysing each particular clinical entity: SJS-TEN (62.5% vs 87.5%), MPE (15% vs 47.4%) and AGEP (33% vs 80%). When including the effector cell subpopulations involved in each clinical entity, CD3⁺CD4⁺T_H1 or CD3⁺NK cells in SJS-TEN, CD3⁺CD4⁺T_H1+NK cells in MPE and CD3⁺NK cells in AGEP, we could significantly increase the sensitivity of the *in vitro* test to 100%, 68.4% and 100%, respectively, with an overall sensitivity of 87% and 85% of specificity in NIDHR.

Abbreviations: AGEP, Acute generalized exanthematous pustulosis; APC, Antigen-presenting cell; ASPS, Algorithm of the Spanish Pharmacovigilance System; AUC, Area under curve; BL, Betalactam; CFSE, Carboxyfluorescein diacetate succinimidyl ester; C-LTT, Conventional lymphocyte transformation test; dDC-LTT, Drug-primed-mo-DCs lymphocyte transformation test; DHRs, Drug hypersensitivity reactions; DPT, Drug provocation test; DRESS, Drug reaction with eosinophilia and systemic symptoms; EAACI, European Academy of Allergy and Clinical Immunology; ESCD, European Society of Contact Dermatitis; FDE, Fixed drug eruption; IDHR, Immediate drug hypersensitivity reaction; IDT, Intradermal test; LTT, Lymphocyte transformation test; mo-DC, Monocyte-derived dendritic cell; MPE, Maculopapular exanthemas; NIDHR, Non-immediate drug hypersensitivity reaction; PBMC, Peripheral blood mononuclear cell; PHA, Phytohaemagglutinin; PI, Proliferation index; PT, Patch test; RCM, Radiocontrast media; ROC curve, Receiver operating characteristic curve; SJS, Stevens-Johnson Syndrome; ST, Skin test; TEN, Toxic epidermal necrolysis.

Ruben Fernandez-Santamaria, Gador Bogas, Francisca Palomares, Maria José Torres and Cristobalina Mayorga are equal contribution

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ORIGINAL ARTICLE

Drug Allergy, Insect Sting Allergy, and Anaphylaxis

Synthetic antigenic determinants of clavulanic acid induce dendritic cell maturation and specific T cell proliferation in patients with immediate hypersensitivity reactions

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Abstract

Background: Immediate drug hypersensitivity reactions (IDHRs) to clavulanic acid (CLV) have increased in the last decades due to a higher consumption alongside amoxicillin (AX). Due to its chemical instability, diagnostic procedures to evaluate IDHRs to CLV are difficult, and current in vitro assays do not have an optimal sensitivity. The inclusion of the specific metabolites after CLV degradation, which are efficiently recognised by the immune system, could help to improve sensitivity of in vitro tests.

Methods: Recognition by dendritic cells (DCs) of CLV and the synthetic analogues of two of its hypothesised antigenic determinants (ADs) was evaluated by flow cytometry in 27 allergic patients (AP) and healthy controls (HC). Their ability to trigger the proliferation of T cells was also analysed by flow cytometry.

Results: The inclusion of synthetic analogues of CLV ADs, significantly increased the expression of maturation markers on DCs from AP compared to HC. A different recognition pattern could be observed with each AD, and, therefore, the inclusion of both ADs achieves an improved sensitivity. The addition of synthetic ADs analogues increased the proliferative response of CD4⁺Th2 compared to the addition of native CLV. The combination of results from both ADs increased the sensitivity of proliferative assays from 19% to 65% with a specificity higher than 90%.

Conclusions: Synthetic ADs from CLV are efficiently recognised by DCs with ability to activate CD4⁺Th2 cells from AP. The combination of analogues from both ADs,

Abbreviations: ADf, antigenic determinant; AP, allergic patients; APC, antigen presenting cell; AX, amoxicillin; BAT, basophil activation test; BCR, B cell receptor; BL, betalactams; BPOL, benzylpenicilloyl octa-L-lysine; CCR7, C-C chemokine receptor type 7; CFSE, carboxyfluorescein succinimidyl ester; CLV, clavulanic acid; DC, dendritic cell; DPT, drug provocation test; EAACI, European Academy of Allergy and Clinical Immunology; GM-CSF, granulocyte-macrophage colony stimulating factor; HC, healthy control; HRT, histamine release test; HRUM, Hospital Regional Universitario de Málaga; HSA, human serum albumin; ICON, Internal Consensus on drug allergy; IDHR, immediate drug hypersensitivity reaction; LPS, lipopolysaccharide; LTT, lymphocyte transformation test; MD, minor determinant; MHC, major histocompatibility complex; MI, maturation Index; moDCs, monocyte-derived dendritic cells; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PI, proliferation index; PV, penicillin V; sIgE, specific immunoglobulin E; SPT, skin prick test; ST, skin test; TCR, T cell receptor.

RFS, GB and MIM; TDF, CM and MJT contributed equally to this work.

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