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Role of purinergic receptor P2X7R signaling in exosome release and trained immunity in transplantation

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Hyshem Lancia. Role of purinergic receptor P2X7R signaling in exosome release and trained immunity in transplantation. Human health and pathology. Université Grenoble Alpes [2020-..], 2023. English. NNT : 2023GRALS037 . tel-04766528

HAL Id: tel-04766528

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THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ GRENOBLE ALPES

École doctorale : ISCE - Ingénierie pour la Santé la Cognition et l'Environnement

Spécialité : BIS - Biotechnologie, instrumentation, signal et imagerie pour la biologie, la médecine et l'environnement

Unité de recherche : Translational Innovation in Medicine and Complexity

Rôle de la signalisation du récepteur purinergique P2X7R dans la libération d'exosomes et l'immunité entraînée en transplantation

Role of purinergic receptor P2X7R signaling in exosome release and trained immunity in transplantation

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Remerciements

Je tiens à exprimer ma profonde gratitude envers toutes les personnes qui ont contribué de près ou de loin à la réalisation de cette thèse. Le chemin qui m'a conduit jusqu'à ce travail de recherche a été long et parsemé de défis, mais il a également été enrichissant grâce au soutien inestimable que j'ai reçu.

Tout d'abord, je souhaite exprimer ma reconnaissance envers mes directeurs de thèse, le Dr. Gilles Benichou, le Dr. Curtis Cetrulo et le Dr. Michel Sève.

Gilles, merci infiniment de m'avoir guidé toutes ces années. Les connaissances que vous m'avez transmises, tant sur le plan intellectuel que personnel, me sont d'une valeur inestimable. Merci pour votre générosité et votre disponibilité. Vous êtes un véritable mentor pour moi. Apprendre à vos côtés est un véritable honneur.

Curt, je vous suis profondément reconnaissant de votre soutien sans faille, de votre enthousiasme et de votre support. Faire partie de votre laboratoire m'a comblé de joie.

Michel, merci infiniment pour vos conseils, votre bienveillance et votre accessibilité. Vous avez rendu cette thèse possible.

Dr. Alexandre Lellouch, je tiens à vous exprimer ma profonde gratitude. Merci d'avoir cru en moi, merci pour votre soutien et merci d'avoir ouvert des portes qui m'ont permis de découvrir de nouveaux horizons et de réaliser des rêves auxquels je n'osais à peine penser.

Je remercie également les membres de mon comité de suivi de thèse, le Dr. Jean Kanellopoulos et le Dr. Alessandro Alessandrini, pour leur expertise et leurs commentaires constructifs qui ont contribué à améliorer la qualité de ce travail.

Mark Randolph (alias King Mark, alias Captain Mark), merci beaucoup de défendre les membres de votre équipe sans relâche. Vous êtes un exemple de leadership.

Natacha Carnel-Amar, merci pour votre soutien tout au long de ma thèse. Merci pour votre aide, vous êtes une merveilleuse camarade et une merveilleuse amie.

Maeva Chauvin, merci infiniment pour votre soutien moral et votre positivité en toutes circonstances. Merci d'avoir joué le rôle de post-doc quand j'en avais besoin. Vous êtes une véritable amie.

Marion Goutard et Pierre Tawa, merci pour votre amitié et votre générosité. Je n'oublierai jamais les moments passés ensemble.

Alec Andrew, merci beaucoup, il n'y a aucun problème que tu ne saches résoudre. Merci mon ami de me comprendre autant.

Yanis Berkane et Laura Charlès, un grand merci de m'avoir accompagné au long de cette dernière année. Je suis très fier de vous compter parmi mes amis.

À Karen et Mathieu, merci d'avoir été là au moment où j'en avais le plus besoin. Votre amitié et votre soutien infaillible ont été un réconfort précieux.

Aux membres du Centre de Recherche en Transplantation du Massachusetts General Hospital / Harvard Medical School sous la direction du Dr. Joren Madsen, merci infiniment de m'avoir accueilli et formé. C'est un honneur d'avoir évolué avec vous ces dernières années.

Je tiens également à remercier mes parents pour leur soutien infini et l'éducation dont j'ai bénéficié.

Enfin, je tiens à remercier ma femme pour sa patience infinie et son amour inconditionnel.

Hyshem

Acknowledgments

I would like to express my deep gratitude to all the individuals who have contributed, in one way or another, to the completion of this thesis. The journey that led me to this research work has been long and filled with challenges, but it has also been enriching thanks to the invaluable support I have received.

First and foremost, I want to extend my appreciation to my thesis advisors, Dr. Gilles Benichou, Dr. Curtis Cetrulo, and Dr. Michel Sève.

Gilles, thank you immensely for guiding me all these years. The knowledge you have imparted to me, both intellectually and personally, is of immeasurable value. Thank you for your generosity and availability. You are a true mentor to me, and learning alongside you is a genuine honor.

Curt, I am deeply grateful for your unwavering support, enthusiasm, and assistance. Being part of your laboratory has filled me with joy.

Michel, thank you so much for your guidance, kindness, and accessibility. You made this thesis possible.

Dr. Alexandre Lellouch, I want to express my profound gratitude to you. Thank you for believing in me, for your support, and for opening doors that allowed me to explore new horizons and achieve dreams I could scarcely imagine.

I also thank the members of my thesis advisory committee, Dr. Jean Kanellopoulos and Dr. Alessandro Alessandrini, for their expertise and constructive feedback that contributed to improving the quality of this work.

Mark Randolph (aka King Mark aka Captain Mark), thank you very much for tirelessly advocating for your team members. You are an exemplary leader.

Natacha Carnel-Amar, thank you for your support throughout my thesis. Thank you for your help; you are a wonderful colleague and a wonderful friend.

Maeva Chauvin, thank you immensely for your moral support and positivity through thick and thin. Thank you for stepping into the role of a post-doc when I needed it. You are a true friend.

Marion Goutard and Pierre Tawa, thank you for your friendship and generosity. I will never forget the moments we shared.

Alec Andrew, thank you very much; there is no problem you cannot solve. Thank you, my friend, for understanding me so well.

Yanis Berkane and Laura Charlès, a big thank you for accompanying me throughout this past year. I am very proud to have you as my friends.

To Karen and Mathieu, thank you for being there when I needed it most. Your friendship and unwavering support have been a precious comfort.

To the members of the Massachusetts General Hospital / Harvard Medical School, Center for Transplantation Sciences under the direction of Dr. Joren Madsen, thank you immensely for welcoming me and training me. It is an honor to have grown alongside you in recent years.

I also want to thank my parents for their endless support and the education I received.

Finally, I want to thank my wife for her infinite patience and unconditional love.

Hyshem

Abstract:

After skin allotransplantation, recipient antigen presenting cells (APCs) regularly acquire allogeneic MHC molecules from donor cells. Subsequently, they display on their surface either intact donor MHC molecules (MHC cross-dressing) or processed donor peptides bound to self-MHC molecules (indirect presentation). Intercellular transfer of donor MHC molecules is mediated primarily by extracellular vesicles (EVs) released by the allograft. Both presentation of whole donor MHC proteins and peptides by recipient APCs (semi-direct and indirect pathways) are known to contribute to the pro-inflammatory T cell response leading to allograft rejection. At the same time, there is ample evidence showing that initiation of adaptive alloimmunity depends on early innate inflammation caused by tissue injury and subsequent activation of myeloid cells (macrophages and dendritic cells) recognizing danger-associated molecular patterns (DAMPs). Among these DAMPs, extracellular ATP, which binds to P2X7 receptors, is known to play a key role in innate inflammation by activating NLRP3 inflammasome and subsequent production and release of inflammatory cytokines and EVs. This prompted us to evaluate the influence of innate inflammation triggered by ATP-mediated signaling of P2X7 receptors on EV release by donor cells after skin transplantation in mice.

Another aspect of the role of innate immunity in transplant rejection is the induction of trained immunity. Recently, it has been shown that macrophages exposed to various stimuli differentiate into memory-like macrophages capable of responding faster and stronger upon exposure to a second stimulus of the same or a different kind. This phenomenon is called trained immunity. In this study, we investigated the role of P2X7 receptor signaling in macrophage training and its influence on the inflammatory response and rejection of a skin allograft in mice.

Our results show that inhibition of P2X7R signaling prevented donor exosome release and macrophage training in vitro and in vivo and significantly prolonged the survival of skin allografts in mice. This study reveals a new aspect of the role of innate immunity in the initiation of adaptive alloimmunity by T cells and allograft rejection. In addition, our

study set the path for the development of P2X7R-based therapies in clinical transplantation.

RÉSUMÉ:

Après une allogreffe de peau, les cellules présentatrices d'antigènes (CPA) du receveur acquièrent régulièrement des molécules du CMH allogénique provenant des cellules du donneur. Par la suite, elles les exposent à leur surface soit sous forme de molécules intactes du CMH du donneur ("cross dressing" du CMH) soit sous forme de peptides du donneur traités et liés aux molécules du CMH du receveur (présentation indirecte). Le transfert intercellulaire des molécules du CMH du donneur est principalement médié par des vésicules extracellulaires (VE) libérées par la greffe. Il est bien établi que la présentation des protéines entières du CMH du donneur ainsi que des peptides par les CPA du receveur (voies semi-directe et indirecte) contribue à la réponse pro-inflammatoire des lymphocytes T conduisant au rejet de la greffe. Dans le même temps, de nombreuses preuves montrent que le déclenchement de l'allo-immunité adaptative dépend de l'inflammation innée précoce provoquée par des lésions tissulaires et de l'activation subséquente des cellules myéloïdes (macrophages et cellules dendritiques) reconnaissant des motifs moléculaires associés au danger (DAMP). Parmi ces DAMP, l'ATP extracellulaire, qui se lie aux récepteurs P2X7, est connu pour jouer un rôle clé dans l'inflammation innée en activant l'inflammasome NLRP3 et en induisant la production et la libération de cytokines inflammatoires et de VE. Cela nous a poussés à évaluer l'influence de l'inflammation innée déclenchée par la signalisation médiée par l'ATP des récepteurs P2X7 sur la libération de VE par les cellules du donneur après une transplantation de peau chez les souris.

Un autre aspect du rôle de l'immunité innée dans le rejet de greffe est l'induction de l'immunité entraînée. Récemment, il a été démontré que les macrophages exposés à divers stimuli se différencient en macrophages de type mémoire capables de répondre plus rapidement et plus intensément lorsqu'ils sont exposés à un deuxième stimulus du même type ou différent. Ce phénomène est appelé immunité entraînée. Dans cette étude, nous avons examiné le rôle de la signalisation des récepteurs P2X7 dans la transformation des macrophages en macrophages entraînés et son influence sur la réponse inflammatoire et le rejet d'une allogreffe de peau chez les souris.

Nos résultats montrent que l'inhibition de la signalisation du récepteur P2X7 empêche la libération des exosomes du donneur et la formation des macrophages in vitro et in vivo, prolongeant ainsi significativement la survie des allogreffes de peau chez les souris. Cette étude révèle un nouvel aspect du rôle de l'immunité innée dans le déclenchement de l'allo-immunité adaptative par les lymphocytes T et le rejet de la greffe. De plus, notre étude ouvre la voie au développement de thérapies basées sur les récepteurs P2X7 dans la transplantation clinique.

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INTRODUCTION

The purinergic receptor 7 (P2X7R)

Generalities

The P2X7 receptor (P2X7R) belongs to the ionotropic P2X receptor subfamily (Burnstock 2006) (Table 1). P2X receptors are ligand-gated trimeric ion channels that, upon activation, exhibit non-selective permeability to cations such as sodium (Na⁺), potassium (K⁺), and calcium (Ca²⁺) (Khakh 2001; Khakh and North 2006, 2012). This receptor family encompasses seven distinct subtypes referred to as P2X1 through P2X7. Functional P2X receptor channels are formed by the assembly of trimeric complexes, which may consist of either homotrimers or heterotrimers (Barrera et al. 2005; Kawate et al. 2009; Lewis et al. 1995; Nicke et al. 1998; Torres, Egan, and Voigt 1999; Saul et al. 2013).

Purinergic Receptors

<i>Family</i>	Adenosine/P1 receptors (P1Rs)	P2 receptors (P2Rs)	
<i>Subfamily</i>	-	P2Y	P2X
<i>Receptor Subtypes</i>	A1, A2A, A2B, A3	P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14	P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7
<i>Structure</i>	G protein-coupled receptors	G protein-coupled receptors	Ligand ion channels
<i>Ligands</i>	Adenosine	ATP, ADP, UTP, UDP	ATP

Table 1. Classification of purinergic receptors and their endogenous ligands. Table adapted from (Erlinge and Burnstock 2008)

P2X receptors are extensively distributed in excitatory and non-excitatory cell types, such as neurons, glial cells, platelets, epithelial cells, and macrophages. They play pivotal roles in many physiological and pathological processes, including but not limited to inflammation, immunomodulation, cardiovascular regulation, synaptic transmission, pain perception, and tumorigenesis (Khakh and North 2012; Coddou et al. 2011; Surprenant and North 2009; Burnstock 2013; Idzko, Ferrari, and Eltzschig 2014; Burnstock 2016; Habermacher et al. 2016) (Figure 1.).

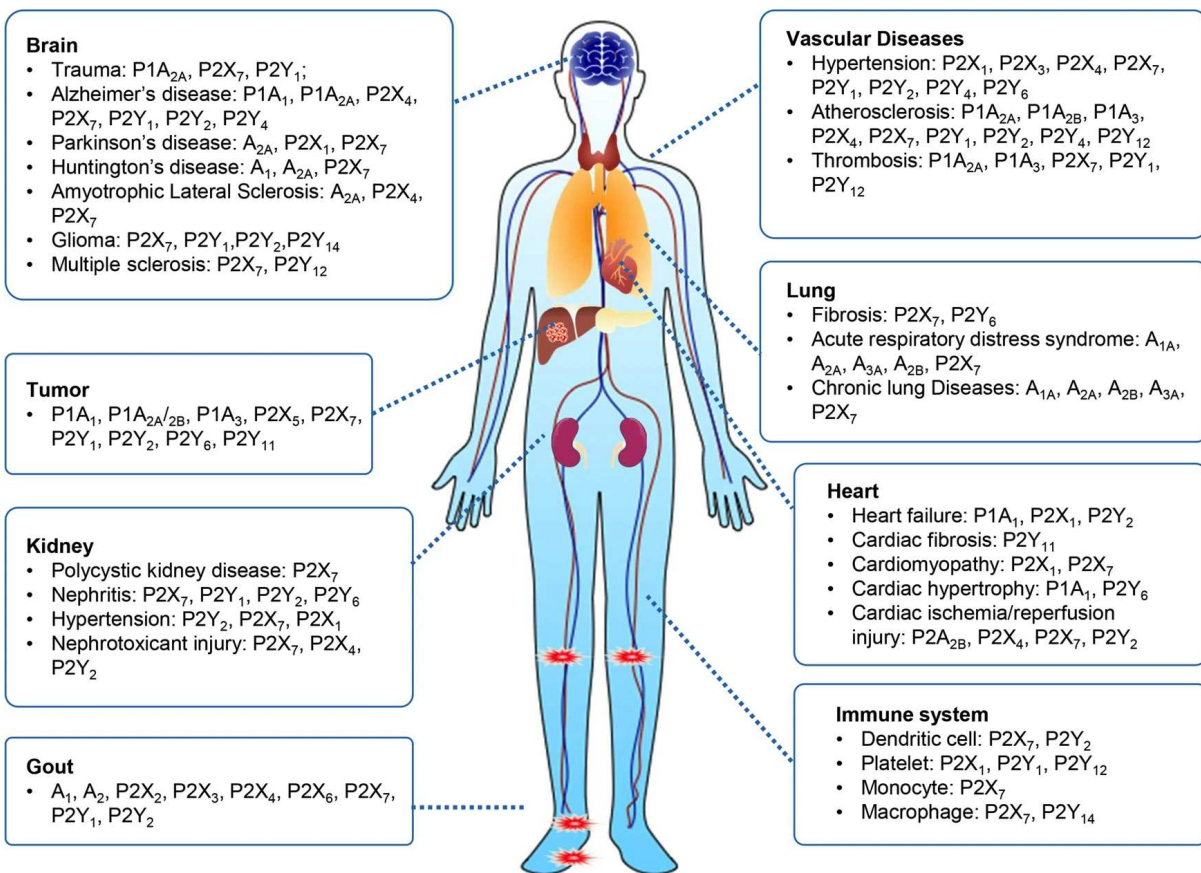


Figure 1. The involvement of purinergic signaling in human pathologies. Figure from (Huang et al. 2021)

Among the family of purinergic receptors, one stands out from the crowd: the Purinergic receptor-7 (P2X7R). As of August 2023, P2X7R stands out as the most extensively researched purinergic receptor, with over 700 studies documented in Medline. A few reasons can explain the interest for this receptor. Historically, P2X7R holds the distinction

of being the first P2 receptor to undergo comprehensive investigation within a well-defined immune cell population, specifically macrophages (el-Moatassim and Dubyak 1992; Murgia et al. 1993), and to be implicated in immune cell responses (Lin, Krishnaraj, and Kemp 1985; Dubyak, Cowen, and Lazarus 1988). Moreover, P2X7R was also the first P2XR described to trigger an immune response, including NLRP3 activation and subsequent interleukin-1 β (IL-1 β) release (Ferrari et al. 1996; Ferrari et al. 1997). Additionally, P2X7R actively participates in diverse processes invoked in response to cellular damage, with a primary objective of reestablishing homeostatic equilibrium. These processes notably include wound healing and the regulation of cellular growth (*Di Virgilio and Adinolfi 2017; Junger 2011*).

Structure and expression of P2X7 Receptor

Cellular and tissue distribution

The P2X7 receptor is commonly characterized as a cell-surface receptor. It exhibits prominent expression in various immune cells, including macrophages, monocytes, dendritic cells, natural killer cells, B lymphocytes, T-cells, mast cells, and microglia. Additionally, P2X7 receptors are found in epithelial cells, oligodendrocytes within the Central Nervous System (CNS), and Schwann cells in the Peripheral Nervous System (PNS) (Buell, Chessell, et al. 1998; Di Virgilio, Schmalzing, and Markwardt 2018; Sluyter 2017; Di Virgilio, Sarti, and Grassi 2018; Kaczmarek-Hajek et al. 2018).

The presence of P2X7 receptors on neurons remains a subject of debate (Illes, Khan, and Rubini 2017; Miras-Portugal et al. 2017). Multiple studies report P2X7 expression and functionality in astrocytes (Ballerini et al. 1996; Sperlagh and Illes 2014), although contradictory evidence also exists (Jabs et al. 2007).

Emerging scientific evidence suggests the presence of P2X7 receptors within intracellular compartments of cells (Gu et al. 2000). Despite the uncertain functional roles of these intracellular receptors, it is plausible that intracellular pools of P2X7 receptors may play a role in enhancing cell-surface receptor expression during cellular differentiation events, as evidenced in the differentiation process of human monocytes into macrophages (Gudipaty et al. 2001).

However, when human monocytes are subjected to P2X7 receptor activation, there is no discernible alteration in the expression of cell-surface P2X7 receptors. This observation suggests that the rapid insertion of receptors from this intracellular reservoir is unlikely to occur following the receptor activation (Gudipaty et al. 2001).

Structure of P2X7R

P2X7R is an ATP-gated ion channel, initially identified as the cytolytic P2Z receptor owing to its requirement for elevated ATP concentrations (greater than 100 μ M) (Gordon 1986; Di Virgilio 1995) to initiate activation. It underwent a reclassification due to its substantial amino acid sequence similarity with the other six P2XR receptors. Legitimately, P2XZR was reclassified as P2X7R (Surprenant et al. 1996) (Table 2).

	P2RX2_ HUMAN	P2RX3_ HUMAN	P2RX7_ HUMAN	P2RX6_ HUMAN	P2RX5_ HUMAN	P2RX1_ HUMAN	P2RX4_ HUMAN
sp Q9UBL9 P2 RX2_HUMAN	100.00	47.07	36.28	38.33	40.65	38.32	46.56
sp P56373 P2 RX3_HUMAN	47.07	100.00	38.96	39.36	43.70	45.36	45.95
sp Q99572 P2 RX7_HUMAN	36.28	38.96	100.00	35.51	36.05	40.87	46.03
sp O15547 P2 RX6_HUMAN	38.33	39.36	35.51	100.00	45.89	43.22	45.09
sp Q93086 P2 RX5_HUMAN	40.65	43.70	36.05	45.89	100.00	42.90	52.07
sp P51575 P2 RX1_HUMAN	38.32	45.36	40.87	43.22	42.90	100.00	50.91
sp Q99571 P2 RX4_HUMAN	46.56	45.95	46.03	45.09	52.07	50.91	100.00

Table 2. Percentage of similarity after the alignment of the extracellular portion of human P2X-family receptors protein sequences. Protein sequences of P2X1–7 from human origin were obtained from UniprotKB database and aligned using Clustal Omega

The human P2X7R gene consists of 13 exons and is situated on chromosome 12q24.31 (Buell, Talabot, et al. 1998). Of note, its proximity to the P2X4R gene suggests a possible origin through gene duplication. Moreover, the recombinant P2X7R subunit has been extensively studied not only in humans but also in six other mammalian species, including macaques, dogs, pandas, mice, rats, and guinea pigs. These species exhibit a genetic similarity of over 75% with the human P2X7R gene. In contrast, non-mammalian species like the zebrafish show a much lower genetic similarity, measuring below 40% (Sluyter 2017).

Each subunit of the human recombinant P2X7R is a 595-amino acid protein. It comprises a substantial extracellular loop housing two ATP-binding sites, along with two alpha-helical transmembrane domains, and intracellular N- and elongated C-terminal tails (Karasawa and Kawate 2016; Mansoor et al. 2016; Hattori and Gouaux 2012; Kawate et al. 2009) (Figure 2). Recently, the tridimensional structure of the subunit has been fully revealed by crystallography (McCarthy, Yoshioka, and Mansoor 2019). It is widely acknowledged that a functional P2X7R comprises three subunits (Figure 2). The P2X7 subunits predominantly undergo assembly as homotrimeric receptors (Nicke 2008) ; however, in specific instances, they exhibit the capacity to assemble with P2X4 subunits, resulting in the formation of heterotrimeric receptors (Perez-Flores et al. 2015).

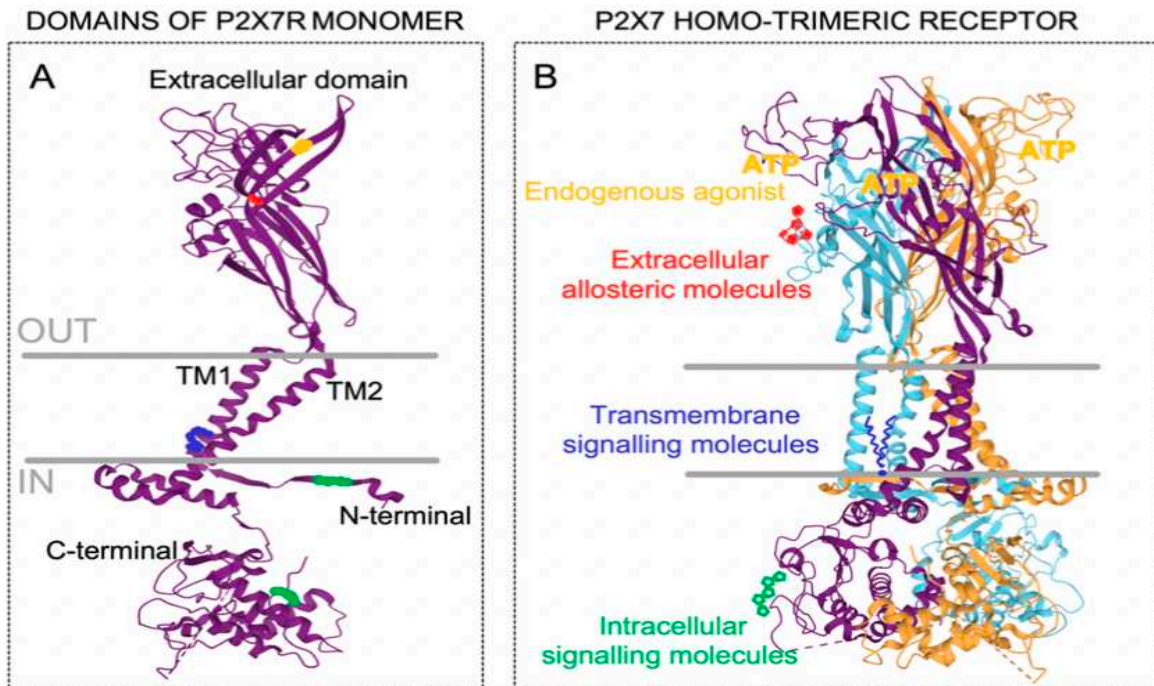


Figure 2. Topology of the P2X7 receptor (P2X7R). from (Martinez-Cuesta et al. 2020).

The tridimensional arrangement of P2X7R subunits is reminiscent of the anatomical structure of a dolphin. In this analogy, the extracellular region corresponds to the body, inclusive of the head and fins, while the two transmembrane helices (TM1 and TM2) assume the role of the tail (figure 3).

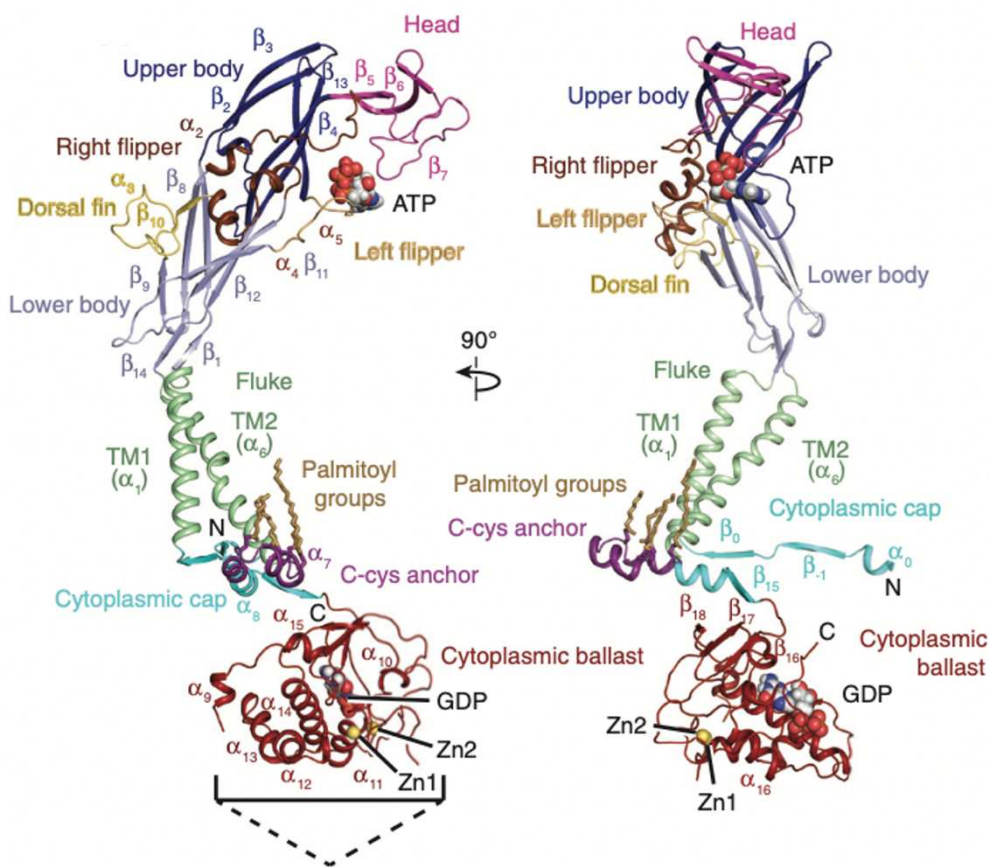


Figure 3. The P2X7 monomer represented as secondary structure. from (McCarthy, Yoshioka, and Mansoor 2019)

The three ATP-binding sites, originating from the ATP-binding regions of adjacent monomeric pairs (Figure 4), conform to the activated state of P2X7R. Activation in this context necessitates the presence of three ATP molecules to initiate gating (Hattori and Gouaux 2012; Grimes and Young 2015). Consequently, these ATP-bound sites facilitate the opening of the lower body domain pore, which is formed by the central TM segments within each of the three pairs of TM domains. Furthermore, in close proximity to these ATP-binding sites, three additional drug-binding sites (Figure 4) are believed to host various compounds possessing inhibitory and/or allosteric modulatory effects on P2X7R, including the inhibitor A438079 utilized in our investigation (Allsopp et al. 2018).

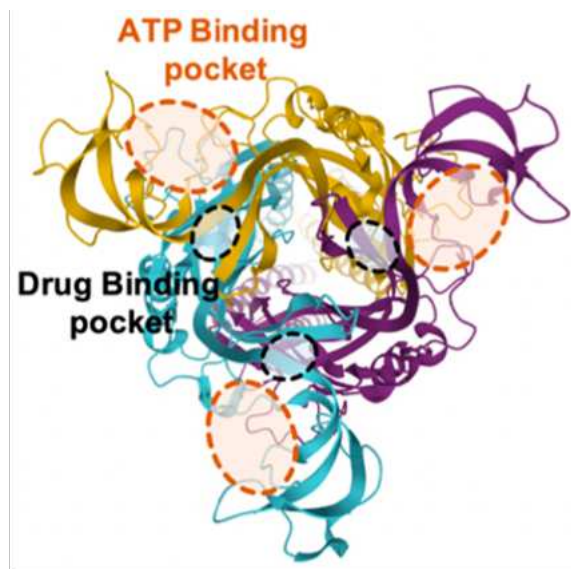


Figure 4. An overhead perspective of the P2X7R configuration, encompassing the three extracellular ATP-binding sites (Martinez-Cuesta et al. 2020).

Furthermore, the intracellular N- and C-terminus of the P2X7 receptor (P2X7R) subunits are known to exert a significant influence on the receptor's biological functions. Nevertheless, the precise structural details of these termini remained obscure for a long time, primarily due to technical limitations that hindered comprehensive research into their architecture. However, recent advancements in cryo-electron microscopy techniques have enabled the examination of the complete P2X7 receptor, shedding light on the structural characteristics of its cytoplasmic domain.

These investigations elucidated how palmitoylation directly modulates the functionality of P2X7 to prevent receptor desensitization and unveiled the presence of a dinuclear zinc ion complex and a high-affinity guanosine nucleotide binding site within the cytoplasmic domain of the receptor (Figure 3).

Moreover, the P2X7R subunit prominently displays a longer C-terminus featuring an additional 200 amino acid residues in comparison to other P2XRs (Figure 2) (Costa-Junior, Sarmiento Vieira, and Coutinho-Silva 2011). Within this extended region, it

encompasses a distinctive cysteine-rich domain and a lipopolysaccharide-binding domain exclusive to P2X7R. It is of significant note that this extended C-terminus plays a central role in regulating the activation of signaling pathways and facilitating post-translational modifications (Costa-Junior, Sarmiento Vieira, and Coutinho-Silva 2011).

Conversely, the N-terminus, (figure 2), hosts a unique protein kinase C site, responsible for governing the desensitization of purinergic receptors (Amstrup and Novak 2003). Furthermore, it is implicated in the regulation of calcium ion (Ca²⁺) flux through the channel (Liang et al. 2015), while also exerting control over receptor gating and contributing to its activation (Allsopp and Evans 2015).

P2X7 Receptor Polymorphisms

The human P2X7 receptor (P2X7R) exhibits a remarkably high level of polymorphism, with over 150 identified single-nucleotide polymorphisms (SNPs) located in both the extracellular loop and the C-terminal domain (Di Virgilio et al. 2017; Jiang et al. 2013; Roger et al. 2010; Fuller et al. 2009). These genetic variations can give rise to a wide array of unpredictable functional consequences from an augmentation in ATP-activated ion channel activity (Stokes et al. 2010) to the development of a chronic pain disorders (Ren and Illes 2022).

In addition to these P2X7R polymorphisms, there are nine distinct splice variants derived from the canonical P2X7R subunit, known as P2X7A. These splice variants are classified as P2X7B through P2X7J, each of which imparts unique characteristics to the receptor (Di Virgilio et al. 2017; Peverini et al. 2018) (Figure 5). For example, P2X7B can generate functional ion channels but lacks the ability to form pores, which remains a subject of ongoing debate concerning receptor activation mechanisms (Adinolfi et al. 2010). On the other hand, P2X7H appears incapable of assembling into a functional receptor (Cheewatrakoolpong et al. 2005). In contrast, P2X7J has the capacity to interact with P2X7A subunits, resulting in the formation of a non-functional heterotrimeric receptor with a cell-protective mechanism (Feng et al. 2006).

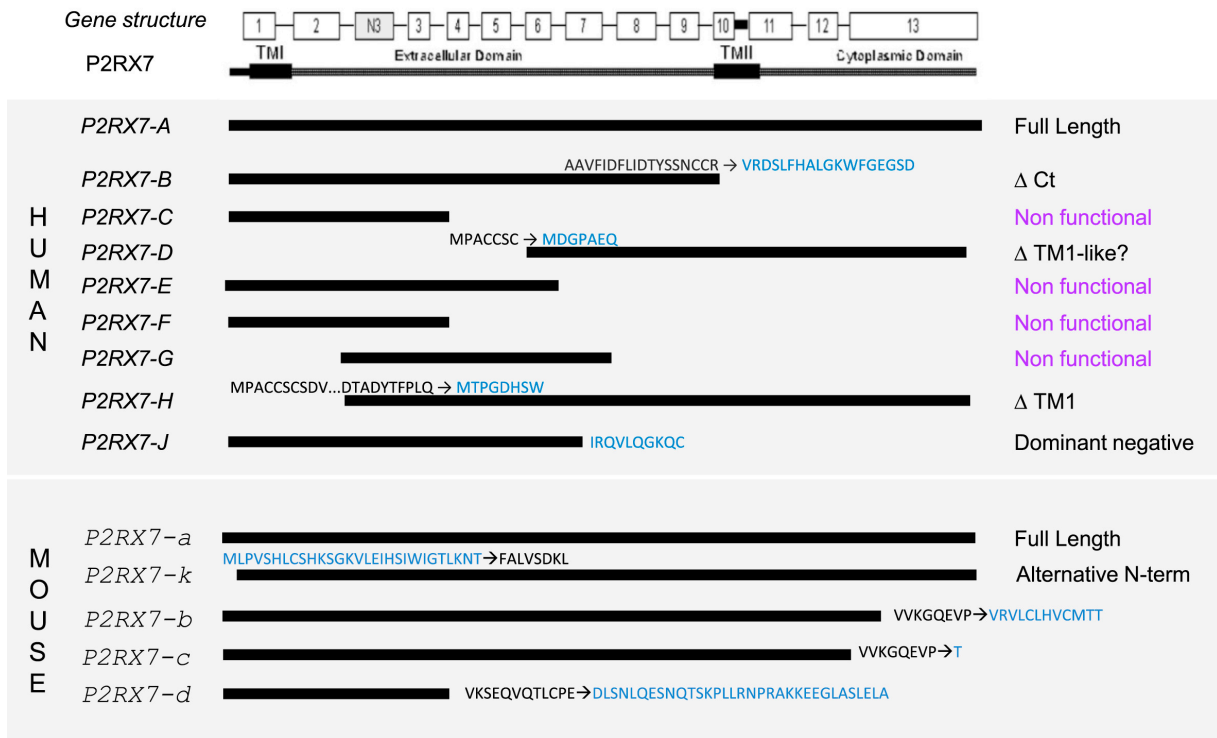


Figure 5. Human and mouse *P2RX7* splice variants from (Benzaquen et al. 2019)

P2X7R activation

The P2X7R receptor is commonly characterized as a cell surface receptor with its ATP-binding site exposed in extracellular. Given that ATP is typically found inside cells, the ATP that is released into the extracellular space becomes a significant signaling molecule (Cauwels et al. 2014; Ravichandran 2011; Elliott et al. 2009). Consequently, the activation of P2X7R necessitates elevated levels of extracellular ATP, typically EC_{50} of ~0.3 to 1 mM (Surprenant et al. 1996; Karasawa and Kawate 2016; Bradley et al. 2011; Chessell et al. 1998). As a result, the mechanisms underlying ATP release are intricately linked to the receptor's functionality.

Unregulated ATP release occurs as a consequence of cellular damage or dying cells, typically brought about by non-specific mechanisms, such as the disruption of the cell membrane. Conversely, controlled ATP release serves as a crucial regulatory mechanism, allowing both inflammatory and non-inflammatory cells to modulate their

proximal activation through autocrine/paracrine signaling (Dosch et al. 2018). This controlled release is facilitated by specific cellular structures, including exocytotic granules, plasma membrane-derived microvesicles, and various membrane channels, such as connexins (e.g., connexin-43), pannexins (e.g., pannexin-1), calcium homeostasis modulator channels (CALHM), and the P2X7R receptor (Giuliani, Sarti, and Di Virgilio 2019; Diezmos, Bertrand, and Liu 2016; Fortes et al. 2004; Schenk et al. 2008).

In the context of P2X7R activation, membrane channels like connexins and pannexins play pivotal roles. Connexins can form gap junctions between adjacent cells and also create unopposed hemichannels that permit the passage of small hydrophilic molecules, including ATP and ions, across the cellular bilayer. There is evidence suggesting co-localization of connexin-43 with P2X7R in macrophages, facilitating intercellular communication through gap junction formation mediated by extracellular ATP (Fortes et al. 2004).

The "pannexin-1/ATP/P2X7R axis" has been implicated in P2X7R activation (Iglesias et al. 2008) and has been identified in human inflammatory cells, such as monocytes (Parzych et al. 2017) and macrophages (Pelegriin and Surprenant 2006). Interestingly, PANX-1 forms protein-protein associations with P2X7R, and upon activation by extracellular ATP, it rapidly opens a characteristic ion channel that selectively permits the trafficking of small cations, including calcium ions (Ca^{2+}), within milliseconds. Subsequently, seconds later, a prolonged opening or activation of a large pore ensues, allowing the passage of molecules up to 900 Daltons (Ma et al. 2009) which contributes to the induction of cell death.

P2X7R functions

The P2X7 receptor plays diverse roles not only within a single cell but also across other cell types where it may be present. After binding to ATP, the P2X7 receptor functions as a cationic channel. A brief ATP activation lasting just a few seconds opens a cationic pathway, enabling an influx of Na^{+} and Ca^{2+} while facilitating the efflux of K^{+} (North and Surprenant 2000). Notably, this receptor is virtually impermeable to anions. Consequently,

there is a substantial rise in intracellular Na⁺ concentration, resulting in membrane depolarization. The increased intracellular Ca²⁺ concentration triggers Ca²⁺-dependent signaling pathways. Simultaneously, the efflux of K⁺ reduces its cytosolic levels, thus promoting the activation of other vital processes, such as the assembly and activation of the NLRP3 inflammasome (Sanz and Di Virgilio 2000).

It is noteworthy that, unlike most receptors, mammalian P2X7 receptors are resistant to desensitization when repeatedly stimulated by agonists. Instead, this leads to a gradual augmentation in receptor activity, a property known as facilitation (Roger, Pelegrin, and Surprenant 2008).

The release of TNF- α , IL1 β , IL18 and IL6

Activation of the P2X7R receptor induces a series of molecular events in human macrophages. Specifically, it leads to the activation of cyclooxygenase (COX) and the upregulation of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) kinases, along with phospholipase A2 (Chotjumlong et al. 2013; Wan et al. 2014). This cascade of events ultimately results in the activation of Jun N-terminal kinase (JNK) and p38 through a mechanism that depends on protein tyrosine kinase activity. This process also involves the release of tumor necrosis factor (TNF- α) (Suzuki et al. 2004).

Studies on other cell types suggest the presence of a potential tyrosine phosphorylation site within the intracellular C-terminal domain of the P2X7 receptor, which plays a significant role in mediating the release of TNF- α induced by P2X7R activation (Dalgarno et al. 2018).

The activation of the P2X7 receptor pathway is closely associated with the regulation of potassium ions (K⁺) flux, particularly within the context of the NLRP3 inflammasome signaling pathway (Adinolfi et al. 2018). This phenomenon hinges on the reduction of intracellular K⁺ concentrations, which subsequently triggers the assembly and activation of the NLRP3 inflammasome complex. This activation process is orchestrated by the

cytoplasmic kinase known as never-in-mitosis A-related kinases (NEK), which catalyzes the cleavage of pro-caspase-1 into its active form, caspase-1. This enzymatic activity, in turn, leads to the maturation of pro-interleukin (pro-IL)-1 β and pro-IL-18 into their active cytokine forms, IL-1 β and IL-18, respectively (Adinolfi et al. 2018) (Figure 6).

Furthermore, the activation of the P2X7R receptor also plays a role in the release of cytokines IL-6 and IL-1 β , although this process occurs via an inflammasome-independent pathway (Gicquel et al. 2015; Englezou et al. 2015). The synthesis of immature pro-IL-1 β requires the activation of the nuclear factor nuclear factor κ B (NF- κ B), and this factor appears to be intricately involved in the regulation of cytokine gene expression mediated by P2X7R (Peng et al. 2015). Additionally, a study revealed an interaction between the adaptor protein MyD88 and the C-terminal domain of the P2X7R subunit, which triggers NF- κ B activation (Liu, Xiao, and Li 2011).

Beyond NLRP3, other NLRP proteins also contribute to these cellular processes. For instance, NLRP2 forms a complex with P2X7R, an association linked to pannexin-1 (panx-1), facilitating the maturation and release of IL-1 β (Salaro et al. 2016). Moreover, the NLRP3/inflammasome axis has been associated with pyroptosis, another way to release pro-inflammatory cytokines.

It's important to acknowledge that the variability in transduction pathways initiated by P2X7R activation should be interpreted within the structural diversity of P2X7R domains, which are surrounded by a distinct molecular milieu in each specific cell type, contributing to the diverse responses observed in various cellular contexts.

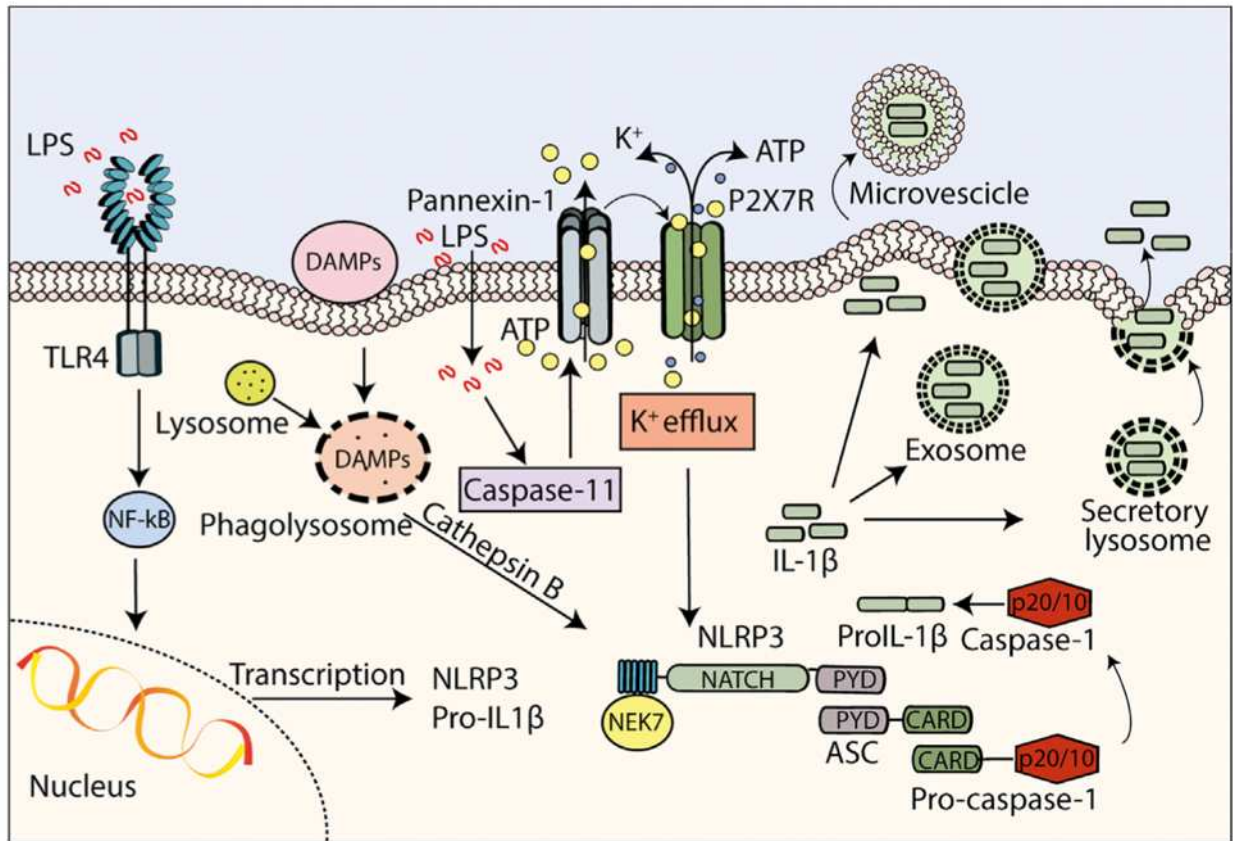


Figure 6. Central Role of P2X7R in cytokines released from (Di Virgilio et al. 2017)

The release of extracellular vesicles

Numerous mechanisms for cytokine release have been documented in scientific literature subsequent to P2X7R activation. These include exocytosis of secretory lysosomes, passive efflux through the hyperpermeable plasma membrane via Gasdermin D pores. It is also essential to note another significant pathway for cytokine release, which involves the shedding of plasma membrane-derived microvesicles and the exocytosis of exosomes derived from multivesicular bodies (MVBs) (Dubyak 2012) (Figure 7).

Indeed, P2X7R activation substantially enhances the release of membrane vesicles from the plasma membrane (PM). These extracellular vesicles (EVs), are circular membrane structures enriched with bioactive molecules crucial for mediating intercellular communication (Bianco et al. 2009).

Notably, P2X7R distinguishes itself from other members within the P2X receptor family through its distinctive cytoplasmic C-terminus, housing several binding sites for Src kinase proteins (Pfeiffer et al. 2004). These Src kinases phosphorylate and activate ROCK (Rho-associated protein kinase) and p38 MAP kinases. These signaling proteins instigate the localized disassembly of cytoskeletal components and facilitate the translocation of acid sphingomyelinase (A-SMASE) to the PM. A-SMASE, in turn, catalyzes the hydrolysis of sphingomyelin, a phospholipid abundant in the outer leaflet of the PM, into ceramide. This enzymatic process promotes the formation of blebs and the subsequent shedding of EVs (Bianco et al. 2009) (Figure 7).

In addition to the generation of large EVs, P2X7R stimulation also initiates the release of small EVs, commonly known as exosomes. These exosomes originate within the endocytic compartment, specifically from multivesicular bodies, which subsequently merge with the PM (Ruan et al. 2020) (Figure 7).

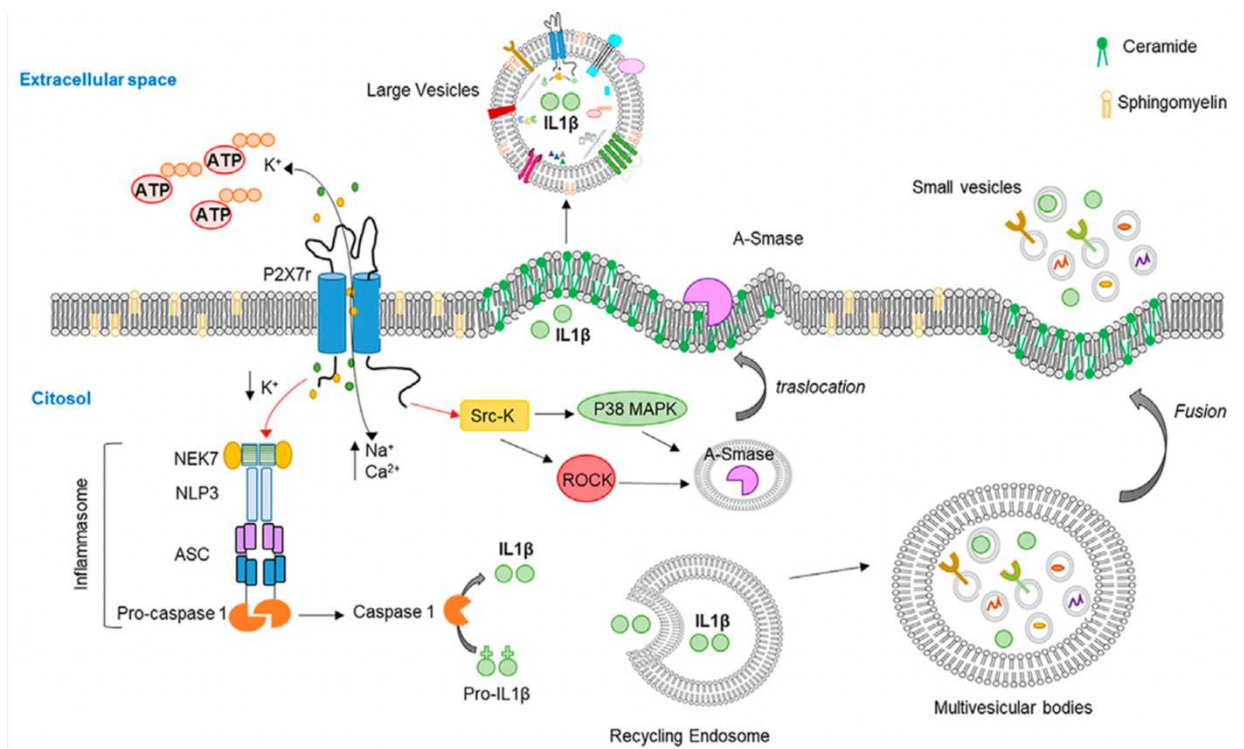


Figure 7. ATP-dependent Extracellular Vesicle biogenesis from (Lombardi et al. 2021)

Extracellular Vesicle in Transplantation

Extracellular vesicle characterization

Extracellular vesicles (EVs) are lipid bilayer particles, with dimensions ranging from 50 to 2000 nanometers, which serve as essential mediators of intercellular communication across various cell types (Yanez-Mo et al. 2015). Since their initial discovery in the 80's (Harding and Stahl 1983; Pan and Johnstone 1983), a plethora of EV subtypes have been characterized, leading to the adoption of diverse classification schemes. One such classification, based on the biogenesis spectrum, distinguishes two principal EV types (Vlassov et al. 2012).

The first type, known as exosomes, originates from endosomal pathways. They originate within multivesicular bodies, formed through the inward invagination of endosomal membranes. These structures subsequently migrate and fuse with the plasma membrane, facilitating the release of exosomes into the extracellular milieu (van Niel et al. 2022; Mathieu et al. 2019; Jeppesen et al. 2019).

The second type of EVs delineated by this classification are microvesicles, which are released directly into the extracellular space following the budding of the cell's plasma membrane (Thery et al. 2018; Gyorgy et al. 2011).

As the availability of specific molecular markers for discerning these distinct biogenesis pathways remains limited, researchers predominantly rely on biophysical or biochemical properties for the identification and segregation of EVs (Thery et al. 2018). In transplant research, the primary focus centers on three categories of EVs: exosomes or small EVs, microvesicles, and apoptotic bodies. Microvesicles and apoptotic bodies are classified within the large EVs category due to their variable sizes, which are bigger than the exosomes (Lee, El Andaloussi, and Wood 2012).

Except for apoptotic bodies that contain DNA (Elmore 2007), there are no substantial variations in the overall composition of extracellular vesicles (EVs), as they consistently contain cytoplasmic proteins, lipids, mRNA, miRNA, and receptors across all subtypes.

Exosomes or small EVs, exhibit dimensions ranging from 50 nm to 150 nm and are prevalent in various biological fluids. In contrast, microvesicles and apoptotic bodies, categorized as large EVs, have sizes spanning from 200 nm to 800 nm and 50 nm to 5000 nm, respectively, and are comparatively less abundant in biological fluids.

EVs participate in a wide array of biological processes. Indeed, EVs represent a pivotal avenue for intercellular communication and contribute to the elimination of superfluous molecules from cells, alterations in the extracellular environment, cellular adaptation, angiogenesis, coagulation, and the activation of immune functions (Yanez-Mo et al. 2015; Yuana, Sturk, and Nieuwland 2013; Tarabozetti et al. 2002; Kim et al. 2004; Yang et al. 2008; Berckmans et al. 2011; Davila et al. 2008; Benichou et al. 2020).

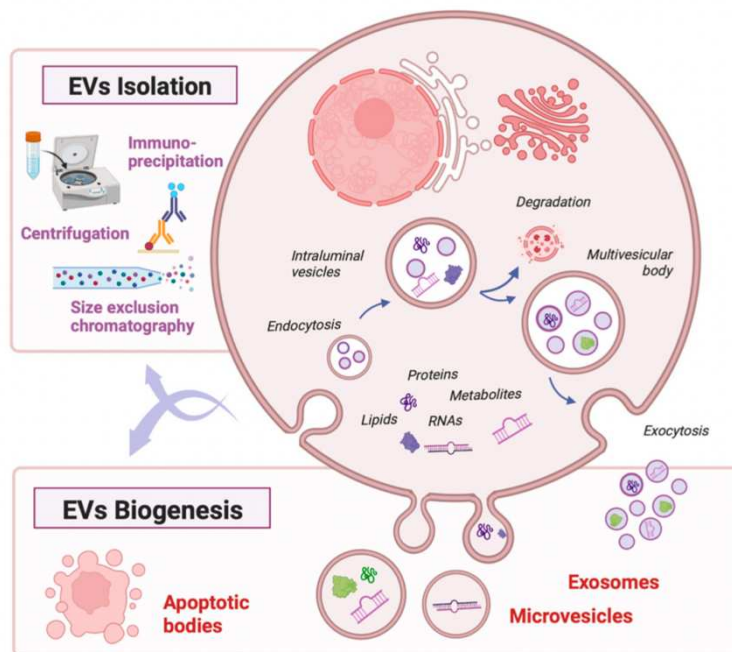


Figure 8. EV biogenesis, cargo, and isolation methods from (Romero-Garcia et al. 2023)

EVs and innate immunity

Immune cells involved in an inflammatory response produce extracellular vesicles (EVs) that wield a significant influence in amplifying the inflammatory cascade. Notably, EVs serve as molecular carriers, transporting bioactive lipid mediators like eicosanoids and enzymes required for leukotriene biosynthesis, thus manifesting a chemotactic effect (Boilard 2018; Esser et al. 2010). For instance, there is documentation indicating that ceramide-enriched EVs emanating from hepatocytes stimulate macrophage chemotaxis to the liver (Kakazu et al. 2016). Furthermore, other studies have shown that EVs originating from macrophages possess the capability to induce neutrophil chemotaxis in controlled in vitro settings (Esser et al. 2010).

EVs operate within a multifaceted framework, fulfilling a dual role (Burgelman, Vandendriessche, and Vandenbroucke 2021). On one hand, they harbor proinflammatory cytokines, along with damage-associated molecular patterns (DAMPs) like ATP, contributing to the transformation of macrophages into the M1 phenotype, characterized by pro-inflammatory cytokine secretion (Essandoh et al. 2015). Simultaneously, they play a role in the differentiation of naive T-cells into T-helper cells and facilitate the chemotaxis of various leukocytes (Gao et al. 2019).

On the other hand, EVs also exhibit anti-inflammatory properties that are indispensable for tissue repair and the attenuation of inflammatory responses. In this context, EVs down-regulate complement factors, diminish chemotactic signals, and reduce the infiltration of leukocytes by modulating the expression of adhesion molecules on endothelial cells (Tu et al. 2020). Additionally, as a safeguard against potential hyper-inflammation, EVs released subsequent to inflammasome activation carry interferon- β (IFN- β), a cytokine that impedes the activation of the NLRP3 inflammasome in bystander cells (Budden et al. 2021).

EVs and adaptive immunity

EVs, T cell, and B cell development

Extracellular vesicles (EVs) appear to exert a substantive influence on the development of T cells. Researchers have conducted an intricate examination of the EVs released by thymic epithelial cells, revealing that these EVs carry MHC-peptide complexes, including tissue-restricted antigens (TRAs), which are well-established as critical components in the process of thymocyte selection.

The participation of these EVs derived from thymic epithelial cells likely holds significance in both negative and positive thymic selection. Specifically, these EVs serve as vehicles for the dissemination of self-antigens such as TRAs, thereby making these important molecules widely accessible to the pool of developing thymocytes. This accessibility, in turn, plays a role in enhancing the maturation process of thymocytes (Skogberg et al. 2015).

Moreover, studies have shed light on the role of EVs in improving intercellular communication within the thymus and demonstrate that EVs originating from thymic epithelial cells trigger the maturation of single-positive thymocytes, whether they are CD 4+ or CD 8+ (Lundberg et al. 2016).

The development and maturation of B cells are also subject to the influence of extracellular vesicles (EVs). Specifically, in the bone marrow environment, precursor B cells have been observed to release EVs containing the CD24 molecule following engagement with CD24 through antibody-mediated interactions. CD24 is a well-known contributor to the processes associated with B cell development and selection (Ayre et al. 2015). Furthermore, studies have provided evidence indicating that the activation of CD24 or the B cell receptor (BCR) on a donor cell triggers the release of EVs carrying both CD24 and BCR. These EVs serve as conveyors for the transfer of CD24 and a fully functional BCR to recipient cells. Consequently, this transfer process endows the recipient cells with additional BCR and increases their sensitivity to CD24 antibodies, which plays a pivotal role in the induction of apoptosis (Phan et al. 2021).

EVs, antigen presentation on transplantation

Article: Pathways of Antigen Recognition by T Cells in Allograft Rejection (see appendix)

Authorship: Hyshem H. Lancia, MS, Natacha Carnel, PharmD, MS, Claire Guinier, MD, and Gilles Benichou, PhD

Abstract:

The adaptive immune response leading to the rejection of allogeneic transplants is initiated and orchestrated by recipient T cells recognizing donor antigens. T-cell allorecognition is mediated via 3 distinct mechanisms: the direct pathway in which T cells recognize allogeneic major histocompatibility complex (MHC) molecules on donor cells, the indirect pathway through which T cells interact with donor peptides bound with self-MHC molecules on recipient antigen-presenting cells, and the recently described semidirect pathway whereby T cells recognize donor MHC proteins on recipient antigen-presenting cells. In this article, we present a description of each of these allorecognition pathways and discuss their role in acute and chronic rejection of allogeneic transplants (Carnel et al. 2023).

EVs as biomarkers of allograft rejection

EVs are cargo particles carrying intracellular and membrane proteins reflecting the phenotype of their parental cells. As EVs stably circulate in all body fluids, such as blood or urine, they represent a good source of biomarkers. Furthermore, the collection and isolation of EVs are less invasive than biopsies (Ciferri, Quarto, and Tasso 2021).

In the case of heart transplantation, rejection of the transplanted organ induces changes in the composition of EVs. Researchers collected plasma from patients undergoing heart transplants and isolated EVs by differential centrifugation. After analysis of the composition, they found differences regarding immune and non-immune markers during an episode of acute cellular rejection and antibody-mediated rejection (Castellani et al. 2020). Additionally, another study compared the composition of EVs from serum between heart failure patients and healthy controls. They found differences between these conditions (Kennel et al. 2018). Collectively, EVs can serve as valuable biomarkers for

characterizing both immune and non-immune alterations within an organ, as well as for assessing the rejection status of a transplanted organ.

Trained immunity

Definition

Trained immunity can be defined as a long-term functional reprogramming of innate immune cells, triggered by exogenous or endogenous agents leading to an enhanced response toward a second challenge after the return to a non-activated state (Netea, Quintin, and van der Meer 2011) (figure 9).

The concept of trained immunity was highlighted in 2012 with the *Bacillus Calmette–Guérin* (BCG) vaccine off-target protective effects were published (Kleinnijenhuis et al. 2012). BCG vaccination of SCID mice admitting an absence of adaptive immune response due to the lack of mature T cell and B cell showed a protection against *Mycobacterium tuberculosis* as accepted but also a cross-protection against lethal *Candida albican* infection. Other studies before reported the same observation without mentioning the term “trained immunity” and allocated this cross-protection specifically to macrophages and the production of pro-inflammatory cytokines (Bistoni et al. 1986) (Vecchiarelli et al. 1989). Moreover, endogenic motifs, such as DAMPs (for example, vimentin, high mobility group box 1) and cytokines (for example, GM-CSF) can induce trained immunity (Ochando et al. 2020). More specifically, the induction of trained immunity by PAMPs and DAMPs cause long-term epigenetic reprogramming at the promoters of inflammatory genes (for example, IL-1 β , IL-6, and (TNF- α) and also induces a metabolic rewriting (Kleinnijenhuis et al. 2012).

Innate immune cells model will be considered to have undergone trained immunity if the restimulation of these cells after a resting period enhanced the responsiveness of these cells compared to the first stimulation (Ochando et al. 2023).

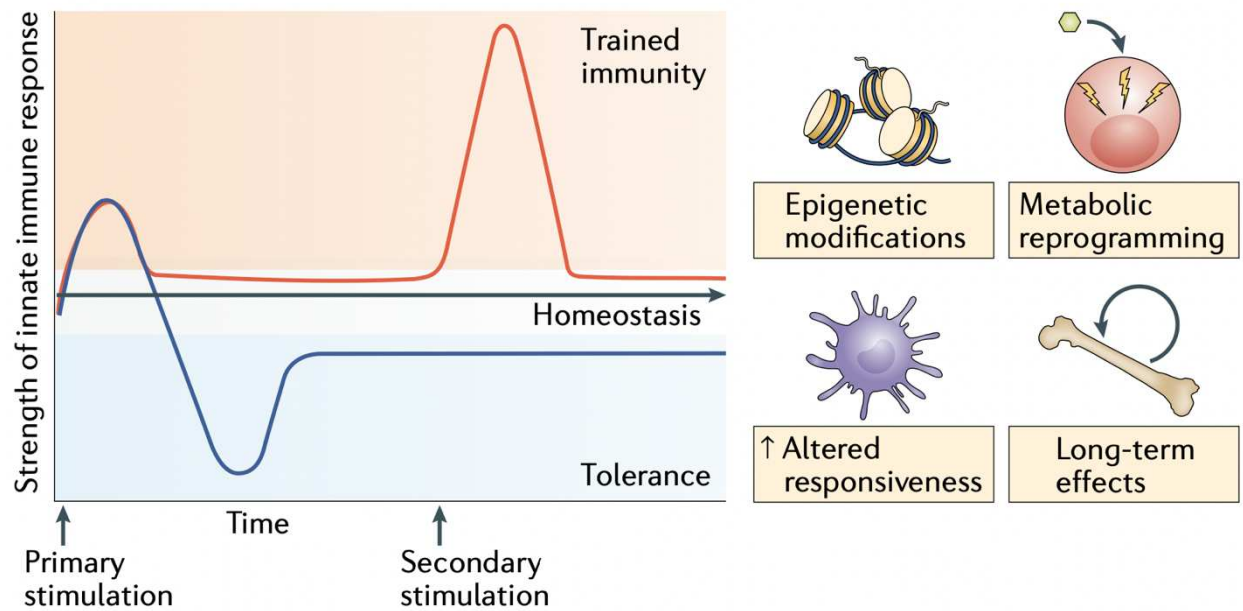


Figure 9. Trained immunity and tolerance from (Netea et al. 2020)

Epigenetic reprogramming

A key element underlying trained immunity in innate immune cells is the epigenetic reprogramming. Indeed, histone modification induced by the first stimulation induces a long-term chromatin decompaction, making genes highly accessible for transcription during a second stimulation. More specifically, two epigenetic markers are triggered by trained immunity. The first one is the acetylation of histone 3 lysine 27 (H3K27ac). The second mark is the mono-methylation on histone 3 lysine 4 (H3K4me) and the trimethylation on the same site (H3K4me3) (Figure 10). These modifications result in enhanced transcription of pro-inflammatory cytokines IL-1 β , TNF- α , and IL-6 in trained macrophages (Zhao et al. 2019).

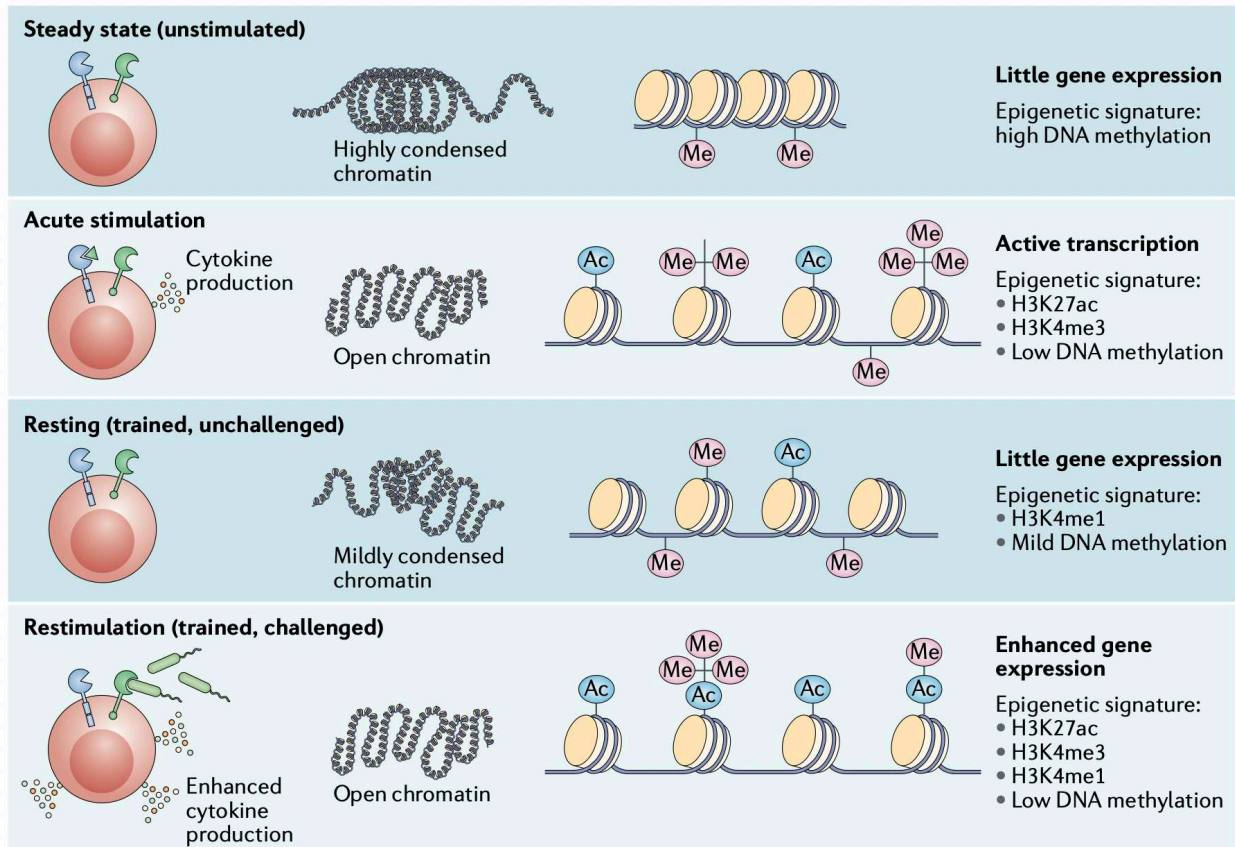


Figure 10. Epigenetic reprogramming underlies the induction of trained immunity from (Netea et al. 2020)

Immunometabolism

Resting immune cells use oxidative phosphorylation and fatty acid oxidation to produce low energy to fulfill their basic needs. The activation of innate immune cells will require extra energy production. The cells will generate energy through aerobic glycolysis, glutaminolysis, cholesterol metabolism, and fatty acid synthesis. Trained macrophages undergoing an epigenetic change will increase their metabolic needs by increasing their aerobic glycolysis via

dectin-1-AKT-HIF-1 α axis and the activation of the mammalian target of rapamycin (mTOR) (Fanucchi et al. 2021; Cheng et al. 2014). More specifically, mTOR acts as a sensor and evaluates the energy level, the oxygen level, and intracellular nutriment, then activate transcriptional regulators (for example, transcriptional repressor protein YY1 and

HIF-1 α), resulting in glycolysis and mitochondrial oxidative metabolism to meet cellular needs (Laplante and Sabatini 2012). As shown by Dr. Kaufmann et al., mitochondrial respiration and lactate production are a result of glycolysis increase triggered by monocyte training (Kaufmann et al. 2018) (Figure 11).

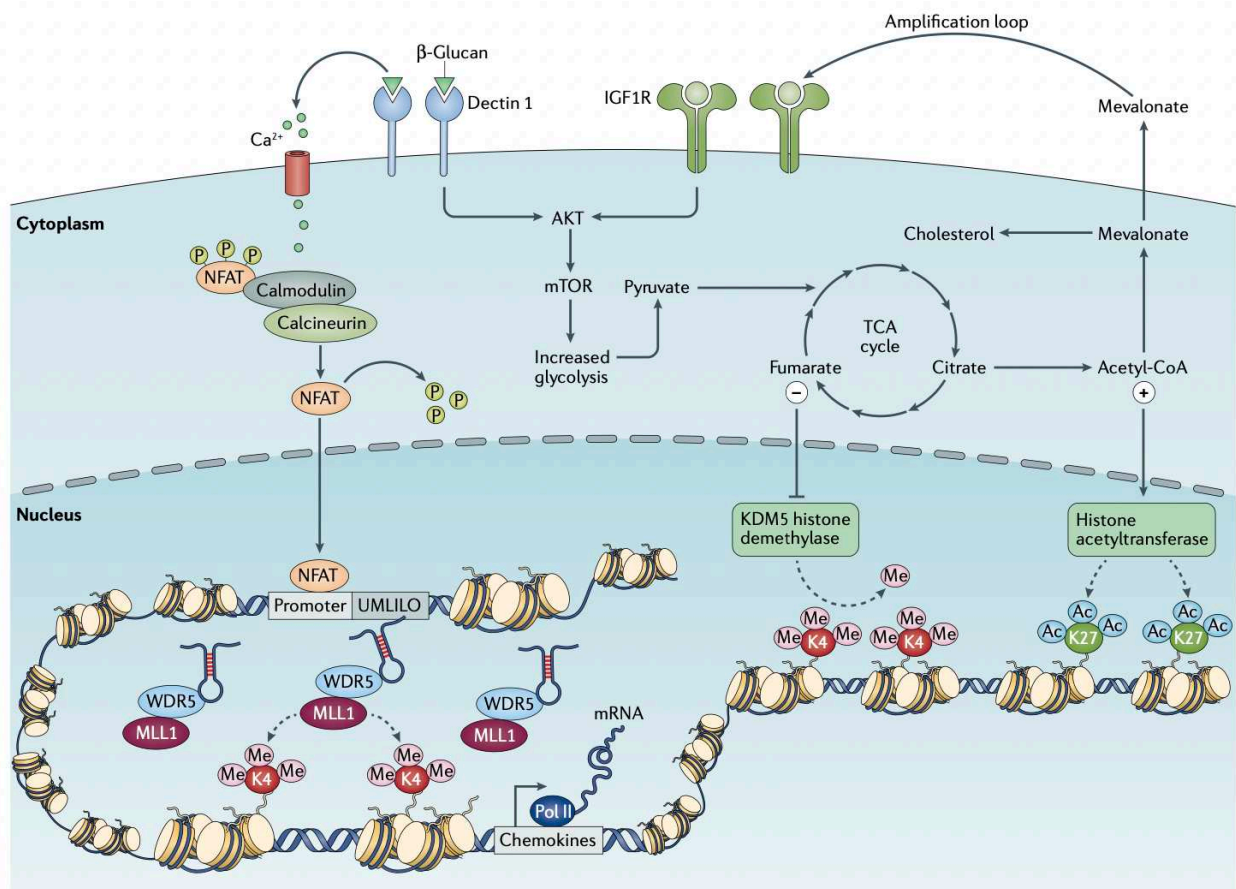


Figure 11. The interplay between epigenetics and metabolic processes form (Netea et al. 2020)

Central and Peripheral trained immunity

The short lifespan of myeloid cells like monocytes, activated macrophages, and DC (Patel et al. 2017; Parihar, Eubank, and Doseff 2010; Merad et al. 2013) (half-life less than 7 days) seems contradictory with the training remaining for years (Rieckmann et al. 2017).

Several works have been published clearing up this aspect, showing the training of bone marrow progenitor cells sustaining the training state of differentiated cells resulting from it. The training of undifferentiated cells located in the bone marrow is called: central training (Chavakis, Mitroulis, and Hajishengallis 2019). Dr Chavakis' lab shows that injecting β -glucan into mice triggered trained immunity of hematopoietic progenitors in the bone marrow and increased the myelopoiesis (Mitroulis et al. 2018).

Peripheral-trained immunity describes the training of innate immune cells localized in the tissue. Different pathways can conduce mature myeloid cells to be trained in the tissue. Trained immune cells can derive from a central trained progenitor and migrate to the tissue; for example, a trained monocyte infiltrates a tissue and differentiate into a trained macrophage. Additionally, some organs or tissues like the lungs or skin are constantly exposed to a wide range of stimuli due to their direct contact with the outside world. As a matter of fact, lungs or skin are exposed to immune training stimuli. In a viral-challenged experiment, chronically infected mice with gammaherpes virus showed a development inhibition of allergic asthma induced by lung exposition with dust-mite. This protective effect is assigned to the generation and maintenance of monocyte-derived trained macrophages following repeated exposure to the virus (Machiels et al. 2017). Moreover, the infection of mice with adenovirus produces long-lived trained alveolar macrophages showing an enhanced antibacterial immunity effect (Yao et al. 2018).

Organ transplantation

Trained immunity can be triggered by the same factors involved in the process of transplantation rejection.

Ischaemia–reperfusion injury

The organ's blood supply will be interrupted between the procurement and the transplantation phase. During this period, the organ will be plunged into an ischemia phase, triggering hypoxia and tissue injuries. After the transplantation, the organ will release some physio-pathological factors, DAMPs due to the reperfusion injuries (for

example, ROS, ions alteration, endothelial cell dysfunction, and cell death) in the extracellular space and the bloodstream (Eltzschig and Eckle 2011). ATP released by damaged cells binds to P2X7R, inducing a modification of the innate immune cell metabolism and the release of proinflammatory cytokines (for example, IL-1 β , TNF- α , and IL-6). The release of pro-inflammatory cytokines by activated macrophages through P2X7R is associated with graft rejection (Iyer et al. 2009; McDonald et al. 2010; Amores-Iniesta et al. 2017; Barbera-Cremades et al. 2017). In a model mouse allo-heart transplantation, Dr. Vergani's lab used an inhibitor of P2X7R and showed a prolongation of heart survival. The treatment with the P2X7R inhibitor, oATP, used in this study is controversial because knowing to have an interaction with P2X1R and P2X4R. Nevertheless, the study showed a reduced number of effector T cells, specifically the number of Th1 and Th17, compared to the untreated control (Vergani et al. 2013). Thrombospondin 1 (TSP1) is a matricellular protein produced by macrophages (Litvinukova et al. 2020) and involved in cell glucose metabolism (Gutierrez and Gutierrez 2021). TSP1 is overexpressed during IRI and mediates liver, kidney, and flap injury (Kelm et al. 2020; Isenberg et al. 2008; Maxhimer et al. 2009; Thakar et al. 2005). TSP1 activates macrophage inflammasome via TLR4 (like LPS) or CD47 and produces the release of proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) (Li et al. 2013; Stein et al. 2016; Yamauchi et al. 2002).

The blockade of pro-inflammatory cytokines release triggered by IRI showed a modulation of the adaptive graft leukocyte infiltration and a beneficial effect on graft survival (Jong et al. 2016; Uehara et al. 2018; Solhjoui et al. 2017). Altogether, IRI plays a major role in the activation of the innate immune cells via mediators triggering also trained immunity (Fagenson et al. 2020) and directly influencing the graft survival (Zhao et al. 2018).

Innate allorecognition

Current immunosuppression drugs used to modulate the rejection of a transplanted organ focus on the adaptive system. Indeed, the humoral and cellular immunity conducted by B cells and T cells against the transplanted organ leads to its rejection (Nankivell and Alexander 2010). However, this strategy used in the clinic remains suboptimal regarding

the toxicity of the treatment, the long-life posology, and the transplant outcome (Wojciechowski and Wiseman 2021). As a matter of fact, innate immune cells seem to be relegated to the background despite their important role during allograft rejection (Ochando et al. 2019). It appears that a combined strategy targeting innate and adaptive immunity could drastically improve patient outcomes.

The tissue and vascular injuries produced by the surgical trauma during transplantation drive the infiltration of inflammatory monocyte in the graft (Garcia et al. 2010). Indeed, proinflammatory cytokines like IL-1 β or TNF- α released by macrophages in the tissue activate the surrounding endothelium. The expression of adhesion molecules by the activated endothelium captures circulating monocytes and supports them until the transmigration step (Gerhardt and Ley 2015). In the transplanted tissue, these monocytes will recognize DAMPs and allo-antigens, motivating their differentiation into macrophages. Interestingly, a phenomenon of monocyte/macrophage memory has been described by Dr. Lakki's lab. After the immunization of a lymphocyte RAG^{-/-} mouse with splenocytes or skin graft from another allo RAG^{-/-} mouse, recipients re-challenged with the same allo-splenocytes 4 weeks later showed a monocytes and macrophages reaction (infiltration and phagocytosis), more important at the site of injection than the control mouse (Zecher et al. 2009). Later, the team identified a polymorphism in the membrane protein SIRPa between donor and recipient mice, sensed by recipient monocytes via CD47. Recently, the same team identified the paired immunoglobulin-like receptors-A (PIR-A) located on monocytes and macrophages as a receptor of MHC-I. In the case of an allogeneic exposure, recipient monocytes, and macrophages will develop a memory response through the recognition of the allo-MHC I by PIR-A. The inhibition of this pathway improved the outcome of an allo-kidney or an allo-heart transplantation (Dai et al. 2020).

Dr. Ochando's lab showed in a mouse model of vascularized allo-heart transplantation, vimentin and HMGB1 excreted by the stressed endothelium after the IRI bind macrophage receptors Dectin-1 and TLR-4, inducing the training of these cells (Braza et al. 2018). The lab developed a nanoparticle carrying mTOR inhibitor that inhibits the training of macrophages and underlying their production of pro-inflammatory cytokines. Targeted macrophages switched to a regulatory macrophage promoting CD4⁺ Treg expansion and

preventing the activation of alloreactive T cell. The inhibition of macrophage training via mTORi nanoparticle combined with a CD40-TRAF6 inhibitor nanoparticles inhibiting the co-stimulation results in an indefinite heart allograft survival in mice (Braza et al. 2018). Altogether, it seems that trained macrophages enhanced the rejection of the transplanted organ, and innate immune memory compromised the graft survival.

OBJECTIVE

Objective

Rationale

The innate inflammatory immune response triggered by tissue injury during organ transplantation negatively influences graft survival (Ochando et al. 2019; Zhao et al. 2020). Host monocytes infiltrate the allograft early after transplantation (Ordikhani et al. 2020) and become activated via recognition of danger associated molecular patterns (DAMPs) and donor MHC antigens (Oberbarnscheidt et al. 2014). Consequently, macrophages undergo epigenetic reprogramming and secrete pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α and IL-18) rendering them as "trained macrophages" (Divangahi et al. 2021; Ochando et al. 2020). This phenomenon stimulates adaptive inflammatory immunity and is associated with allograft rejection (Divangahi et al. 2021; Ochando et al. 2020).

While recipient trained macrophages are associated with graft loss, donor macrophages present in the allograft secrete extracellular vesicles (EVs) carrying donor MHC antigens (Gonzalez-Nolasco et al. 2018). Recipient macrophages then capture these EVs and display donor MHC molecules on their surface (MHC cross-dressing). It is now clear that presentation of donor MHC molecules by recipient APCs cross-dressed with donor MHC antigens is a key driving force behind activation of allospecific T cells (semi-direct alloresponse) leading to allograft rejection (Marino, Babiker, et al. 2016; Liu et al. 2016; Smyth, Lechler, and Lombardi 2016; Hughes et al. 2020).

Objective

The main objective of this study is to investigate the effect of P2X7R on exosome release and trained immunity with a focus on transplantation. To reach this aim, we investigate the following:

- 1- The role of innate immunity in the release of donor EVs after transplantation and subsequent cross-dressing of recipient APCs with donor MHC molecules.
- 2- The effect of P2X7R on trained macrophages in vitro

3- The role of P2X7R on training immunity in a model of skin graft.

Project 1: ATP-mediated signaling of
P2X7 receptors controls donor
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ATP-mediated signaling of P2X7 receptors controls donor extracellular vesicle release and MHC cross-dressing after allotransplantation.

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Running Title: P2X7 receptors and extra-cellular vesicles in transplantation

Key words: Extracellular vesicles, P2X7 receptors, MHC cross-dressing, transplantation

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Abbreviations

APCs: Antigen presenting cells

BMDMs: Bone marrow-derived macrophages

DAMP: Danger associated molecular patterns

DCs: Dendritic cells

EVs: Extracellular vesicles

gIFN: Gamma interferon

ILVs: Intraluminal vesicles

LPS: Lipopolysaccharides from *Salmonella typhosa*

mAb: Monoclonal antibody

MHC: Major histocompatibility complex

NLRP3: Nod-like receptor protein 3

P2X7R: P2X7 receptor

TCR: T cell receptor

Abstract

After skin allotransplantation, recipient antigen presenting cells (APCs) regularly acquire allogeneic MHC molecules from donor cells. Subsequently, they display on their surface either intact donor MHC molecules (MHC cross-dressing) or processed donor peptides bound to self-MHC molecules (indirect presentation). Intercellular transfer of donor MHC molecules is mediated primarily by extracellular vesicles (EVs) released by the allograft. Both presentation of whole donor MHC proteins and peptides by recipient APCs (semi-direct and indirect pathways) are known to contribute to the pro-inflammatory T cell response leading to allograft rejection. At the same time, there is ample evidence showing that initiation of adaptive alloimmunity depends on early innate inflammation caused by tissue injury and subsequent activation of myeloid cells (macrophages and dendritic cells) recognizing danger associated molecular patterns (DAMPs). Among these DAMPs, extracellular ATP, which binds to P2X7 receptors, is known to play a key role in innate inflammation by activating NLRP3 inflammasome and subsequent production and release of inflammatory cytokines and EVs. This prompted us to evaluate the influence of innate inflammation triggered by ATP-mediated signaling of P2X7 receptors on EV release by donor cells after skin transplantation in mice. In this article, we show that inhibition of P2X7R signaling suppresses both EV release and MHC cross-dressing and prolongs allograft survival in mice. This study reveals a novel aspect of the role of innate in allotransplantation.

Introduction

Recognition of donor MHC antigens by T cells initiates the adaptive immune response leading to acute allograft rejection. T cell allorecognition was originally known to be mediated via two distinct mechanisms called direct and indirect pathways. In the direct pathway, T cells recognize allogeneic MHC proteins displayed on donor antigen presenting cells (APCs) having migrated to the recipient's secondary lymphoid organs (passenger leukocytes) (Steinmuller 1980; Larsen, Morris, and Austyn 1990; Lechler and Batchelor 1982). Alternatively, T cells activated via indirect allorecognition recognize self-MHC molecules complexed with donor peptides processed and presented by recipient APCs (Marino, Paster, and Benichou 2016). Both CD4⁺ and CD8⁺ T cells activated either directly or indirectly can reject a skin allograft (Marino, Paster, and Benichou 2016). On the other hand, direct allorecognition is the driving behind acute cellular rejection of transplants while indirect T cell activation has been associated with alloantibody production and chronic rejection of organ allografts (Baker et al. 2001; Lee et al. 2001).

A few years ago, we reexamined the presence of donor passenger leukocytes in the lymph nodes of skin-grafted mice using imaging flow cytometry and RT-PCR techniques. While we did not detect any passenger leukocytes, we observed significant numbers of recipient cells bound to extracellular vesicles (EVs) expressing donor MHC class I and II molecules on their surface (Marino, Babiker, et al. 2016). Likewise, our study and that of Morelli's group showed high frequencies of recipient APCs displaying donor MHC proteins in the spleen of heart-transplanted mice (Marino, Babiker, et al. 2016; Liu et al. 2016). In the studies by Liu et al. donor EVs had characteristic features of exosomes, which were

internalized or remained attached to the recipient cDCs (Liu et al. 2016). However, it cannot be ruled out that other EVs such as ectosomes (microvesicles) or apoptotic vesicles could also participate in the transfer of MHC molecules between donor and recipient cells after transplantation. This phenomenon referred herein as donor MHC cross-dressing has now been documented in rodents and patients transplanted with various allogeneic solid organs, as well as pancreatic islets and bone marrow cells (Harper et al. 2015; Markey et al. 2014; Brown, Sacks, and Wong 2008; Mastoridis et al. 2021; Liu et al. 2016; Ono et al. 2017; Hughes et al. 2020).

Exchange of proteins between cells of the immune system was documented 40 years ago by Hudson and Bona who documented the acquisition by T cells of surface immunoglobulin molecules from B cells (Hudson and Sprent 1976) and antigens from macrophages (Bona et al. 1973). Furthermore, Frelinger et al. reported in 1974 the transfer of MHC molecules between hematopoietic cells (Frelinger et al. 1974). The first demonstration that APCs cross-dressed in vitro with allogeneic MHC antigens could activate alloreactive T cells was provided by Lechler's laboratory (Herrera et al. 2004). This type of alloresponse is now termed *semi-direct alloresponse*. Finally, Morelli and Thomson have demonstrated that allogeneic EVs can be internalized by APCs, which then process and present allopeptides along with self-MHC molecules (Morelli et al. 2004). This suggests that, in addition to semi-direct alloresponse, EVs may also represent a major source of MHC antigen for T cell activation via indirect allorecognition.

The lack of donor passenger leukocytes in skin-grafted mice and the overwhelming presence of donor MHC-cross-dressed APCs in heart transplanted mice suggest that semi-direct rather than direct alloresponse is the driving force behind allograft rejection. In support of this view, Prunevievieille et al. recently reported that APCs having acquired allogeneic MHC molecules after exposure to exosomes could elicit a potent inflammatory alloresponse both in vitro and in vivo and cause second set allograft rejection in mice (Prunevievieille et al. 2021). Moreover, Zeng et al. reported that in vivo inhibition of small EV biogenesis suppressed donor MHC cross-dressing and significantly delayed cardiac allograft survival in mice (Zeng et al. 2021). Altogether, these studies suggest that semi-direct allorecognition by T cells may be an essential element of the adaptive immune response leading to acute allograft rejection.

In the present study, we examined the role of innate immunity in the release of donor EVs after transplantation and subsequent cross-dressing of recipient APCs with donor MHC molecules. Indeed, potent innate inflammatory responses are induced right after transplant surgery, which are associated with early graft infiltration by polymorphonuclear neutrophils and macrophages (LaRosa, Rahman, and Turka 2007; Ochando et al. 2019; Dai et al. 2020). These phagocytes become activated through recognition of danger associated molecular patterns (DAMPs) (LaRosa, Rahman, and Turka 2007; Land et al. 2016). Among those, extracellular ATP is a major DAMP that is released after tissue injury and induces NLRP3 inflammasome complex formation and activation through binding to purinergic P2X7 receptors (P2X7Rs) on phagocytes (Di Virgilio et al. 2017). One key function of NLRP3 is caspase-1 activation, which drives the release of IL-1 β , IL-6 and IL-

18 pro-inflammatory cytokines. Moreover, several studies have documented the role of P2X7R signaling in the release of EVs by different cell types in health and disease, including immune cells (Di Virgilio et al. 2017). Taken together, these observations prompted us to evaluate the contribution of P2X7R signaling in the release of EVs by leukocytes tested in vitro and in vivo in a transplant model. In this article, we show that inhibition of P2X7R signaling prevented EV release by macrophages activated in vitro. Most importantly, we observed that injection of skin-grafted mice with P2X7R inhibitors prevented donor EV release and donor MHC cross-dressing thus prolonging allograft survival. The implications of these findings for the design of new immune therapies in transplantation are discussed.

Material and Methods

Mice and transplantation procedures

Mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All animal care and handling were performed according to institutional guidelines. Full-thickness skin grafts (2 x 3 cm) were placed on the recipients' flank area according to a technique described elsewhere (Billingham and Medawar 1951).

Preparation of bone marrow-derived macrophages

Bone marrow cells from C57BL/6 were suspended into a double filtrated (0.22 μ m) AIM-V serum-free media supplemented with 20 ng/ml of M-CSF, seeded at a density of 2.5 x 10⁶ cells/ml in a 6 well-plate tissue culture dishes and cultured for 7 days to obtain bone marrow-derived macrophages.

Detection and counting of EVs

Donor EV detection in blood samples of control and transplanted mice was performed using Vesicle Flow Cytometry (CytoFles), as described elsewhere (Danielson et al. 2016; Morales-Kastresana et al. 2019). The number of exosomes and ectosomes present in macrophage culture supernatants was assessed by Nanoparticle Tracking Analysis, which combines light scattering and Brownian motion to detect particles in suspension, as previously described (Prunevieuille et al. 2021).

MHC antigen cross-dressing

The cells were labeled with fluorescent antibodies directed against H-2K^b (AF6-88.5 Biolegend), H-2K^d (SF1-1.1 Biolegend), I-A^b (AF6-120.1 Biolegend), I-A^d (AMS-32.1 eBioscience), as described elsewhere (Marino, Babiker, et al. 2016). Briefly, for IFC, acquisition of cells was performed with the ImageStream instrument (Amnis EMD Millipore), using low speed fluidics, at 40x magnification. Data analysis was performed using IDEAS 6.1 image processing and statistical analysis software (Amnis EMD Millipore).

Statistics

All statistical analyses were performed using STATView software (Abacus Concepts, Inc., Berkeley, CA) and Graphpad Prism, version 5.0b (San Diego California, USA). P-values were calculated using paired t-test. P-values < 0.05 were considered statistically significant.

Results

Inhibition of P2X7R signaling prevents extracellular vesicle release by macrophages activated in vitro.

There are two major types of extracellular vesicles released by viable cells, exosomes and ectosomes (also called microvesicles). Exosomes are nanovesicles (50-100 nm) formed by inward budding of the membrane of late endosomes, which accumulate within the late endosomes as intraluminal vesicles (ILVs) (Kowal, Tkach, and Thery 2014). Exosomes are subsequently secreted by cells through fusion of the endosomal limiting membrane with the plasma membrane (1). On the other hand, ectosomes are bigger vesicular structures (0.1–1.0 μm) shed via outward blebbing of the plasma membrane (Heijnen et al. 1999).

Here, we first tested the influence of P2X7R inhibition on exosome and ectosome release by macrophages activated by Lipopolysaccharides from *Salmonella typhosa* (LPS) in vitro. Bone marrow macrophages (BMDMs) were prepared from C57Bl/6 (B6) mice, as described in the Methods section. BMDMs (5×10^5 cells) were cultured for 24 hours with LPS (500 ng/ml) with or without ATP (2.5 mM, added during the last 30 min of culture). Competitive P2X7R antagonists, oxidized ATP (oATP) or A438079 (referred to as A43 hereafter), were added at different concentrations (25-300 μM) to the culture 10 minutes prior to macrophage stimulation. Next, exosomes and ectosomes were isolated from the culture medium via differential centrifugation, as previously described (Prunevieville et al. 2021). Briefly, a first centrifugation at 2,000 g for 15min was carried out to remove dead cells, followed by a 30-min centrifugation at 16,000 g to isolate ectosomes. Finally,

exosomes were collected following a centrifugation at 100,000 g for 70 min. The nature, size, and concentration of exosomes and ectosomes were evaluated using Nanoparticle Tracking Analysis and western blotting, as described elsewhere (Prunevieuille et al. 2021). Macrophage cell viability was > 95% after 24-hours exposure to LPS, ATP and P2X7R inhibitors (Fig. S1). As shown in Figure 1, both oATP and A43 P2X7R inhibitors suppressed exosome release by macrophages activated by LPS + ATP. Similar results were obtained with ectosome release by activated macrophages (Fig. S2).

Administration of mice with P2X7R inhibitors prevents donor EV release after allotransplantation.

Following their placement, allogeneic skin grafts release EVs that are taken up by APCs in the host's lymphoid organs (Marino, Babiker-Mohamed, et al. 2016; Liu et al. 2016). At the same time, several studies have shown the relevance of blood and urine EVs as potential biomarkers of rejection in mice and patients (Alvarez et al. 2013; Gunasekaran et al. 2018; Lim et al. 2018; Sigdel et al. 2014). Here, we tested whether early innate inflammation associated with the presence of DAMPs is involved in EV release by donor cells after skin transplantation. Extracellular ATP is a major DAMP that is released after transplantation and causes inflammatory cytokine release through its binding to P2X7R and subsequent activation of the NLRP3 inflammasome (Amores-Iniesta et al. 2017). In addition, P2X7R signaling has been documented to play a key role in EV release in several models, including the release of exosomes containing IL-1 β by activated macrophages (Di Virgilio et al. 2017; Qu et al. 2009). These observations prompted us to investigate whether blocking P2X7R signaling in vivo could alter donor EV release in skin-

transplanted mice. To test this, BALB/c mice (H-2^d) were transplanted with a skin patch from a C57Bl/6 mouse (H-2^b) expressing GFP ubiquitously (B6-GFP). Some recipients were injected daily for 7 days (intraperitoneally) with A43 (100 $\mu\text{mol/Kg}$) P2X7R inhibitor. At day 10 post-transplantation, the presence of donor GFP⁺ EVs in the serum of transplanted mice was evaluated by Vesicle Flow Cytometry (CytoFlex), as described in the Methods section and elsewhere (Danielson et al. 2016; Morales-Kastresana et al. 2019). As shown in Figure 2A, no fluorescent EVs were detected in samples from naive BALB/c mice. In contrast, more than 3000 donor EVs per ml were found in the serum of BALB/c mice recipients of a B6 skin graft (Figure 2B). In contrast, samples from BALB/c transplanted mice treated with A43 P2X7R inhibitor contained very few EVs (around 100 more compared to the naive control) (Figure 2A and 2C). Therefore, in vivo treatment of recipients with a P2X7R inhibitor nearly eliminated the release of donor EVs in the blood of skin-transplanted mice.

Administration of mice with P2X7R inhibitors prevents donor MHC cross-dressing after allotransplantation.

We and others have previously reported the presence of large numbers of recipient APCs cross-dressed with donor MHC class I and II molecules in lymph nodes and spleen of mice transplanted with an allogeneic skin patch or heart (Marino, Babiker, et al. 2016; Liu et al. 2016). There is strong circumstantial evidence showing that intercellular transfer of donor MHC molecules is mediated principally via donor EVs trafficking from the graft to host's secondary lymphoid organs or by EVs secreted by donor passenger leukocytes within these organs (Liu et al. 2016; Marino, Babiker, et al. 2016). Since inhibition of

P2X7R signaling suppressed EV release in skin-grafted mice (Fig. 2), we next investigated whether it would impact donor MHC cross-dressing of host's APCs after transplantation. To test this, BALB/c mice received a skin graft from a B6 donor and were injected daily with A43 or oATP P2X7R inhibitor given i.p. for 6 days. At day 7, spleen cells of recipients were collected and the presence of recipient APCs cross-dressed with donor MHC class I (K^b) was evaluated by imaging flow cytometry, as previously described (Marino, Babiker, et al. 2016). As shown in Figure 3, over 4% recipient spleen cells displayed donor K^b molecules in control transplanted mice while only less than 0.2% cross-dressed cells were detected in transplanted mice treated with oATP. Similar results were obtained with A43 inhibitor (data not shown). Figure 3C shows the actual number of cross-dressed cells detected in the spleen of each group of mice. Therefore, inhibition of P2X7R signaling markedly reduced the presence of donor MHC cross-dressed in the spleen of skin-transplanted mice.

Administration of mice with P2X7R inhibitors prolonged skin allograft survival in mice.

BALB/c mice were transplanted with a B6 skin allograft and treated daily for 7 days with A43 P2X7R inhibitor given intraperitoneally at 50, 100 or 150 $\mu\text{mol/Kg}$. As shown in Figure 4, control untreated mice rejected their allograft 8-10 days post-transplantation (MST: 9 days) while mice treated with A43 rejected skin grafts in a delayed fashion (MST:19 days for the group treated with 150 $\mu\text{mol/Kg}$, $p = 0.0016$).

Discussion

There is accumulating evidence suggesting the role of donor extracellular vesicles (EVs) in allograft rejection. Several studies have demonstrated that donor EVs are regularly released by grafted cells and subsequently captured by recipient APCs after transplantation (Gonzalez-Nolasco et al. 2018). This process can lead to presentation of allogeneic MHC molecules alongside with self-MHC proteins on recipient APCs (semi-direct antigen presentation) or processing and presentation of donor MHC peptides bound to self-MHC molecules by host's APCs (indirect antigen presentation) (Marino, Babiker, et al. 2016; Liu et al. 2016; Smyth, Lechler, and Lombardi 2016). The presence of recipient APCs cross-dressed with donor MHC class I and II proteins has now been reported in mouse and human recipients of skin, islet, kidney, liver heart and bone marrow allografts and is therefore a general phenomenon in allotransplantation (Harper et al. 2015; Markey et al. 2014; Brown, Sacks, and Wong 2008; Mastoridis et al. 2021; Liu et al. 2016; Ono et al. 2017; Hughes et al. 2020). At the same time, donor MHC antigen presentation through the semi-direct pathway has been shown to activate alloreactive T cells in vitro and in vivo (Herrera et al. 2004; Prunevieuille et al. 2021). Taken together, these studies suggest donor EVs may contribute to allograft rejection by activating alloreactive T cells via semi-direct and indirect pathways. In the case of skin allografts, we previously documented the absence of donor passenger leukocytes in recipients' secondary lymphoid organs. This suggested that, in this model, semi-direct antigen presentation by recipient APCs cross-dressed with donor MHC proteins rather than direct allorecognition represents the driving force behind T cell-mediated acute rejection. However, this remains to be proven. On the other hand, a few passenger leukocytes (< 100 cells/spleen) were

detected in the spleen of heart-transplanted mice, as originally reported by C. Larsen et al (Larsen, Morris, and Austyn 1990). However, these donor leukocytes are presumably rapidly eliminated by the host's immune system while nearly 10^5 cross-dressed cells per spleen were observed at the time of rejection. At the same time, a recent study by Zeng et al. showed that in vivo inhibition of exosome biogenesis using a neutral sphingomyelinase inhibitor (GW4869) in heart-transplanted mice reduced donor MHC cross-dressing and alloantibody production and significantly prolonged allograft survival (Zeng et al. 2021). These results support the role of EVs and semi-direct alloresponse by T cells in cardiac allograft rejection.

In the present study, we investigated the role of innate inflammation mediated via ATP signaling of P2X7R in EV release and donor MHC cross-dressing in transplanted mice. We hypothesized that early inflammation caused by early activation of graft resident and recipient infiltrating macrophages and DCs by DAMPs is involved in EV secretion after transplantation. Extracellular ATP is a major DAMP that is released during tissue injury and activates the NLRP3 inflammasome thus playing a major role in production and secretion of inflammatory cytokines, including IL-1 β , IL-6 and IL-18 (Di Virgilio et al. 2017). At the same time, several studies support the role of ATP and NLRP3 inflammasome in EV release (Di Virgilio et al. 2017). For instance, ATP has also been shown to promote the release of HIV-laden microvesicles thus spreading HIV infection (Graziano et al. 2015). In addition, P2X7R signaling of macrophages is involved in expression and release of microvesicle-associated tissue factor (TF), thus enhancing pro-thrombotic responses (Moore and MacKenzie 2007). Most relevant to this study, P2X7R signaling not only

governs IL-1 β processing but also its externalization. Indeed, IL-1 β is an atypical cytokine in that it lacks a secretory component and does not follow the classical endoplasmic route for extracellular release. Its secretion via exosomes and plasma membrane-derived ectosomes is mediated via P2X7R signaling (Di Virgilio et al. 2017). Furthermore, Qu's studies showed that MHC class II P2X7R stimulation of macrophages and DCs via P2X7R signaling led to the release of exosomes and ectosomes containing MHC class II molecules (Qu et al. 2009). It is noteworthy, that this phenomenon required the ASC/NLRP3 inflammasome but was independent of caspase-1. Our results showing the effects of P2X7R inhibitors on macrophages exposed to LPS in vitro further demonstrate the role of P2X7R in EV release by activated macrophages.

This study shows for the first time that P2X7R signaling controls donor EV release by donor cells after transplantation. Inhibition of P2X7R signaling was associated with concomitant absence of recipient APCs cross-dressed with donor MHC molecules in the spleen of mice transplanted with a skin allograft. This correlates with a recent study by Barrera-Avalos et al. showing that P2X7Rs are essential for cross-dressing of dendritic cells (Barrera-Avalos et al. 2021). Given that no donor passenger leukocytes had been detected in the spleen of skin-grafted mice, it was likely that lack of cross-dressed cells was due to the lack of donor EV release after transplantation. This resulted in substantial prolongation of allograft survival (MST: 19 days vs. 9 days, $p = 0.0016$). This result correlates with observations reported in a previous study by Amores-Iniesta et al. showing reduced T cell infiltration, reduced necrosis and preserved epidermal and dermal structure at day 7 post-transplantation in skin allografts placed on mice treated with

A438079 (100 umol/kg/day) (Amores-Iniesta et al. 2017). In these mice, a profound suppression of the T cell alloresponse at days 3 and 7 post-transplantation and a significant, although modest, prolongation of skin graft survival (12 days vs. 8 days, $p < 0.01$) were observed (Amores-Iniesta et al. 2017). It is not clear why despite the absence of cross-dressed cells in secondary lymphoid organs of skin-grafted mice, skin grafts were still rejected. It is possible that upon skin graft revascularization, some T cells infiltrate the allograft and become activated in situ by recipient APCs cross-dressed within the graft itself. This concept called "peripheral sensitization" was originally proposed in the 1960's by Medawar and Strober (Medawar 1958; Hume and Egdahl 1955). In support of this view are recent studies from the Lakkis' lab showing the presence of high numbers of recipient cells cross-dressed with donor MHC molecules in kidney and islet allografts early after transplantation (Hughes et al. 2020). It is possible that once cross-dressed with donor MHC class I proteins, these recipient cells, including vascular endothelial cells, could become targets for destruction by alloreactive CD8⁺ cytotoxic T cells, a process contributing to local inflammation and acute rejection. Further studies are needed to address this question.

In summary, this study shows that ATP-mediated signaling of P2X7R controls the production of donor extracellular vesicles and subsequent donor MHC cross-dressing in transplanted mice. This reveals a new aspect of the role of innate immunity in transplantation. Inhibition of P2X7R significantly prolonged the survival of skin allografts in mice. Further studies will be needed to investigate whether P2X7R inhibitors could be

used in combination with other treatments such as T cell costimulation blockade to prevent rejection or promote tolerance induction of allografts.

Acknowledgments

This work was supported by a grant from the NIH to Gilles Benichou, NIH R01DK115618.

Disclosure

The authors have no conflicts of interest to disclose as described by the American Journal of Transplantation

Data availability statement

The data that support the findings of this study are available from the corresponding author, [GB], upon request.

Figures

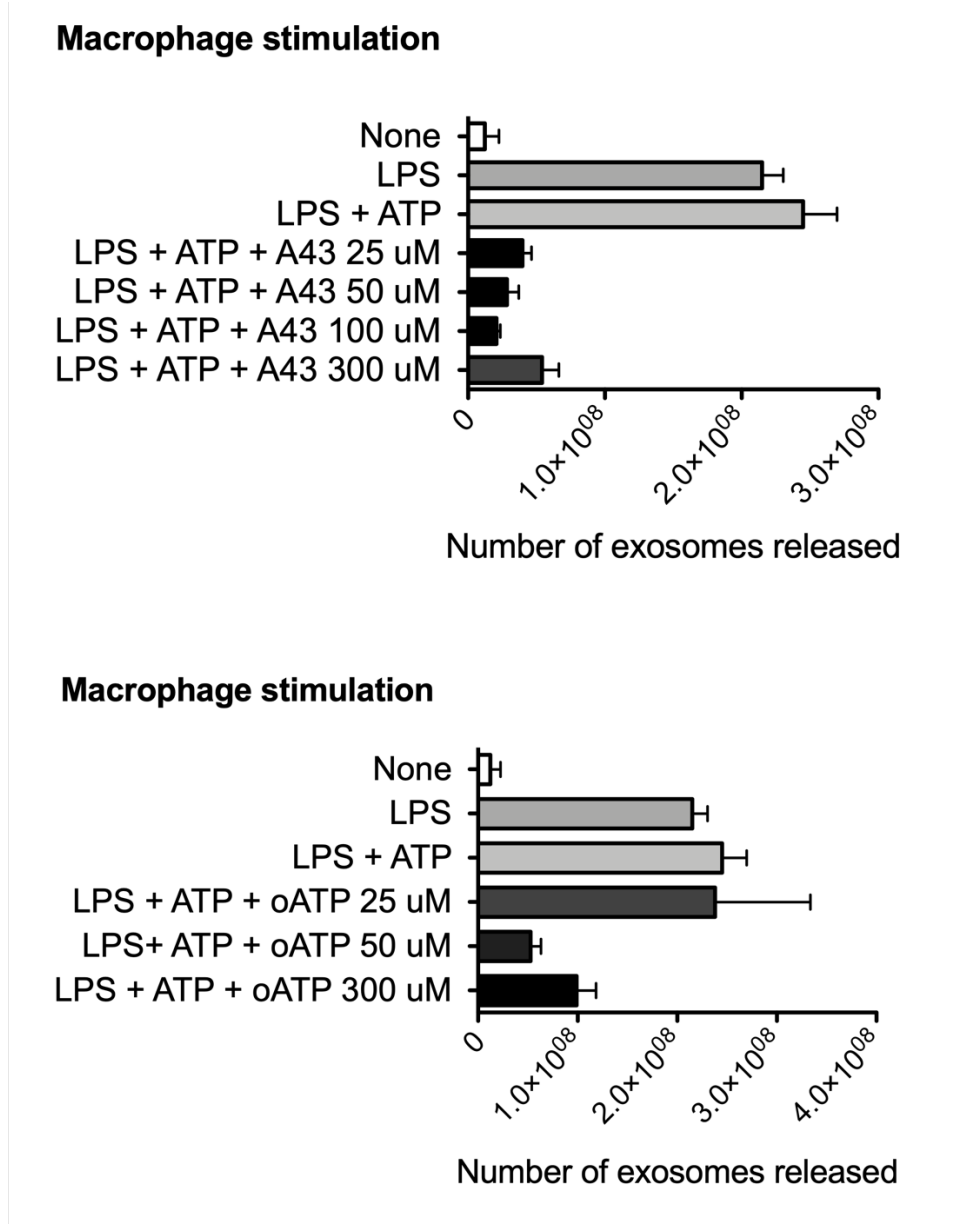


Figure 1. P2XR inhibition prevents EV release by macrophages activated in vitro.

Bone marrow-derived macrophages (5×10^5 BMDMs) were prepared and stimulated in vitro with medium, LPS alone (500 ng/ml) or with LPS + ATP (2.5 uM) for 24 hours (ATP was added during the last 30 minutes of culture). Macrophages were cultured in the

presence or not of A438079 (A43) or oATP P2X7R inhibitors at doses ranging from 25 uM to 300 uM). Next, culture media were collected, and exosomes were isolated, characterized and counted using Nanoparticle tracking, as previously described (Prunevieuille et al. 2021). The results are expressed as number of exosomes released in culture \pm SD.

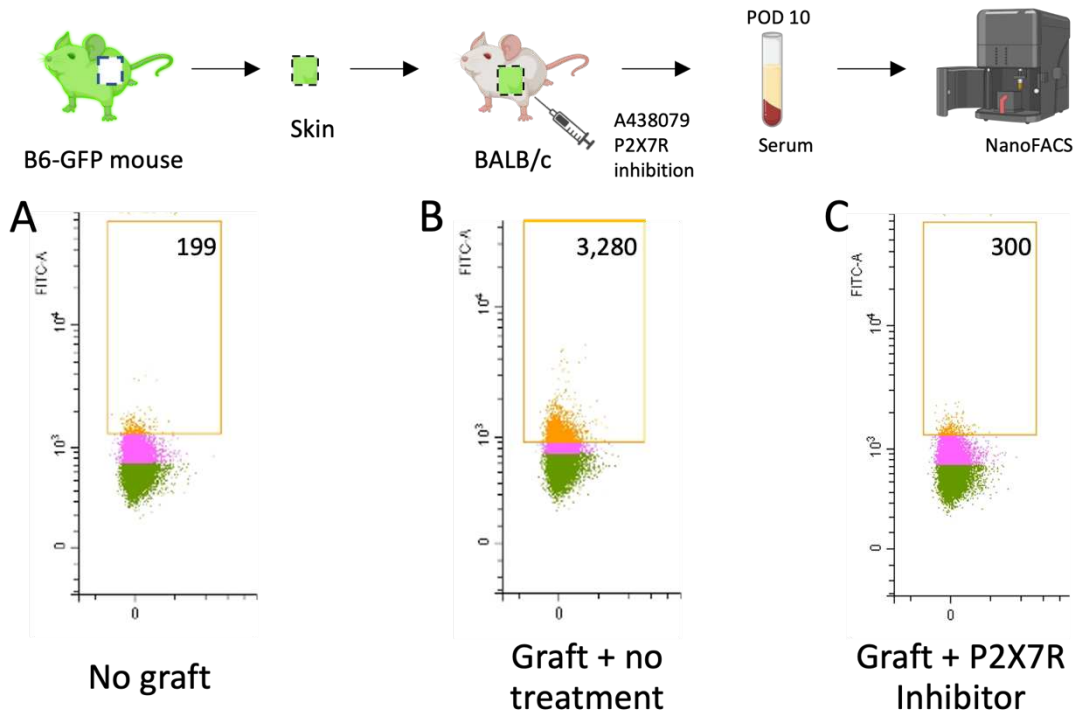


Figure 2. Treatment of mice with P2X7R inhibitors suppresses donor EV release in transplanted mice.

BALB/c mice were transplanted with a skin graft from a C57Bl/6-GFP donor. Recipient mice were either non treated or injected intraperitoneally with A438079 (100 $\mu\text{mol/kg}$) or oATP (2.2 mmol/Kg). Ten days later, recipients' blood was collected and plasma was prepared. The presence of GFP+ EVs was detected using Nanoscale Flow Cytometry (NanoFCM) as described elsewhere (Danielson et al. 2016; Morales-Kastresana et al.

2019). Naive, non-transplanted mice were used as control mice. The results are representative of 3 mice tested individually.

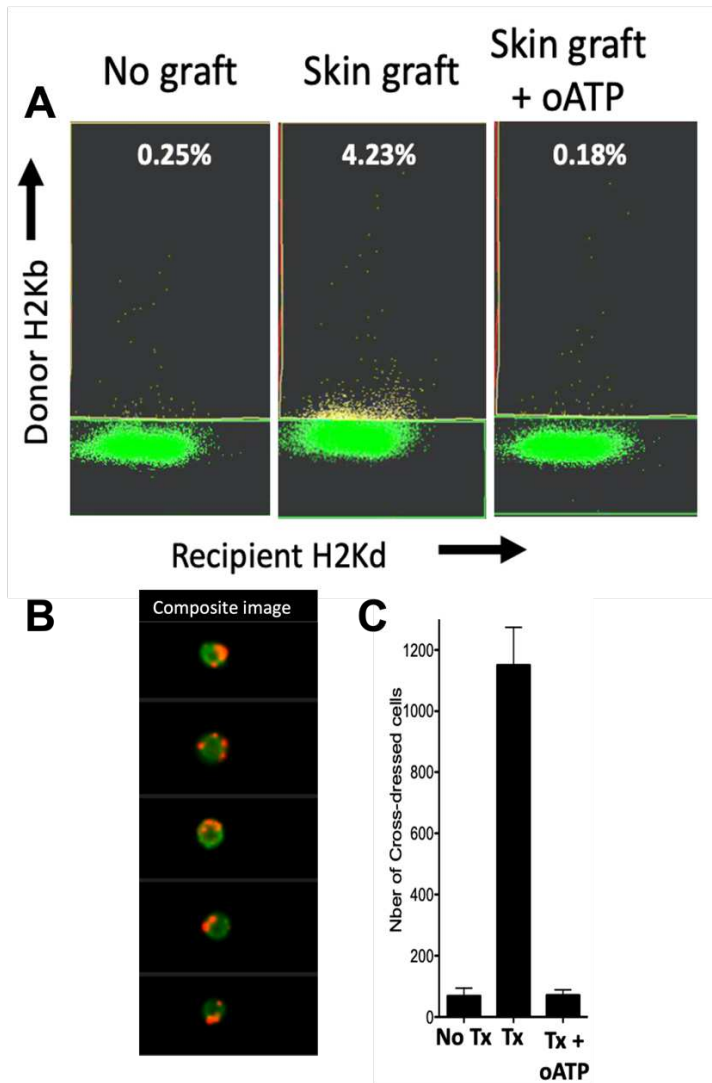


Figure 3. P2X7R inhibitors suppresses the presence of cross-dressed cells in the spleen of transplanted mice.

The spleen of a naïve B6 mouse or allotransplanted with a BALB/c skin and treated with oATP (0.25 mg given daily i.p. for 6 days) or not were collected at seven days after transplantation. The cells were stained with anti-MHC class I K^b antibodies and anti-MHC

class I K^d bound to FITC and APC, respectively. The presence of recipient (K^{b+}) and donor (K^{d+}) cells was assessed using Flow Imaging with low-speed fluidics, at 40x magnification. Data analysis was performed using IDEAS 6.1 image processing and statistical analysis software (Amnis EMD Millipore). **Panel A** shows plots obtained with control recipient cells and cells from transplanted mice treated or not with oATP and tested at day 10 post-transplantation. The results are representative of 3-5 mice per group tested in two separate experiments. **Panel B** shows a representative microscopic analysis of double positive cells (K^{b+}/K^{d+}) observed in panel A. **Panel C** shows the number of recipient cells cross-dressed with donor MHC \pm SD among 3-5 mice tested individually in each group.

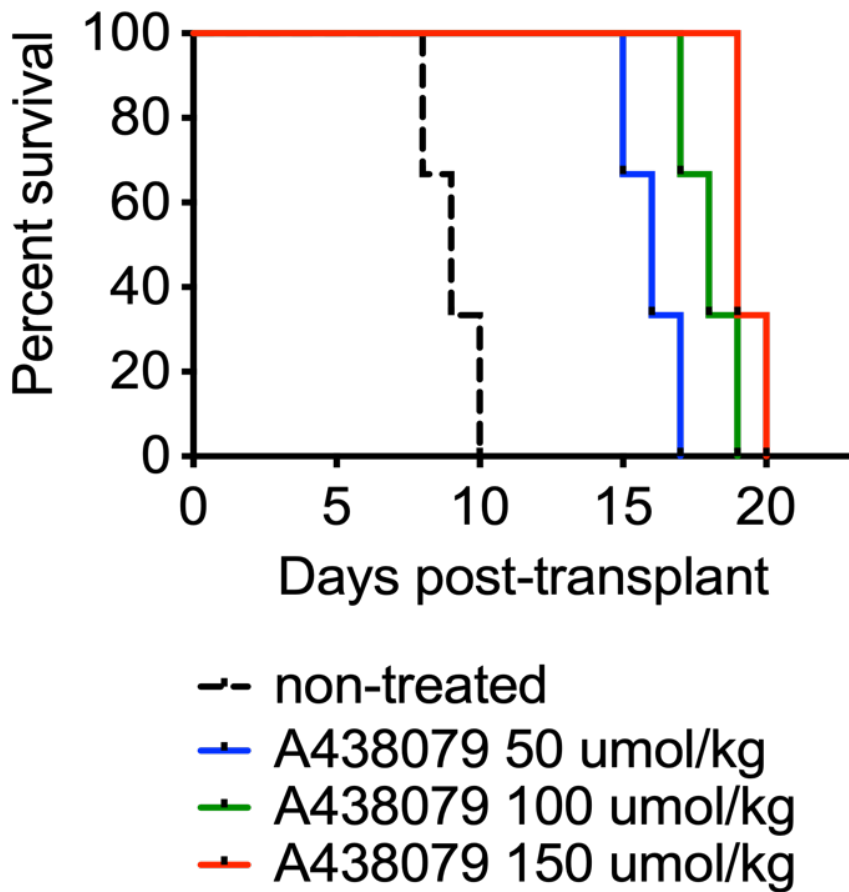


Figure 4. Treatment of mice with P2X7R inhibitors delays skin allograft rejection

B6 mice were injected intraperitoneally with A438079 P2X7R inhibitor given daily at 50, 100 or 150 umol/kg for 7 days starting at the time of placement of a skin allograft from a BALB/c mouse. The results are expressed as percent graft survival and included 3-5 mice in each group.

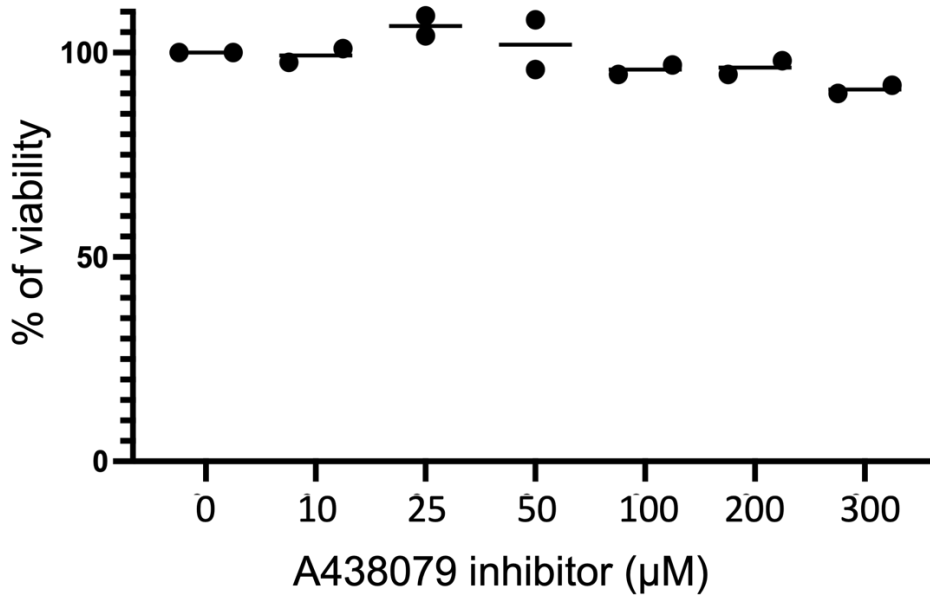


Figure S1. Viability of macrophages treated in vitro with A438079 Inhibitor

Bone marrow-derived macrophages were prepared and cultured for 24 hours in the presence or not with A438079 (A43) P2X7R inhibitor at doses ranging from 10 to 300 µM). Next, the cells were collected and their viability was assessed using live/dead markers by FACS.

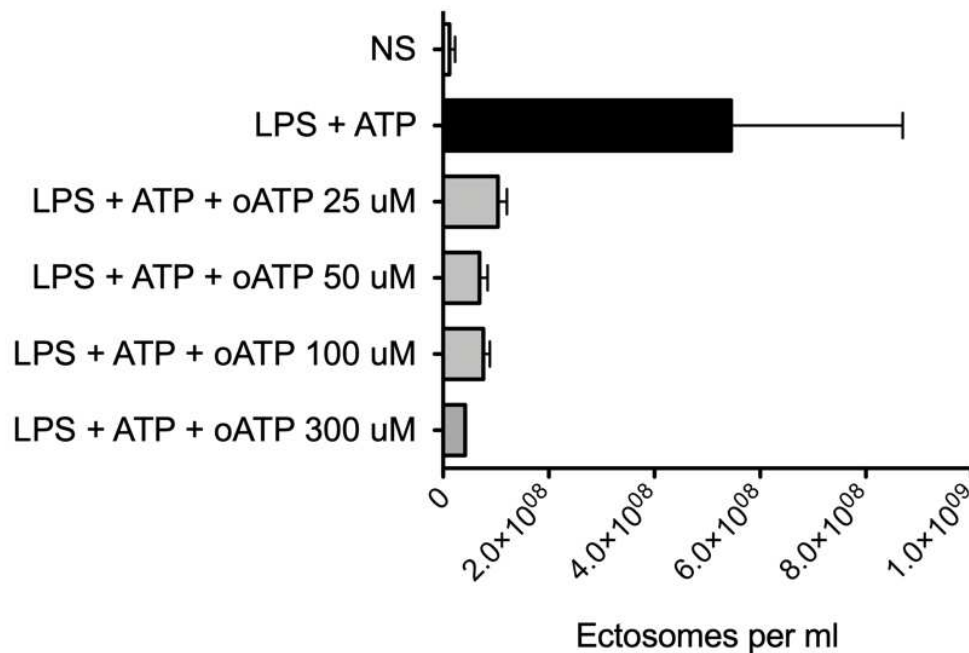


Figure S2. oATP suppresses ectosome release by activated macrophages.

Bone marrow-derived macrophages (5×10^5 BMDMs) were prepared and stimulated in vitro with medium, LPS alone (500 ng/ml) or with LPS + ATP (2.5 μ M) for 24 hours (ATP was added during the last 30 minutes of culture). Macrophages were cultured in the presence or not of A438079 (A43) or oATP P2X7R inhibitors at doses ranging from 25 μ M to 300 μ M). Next, culture medias were collected and ectosomes were isolated, characterized and counted using Nanoparticle tracking, as previously described (Prunevieuille et al. 2021). The results are expressed as number of ectosomes released in culture \pm SD.

The first project focuses on evaluating the influence of innate inflammation triggered by ATP-mediated signaling of P2X7 receptors on extracellular vesicle (EV) release by donor cells after skin transplantation in mice. We demonstrate that inhibiting P2X7 receptor (P2X7R) signaling suppresses both EV release and MHC cross-dressing, leading to prolonged allograft survival in mice.

In this second part, we will explore a new aspect of innate immunity with the concept of trained immunity in transplantation. Indeed, less is known about this phenomenon and its involvement in graft rejection. As demonstrated in the introduction and the first project, blocking P2X7R inhibits the activation of the NLRP3 inflammasome. Interestingly, a recent article exposed the role of the NLRP3 inflammasome, stimulated by the western diet, as a trigger for trained immunity (Christ et al. 2018). The second project will specifically focus on the role of P2X7R in trained immunity and its impact on transplantation.

Project 2: P2X7R inhibition inhibits trained immunity by macrophages activated in vitro and in vivo in skin-transplanted mice.

P2X7R inhibition inhibits trained immunity by macrophages activated vitro and in vivo in skin-transplanted mice.

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Running Title: Inhibition of P2X7 receptors suppresses trained immunity.

Key words: P2X7 receptors, macrophages, trained immunity, transplantation

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Abbreviations

ATP: Adenosine triphosphate

B6: C57BL/6

DAMPs: Damage-Associated Molecular Patterns

IL-6: Interleukin 6

LPS: Lipopolysaccharides from *Salmonella typhosa*

NEK7: NIMA (never in mitosis gene a)-related kinase 1

NF-AT: Nuclear factor of activated T-cells

NLRP3: nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3

P2X7R: P2X7 Receptor

PAMPs: Pathogen-associated molecular patterns

PIR: Paired immunoglobulin-like receptors

TNF α : Tumor necrosis factor α

Abstract

There is now ample evidence showing that innate cells of the immune system can acquire memory functions. For instance, monocytes and macrophages have been shown to undergo epigenetic and metabolic changes after exposure to DAMPs and PAMPs. Consequently, these cells mount stronger effector responses, including inflammatory cytokine release, upon later challenge with the same or other stimuli. This form of heterologous memory called trained immunity can last for years and provide enhanced protection against microbes and tumors. At the same time, P2X7R-mediated NLRP3 inflammasome activation occurring after exposure of macrophages to extracellular ATP plays a major role in innate inflammation. In this study, we investigated the relationships between P2X7R activation and the development of trained immunity. First, we showed that P2X7R inhibition prevented *in vitro* training of mouse pro-inflammatory macrophages exposed to β -glucan. Second, we showed that *in vivo* induction of trained immunity caused accelerated acute rejection of skin allografts in mice. However, short-term treatment of mice with an inhibitor of P2X7R given at the time and post-transplantation restored normal rejection time in mice with trained immunity. The implications of these findings for the development of novel therapies to harness innate pro-inflammatory immunity in transplantation are discussed.

Introduction

Patients undergoing transplant procedures are treated with a cocktail of immunosuppressive drugs, which prevent acute rejection (Nelson et al. 2022) (Sahman, Mugosa, and Rancic 2021). However, continuous and widespread immunosuppression is associated with increased risks of infections, cancer, and nephrotoxicity (Meier-Kriesche, Schold, and Kaplan 2004). In addition, these treatments fail to prevent chronic rejection, which is characterized by graft blood vessel obstruction and tissue fibrosis (McDonald-Hyman, Turka, and Blazar 2015). This underscores the need for the development of more selective immune-based therapies in transplantation.

Innate immunity induced immediately after transplantation is an essential element of the immune process leading to allograft rejection. Donor organ procurement and transplant surgery are associated with ischemia reperfusion injury and cell death (Salvadori, Rosso, and Bertoni 2015; Ochando et al. 2019). This results in local production of danger associated molecular patterns (DAMPs), which stimulate graft-infiltrating innate leukocytes, including PMNs, macrophages and dendritic cells (McDonald et al. 2010; Lee et al. 2011; de Paiva et al. 2009; Dong et al. 2007; Schlichting, Schareck, and Weis 2006). In addition, there is accumulating evidence showing that macrophages can also become activated via recognition of alloantigens through PIR receptors (Dai et al. 2020). Activation of innate immunity results in an inflammatory response that initiates and governs the nature of the subsequent adaptive alloresponse by T and B cells leading to allograft rejection. Recent evidence has been provided showing that in T cell-depleted

recipients of human renal allografts, instances of rejection are characterized by graft infiltration by monocytes and macrophages (Kirk et al. 2003). This observation highlights the critical role played by monocytes and macrophages in initiating and perpetuating the immune response leading to rejection (Xu, Dhanireddy, and Kirk 2006). Therefore, a better understanding of the mechanism underlying the development of innate immunity to DAMPs and alloantigens is critical for the design of new therapies in transplantation.

Immune memory has always been viewed as a hallmark of adaptive immunity. However, recent studies have demonstrated that various cells of the innate immune system, including monocyte/macrophages, neutrophil, NK cells, dendritic cells and can differentiate into cells exerting memory functions after an initial stimulation (Bekkering et al. 2016; Moorlag, Khan, et al. 2020; Moorlag, Rodriguez-Rosales, et al. 2020; Kleinnijenhuis, Quintin, Preijers, Joosten, et al. 2014; Hole et al. 2019). For instance, macrophages exposed to various stimuli have been shown to undergo epigenetic modifications such as histone H3 methylation and acetylation (H3K4Me3, H3K27Ac, respectively) (Saeed et al. 2014) and metabolic changes associated with increased glucose metabolism (Netea et al. 2020). These cells called trained macrophages respond faster and produce higher cytokine levels upon challenge with the same or another stimulus (Drummer et al. 2021). This form of heterologous memory immunity can last from months to years in humans and thereby provide enhanced protection against infections and tumors (Netea et al. 2020; Bekkering et al. 2021). Recent studies from the Ochando's laboratory have provided circumstantial evidence that trained immunity mediated by pro-inflammatory macrophages activated after transplantation contributes the rejection

process (Braza et al. 2018; Ochando et al. 2020). Consequently, prevention or inhibition of trained immunity represents a promising strategy to facilitate survival of allografts.

NLRP3 inflammasome assembly is essentially induced following signaling of P2X7 purinergic receptor (P2X7R) by extracellular ATP (Di Virgilio et al. 2017). P2X7R is a transmembrane ion channel receptor expressed predominantly on immune cells, including macrophages. After binding to ATP, P2X7 receptor functions as a cationic channel (Surprenant et al. 1996; North 2002). A brief ATP activation lasting just a few seconds opens a cationic pathway, enabling an influx of Na^+ and Ca^{2+} while facilitating the efflux of K^+ (North and Surprenant 2000). Consequently, there is a substantial rise in intracellular Na^+ concentration, resulting in membrane depolarization. The increased intracellular Ca^{2+} concentration triggers Ca^{2+} -dependent signaling pathways. Simultaneously, the efflux of K^+ reduces its cytosolic levels, thus promoting the assembly and activation of the NLRP3 inflammasome (Sanz and Di Virgilio 2000). This process is orchestrated by a cytoplasmic kinase known as never-in-mitosis A-related kinases (NEK7), which catalyzes the cleavage of pro-caspase-1 into its active form, caspase-1. Next, caspase 1 promotes the maturation of pro-interleukins pro-IL-1 β and pro-IL-18 into their active cytokine forms, IL-1 β and IL-18, respectively (Adinolfi et al. 2018). Therefore, P2X7R signaling by extracellular ATP is an essential element of the process leading to the production of inflammatory cytokines by activated macrophages. Whether ATP-mediated P2X7R signaling and activation of the inflammasome is required for the training of inflammatory macrophages is an important question that has never not been investigated.

In the present article, we investigated the role of P2X7R in trained immunity by macrophages activated in vitro and in vivo in mice recipients of an allogeneic skin graft. We show that inhibition of P2X7R signaling prevents macrophage trained in vitro following exposure to β -glucan. In addition, we report that in vivo treatment of mice with a P2X7R inhibitor prevented accelerated rejection of skin allografts caused by trained immunity. The implications of these findings for the design of P2X7R-based immunotherapy in transplantation are discussed.

Materials and Methods

Animals

Female Eight- to twelve-week-old C57BL/6 (H-2b), BALB/c (H-2d), were used in the experiments. All were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were consistently housed in sterile, filter-top cages during the experiments, receiving dedicated care from the Center for Comparative Medicine at Massachusetts General Hospital to ensure a pathogen-free environment.

Skin grafting

Full-thickness skin patches (1 cm²) from BALB/c mice (H-2^d) were placed on the flank area of C57Bl/6 fully allogeneic recipients, as described elsewhere (Billingham and Medawar 1951). Skin grafts were considered rejected when 90% necrosis was observed.

Production of bone marrow-derived macrophages and macrophage training in vitro

Bone marrow cells from C57BL/6 were suspended into a double filtrated (0.22 μ m) AIM-V serum-free media supplemented with 20 ng/ml of M-CSF, seeded at a density of 2.5×10^6 cells/ml in a 6 well-plate tissue culture dishes and cultured for 7 days to obtain bone marrow-derived macrophages (BMDM). To induce macrophage training, BMDMs were cultured for 24 hours with 50 μ g/mL of β -glucan (Millipore Sigma), as previously described (Braza et al. 2018; Quintin et al. 2012).

Evaluation of trained macrophages

Cytokine release. Following a resting period of 5 days, trained macrophages were stimulated with 50 ng/mL of LPS (Sigma) for 24 hours. The supernatants were collected and the levels of inflammatory cytokines TNF α and IL-6 using an ELISA kit (R&D systems). **Glucose metabolism.** Culture supernatants from trained macrophages were collected and centrifuged at 2000xg for 15 minutes at +4°C and then stored at -80°C. BMDM glucose metabolism was assessed by measuring the concentration of lactate present in the supernatant using the Lactate Assay Kit (#MAK064-1KT, Millipore Sigma), following the manufacturer's instructions. **Epigenetic modifications.** BMDM were washed with PBS (#25-507, GenClone) and fixed in 4% paraformaldehyde (SC281692, Santa Cruz Biotechnology) for 10 minutes, then permeabilized with 0.1% Triton X-100 (#X100, Millipore Sigma) for 10 minutes. Cells were then incubated for 1 hour in a blocking buffer containing 0.1% Tween20 (#P1379, Sigma-Aldrich) and 1% BSA (#A7906, Millipore Sigma). Subsequently, cells were incubated with an Anti-Histone

H3K4Me3 AF 488 (ab237340, Abcam), Anti-Histone H3K27Ac AF 488 (ab245911, Abcam), or Rabbit IgG Isotype Control AF 488 (53-4616-82, ThermoFisher) for 3 to 4 hours, as previously described (Lungu et al. 2017). Finally, cells were stained with DAPI (#D1306, ThermoFisher) for 3 minutes. Images were acquired using a Keyence BZ-X810 fluorescence microscope and analyzed with ImageJ software (NIH).

Induction of trained immunity in mice

To induce trained immunity in vivo, mice were injected intraperitoneally (i.p.) with 1 mg of β -glucan on day 0 and day 3, as reported elsewhere (Netea et al. 2020).

Results

P2X7R inhibition prevents epigenetic reprogramming of macrophages induced by β -glucan.

We investigated whether inhibition of P2X7R signaling could impact epigenetic modifications induced in macrophages treated in vitro with β -glucan. Indeed, dectin-1 stimulation by PAMPs is among the most extensively studied and well characterized pathways that induce trained immunity. β -glucans are polysaccharides found in fungal cell walls, which are recognized as PAMPs by macrophages via dectin-1, a C-type transmembrane lectin receptor (Brown et al. 2003). Macrophage activated by β -glucan undergo epigenetic changes characterized by mono- and tri-methylation of histone H3 (H3K4me1 and H3K4me3) as well acetylation of H3 (H3K27Ac) (van der Heijden et al. 2018). These epigenetic changes lead to the generation of trained macrophages

exhibiting enhanced inflammatory responses upon challenge with the same or other DAMPs.

Here, to induce trained immunity, bone marrow derived macrophages (BMDM) were prepared from the bone marrow of C57/BL6 (B6) mice and cultured for 24 hours with β -glucan (50 ug/ml), as previously shown to achieve macrophage training (Braza et al. 2018; Stothers et al. 2021). To evaluate the effect of P2X7R inhibition on trained immunity, BMDM were cultured with A438079, a specific and non-competitive inhibitor of P2X7R signaling (Allsopp et al. 2018) for 12 hours prior to β -glucan stimulation. Next, macrophages were washed and rested for 5 days and the presence of epigenetic modifications were investigated (Figure 1A). To detect K4 tri-methylation and K27 acetylation of Histone 3, macrophages were permeabilized and incubated with fluorescent anti-H3K4Me3 alexa-Fluor (AF) 488 (ab237340) and Anti-H3K27Ac AF 488 (ab245911) antibodies, respectively, or Rabbit IgG isotype control AF 488 (ab53-4616-82) for 3 to 4 hours, as previously described (Lungu et al. 2017; Moshareva, Lukyanov, and Putlyaeva 2022; Stepanov et al. 2022). Binding of the antibodies was detected via fluorescence microscopy. As shown in Figure 1, in the absence of β -glucan no methylated or acetylated histones were detected in macrophages. In contrast, upon exposure to β -glucan, histone H3 methylation and acetylation were detected. Most important, preincubation of macrophages with A438079 prevented completely the induction of epigenetics modifications by β -glucan in macrophages (Figure 1). It is important to note that BMDM exposed in vitro to A438079 for the whole duration of the assays displayed > 98% viability (data not shown).

P2X7R inhibition prevents metabolic reprogramming of macrophages induced by β -glucan.

Metabolic rewiring of macrophages, including changes in glucose metabolism and anabolic reassigning of the Krebs cycle to cholesterol and phospholipid synthesis, represents a key feature of trained immunity (Arts, Joosten, and Netea 2016). Indeed, β -glucan induces a switch from oxidative phosphorylation towards aerobic glycolysis (Arts, Joosten, and Netea 2016; Netea et al. 2020). This process occurring through the AKT–mTOR–HIF1 α pathway is associated with reduced basal respiration rate, increased glucose consumption and higher lactate production (Cheng et al. 2014). Metabolic reprogramming of macrophages plays a central role in their activation and pro-inflammatory cytokine secretion (Netea et al. 2020). Based on these principles, we next investigated whether P2X7R inhibition would influence metabolic changes associated with macrophage training in vitro. To test this, BMDM were stimulated with β -glucan for 24 hours. The cells were then washed and rested for 5 days prior to challenge with LPS (50 ng/ml for 24 hours) (Figure 2A). The cell supernatants were then collected, and the presence of lactate was evaluated using a colorimetric assay (Figure 2). To investigate the influence of P2X7R inhibition on glucose metabolism, BMDM were cultured with A438079 at doses ranging from 100 to 300 μ M for 12 hours prior to exposure to β -glucan (Figure 2A). As shown in Figure 2, trained macrophages secreted significant amounts of lactate as compared to cells that had not been exposed to β -glucan. Strikingly, pre-exposure of BMDM to A438079 P2X7R inhibitor completely prevented the onset of these metabolic changes in BMDM (Figure 2B).

P2X7R inhibition prevents the enhanced ability to secrete inflammatory cytokines by macrophages treated with β -glucan.

Both epigenetic and metabolic changes associated with trained immunity result in an enhanced ability of macrophages to secrete pro-inflammatory cytokines, including IL-6 and TNF α upon restimulation (Drummer et al. 2021; Ochando et al. 2023). Enhanced ability to secrete inflammatory cytokines represents actually the hallmark of trained immunity. Here, we tested whether P2X7R inhibition prevented this process. First, BMDM underwent β -glucan-mediated training, as described earlier. The cells were washed and rested for 5 days and then challenged with LPS (50 ng/ml) for 24 hours (Figure 3A). Finally, the presence of IL-6 and TNF α was tested by ELISA, as described in the Methods section. As shown in Figure 3, BMDM cultured with medium, only, and trained BMDM that had not been challenged with LPS did not release any cytokine. As expected, and previously reported (Braza et al. 2018), trained BMDM stimulated with LPS produced significantly higher cytokine amounts than their untrained counterparts ($P < 0.0005$) (Figure 3B-C). Most importantly, pre-exposure of BMDM with A478079 P2X7R inhibitors prevented the effect of training on cytokine release in a dose dependent fashion (Figure 3B-C). Along with the results obtained with epigenetic and metabolic evaluations, we conclude that inhibition of P2X7R signaling prevents trained immunity by macrophages treated with β -glucan.

P2X7R inhibition dampens inflammatory cytokine secretion by already trained macrophages.

In another set of experiments, we investigated the influence of P2X7R on the activation by LPS of already trained BMDM. BMDM were prepared from B6 mice and trained with β -glucan, as described earlier. After the 5-day resting period, trained macrophages were challenged with LPS with or without A438079 P2X7R inhibitor (Figure 4A). The concentration of IL-6 and TNF α was measured in culture supernatants by ELISA. As previously observed, trained macrophages produced higher levels of both cytokines as compared to their non-trained counterparts ($p < 0.0005$) (Figures 3 and 4). The addition of A438079 inhibitor reduced cytokine release in a dose-dependent fashion (Figure 4B-C). It is, however, important to note that P2X7R inhibition did not abolish cytokine secretion but it reduced its level to that observed with untrained macrophages.

P2X7R inhibition reduces the effect of trained immunity in skin-grafted mice.

Based upon our results showing the effects of P2X7R inhibition on macrophage training in vitro, we established a model to evaluate the role of P2X7R signaling on trained immunity induced in vivo in mice. First, we investigated the impact of trained immunity on the rejection of an allograft in mice. B6 (H-2^b) mice received a skin transplant from a fully allogeneic BALB/c (H-2^d) mouse. In addition, these mice were injected twice intraperitoneally with β -glucan (1 mg) or saline on day -7 and day -3 relative to the transplant. Transplanted mice injected with saline rejected their allograft within 10-12 days, as previously reported (Billingham and Medawar 1951; Pilat et al. 2019). On the other hand, in mice that had received β -glucan, skin allografts survived only 5 days. To our knowledge, this is the first demonstration that induction of trained immunity results in accelerated allograft rejection. Most importantly, in transplanted mice that had received

both β -glucan and A438079 agent, skin allografts survived for 10 days i.e., for the same duration as naive mice. Therefore, P2X7R inhibition obliterated the deleterious effects of trained immunity on allograft survival in transplanted mice.

Discussion

There is now ample evidence demonstrating that virtually all innate cells of the immune system are capable of bona fide memory functions (Sherwood et al. 2022). This has been demonstrated in the case of NK cells activated in the absence of self-MHC class I recognition (missing self) as well as monocytes, which can recognize specifically some alloantigens via PIR receptors (Dai et al. 2020). In addition, several studies have documented the ability of monocytes/macrophages to secrete higher cytokine levels upon challenge with the same or another stimulus, including but not restricted to DAMPS and PAMPs (Netea et al. 2020). This phenomenon referred to as *trained immunity* relies on epigenetic and metabolic reprogramming of macrophages (Fanucchi et al. 2021). Trained immunity of pro-inflammatory macrophages can last for months and provides enhanced protection toward microbes, viruses, and tumors (Kleinnijenhuis, Quintin, Preijers, Benn, et al. 2014; Yao et al. 2018; Machiels et al. 2017; Quintin et al. 2012; Saeed et al. 2014; Cirovic et al. 2020; Ding et al. 2023).

It has been previously suggested that the activation of NLRP3 inflammasome is involved in trained immunity (Ochando et al. 2023; Christ et al. 2018). Indeed, NLRP3 activation driven by ATP-mediated signaling of P2X7 receptors by extracellular ATP plays an essential role in the process leading to the production of pro-inflammatory cytokines by

activated macrophages (Wewers and Sarkar 2009; Hanley et al. 2004; Barbera-Cremades et al. 2017). However, the relationships between P2X7R signaling and trained immunity have never been formally investigated. The first set of experiments presented in this paper addressed this question. We observed that P2X7R inhibition prevented both the onset of metabolic and epigenetic changes of macrophages stimulated in vitro with β -glucan, a known inducer of trained immunity. This finding was further confirmed by the observation that macrophages co-treated with P2X7R inhibitor and β -glucan lacked their ability to secrete enhanced levels of IL-6 and TNF α inflammatory cytokines. The mechanisms by which inhibition of P2X7R signaling prevented macrophage training by β -glucan are unclear. It is known that activation of the β -glucan/Dectin-1 axis induces an intracellular calcium influx (Goodridge, Simmons, and Underhill 2007; Elloumi et al. 2012; Xu et al. 2009). This influx, in turn, triggers the translocation of the nuclear factor of activated T cells (NF-AT) into the nucleus, where it binds to DNA and initiates gene transcription (Elloumi et al. 2012). This process is associated with the deposition of epigenetic marks, specifically H3K27ac and H3K4me3, on the promoters of pro-inflammatory genes that are being expressed during trained immunity (Akimzhanov et al. 2008; Quintin et al. 2012; Novakovic et al. 2016). These epigenetic modifications play a pivotal role in long-term unfolding of chromatin structure thus enhancing the transcriptional accessibility of pro-inflammatory genes (Netea et al. 2020). At the same time, P2X7R stimulation plays a key role in intracellular increase of calcium ions (Liang et al. 2015). Based upon these principles, it is plausible that inhibition of P2X7R may impact trained immunity by limiting translocation of NF-AT and subsequent epigenetic changes. In addition, it is possible that ATP is an essential element of the process

associated with trained immunity. Indeed, increased glucose metabolism is likely to result in ATP production, which in turn activates P2X7R signaling, a process required for trained immunity (Sheedy and Divangahi 2021).

The second part of our study focused on the relationships between trained immunity, P2X7R signaling and allograft rejection. While training of pro-inflammatory macrophages is clearly beneficial via boosting protective immunity against microbes and tumors, it is plausible that it could be detrimental in transplantation. This view is supported by a previous report by Ochando's group showing that in vivo modulation of trained immunity with engineered nanobiologic containing mTOR inhibitors promoted allograft acceptance of cardiac allografts in mice (Braza et al. 2018). Here, we showed that mice with trained immunity reject skin allografts in an accelerated fashion. To our knowledge, this is the first report providing direct evidence that trained immunity can impact allograft rejection. We surmise that the pre-existence of trained immunity in transplant recipients might accelerate and amplify graft infiltration by host's macrophages and dendritic cells with enhanced ability to produce inflammatory cytokines. Such potent and rapid innate inflammatory response may enhance subsequent adaptive alloresponses by T and B cells thus accelerating allograft rejection. Further experiments will be required to test these hypotheses. Most importantly, our study showed that the deleterious effect of trained immunity on allograft rejection could be obliterated via administration of recipients with a P2X7R inhibitor given at the time of transplantation. These in vivo observations mirror our findings made in vitro showing that P2X7R inhibition can prevent or suppress trained immunity.

In summary our study showed the presence of trained immunity in transplant recipients can accelerate the onset of acute allograft rejection. This implies that transplant patients with trained immunity due to recent episodes of infections or vaccinations should be considered high risk with regards to early acute rejection. Our study also shows that P2X7R activation is an essential element of the induction and perpetuation of trained immunity. This suggests that short-term treatment with drugs capable of suppressing trained immunity, including P2X7R inhibitors, may be a promising therapy for patients displaying trained immunity prior to transplantation.

Acknowledgments

This work was supported by a grant from the NIH to Gilles Benichou, NIH R01DK115618.

Disclosure

The authors have no conflicts of interest to disclose as described by the American Journal of Transplantation

Data availability statement

The data that support the findings of this study are available from the corresponding author, [GB], upon request.

Figures

Figure 1

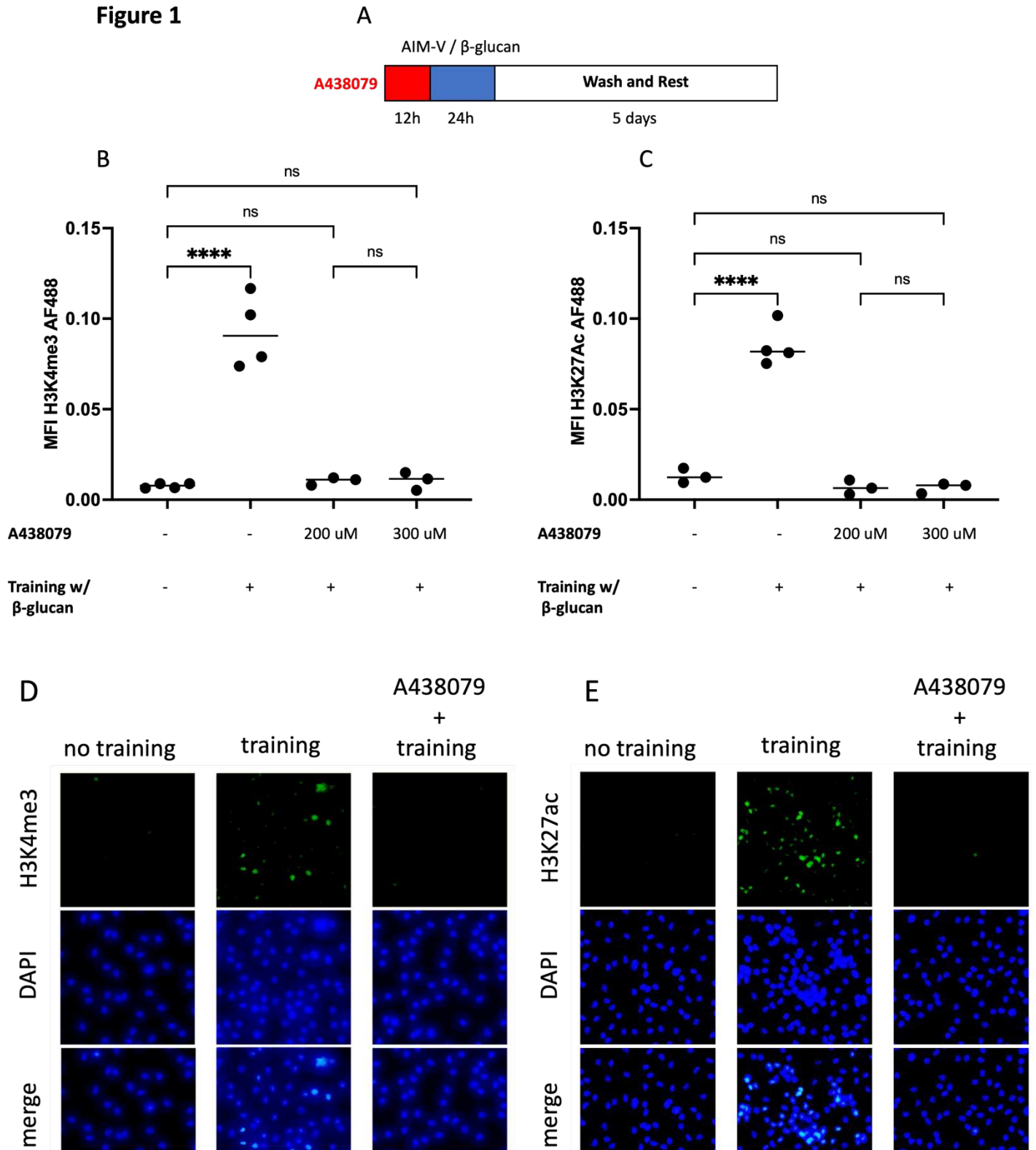


Figure 1. P2X7R inhibition prevents epigenetic reprogramming of macrophages induced by β -glucan.

(A) Illustration of the in vitro training model. Median Fluorescence Intensity of (B) H3K4me3 and (C) H3K27ac of β -glucan trained BMDM previously treated with A438079 (the results are representative of 3 independent experiments, ANOVA, **** $p < 0.0001$). (D-E) Immunofluorescence study showing (D) H3K4me3 and (E) H3K27ac on trained macrophages.

Figure 2

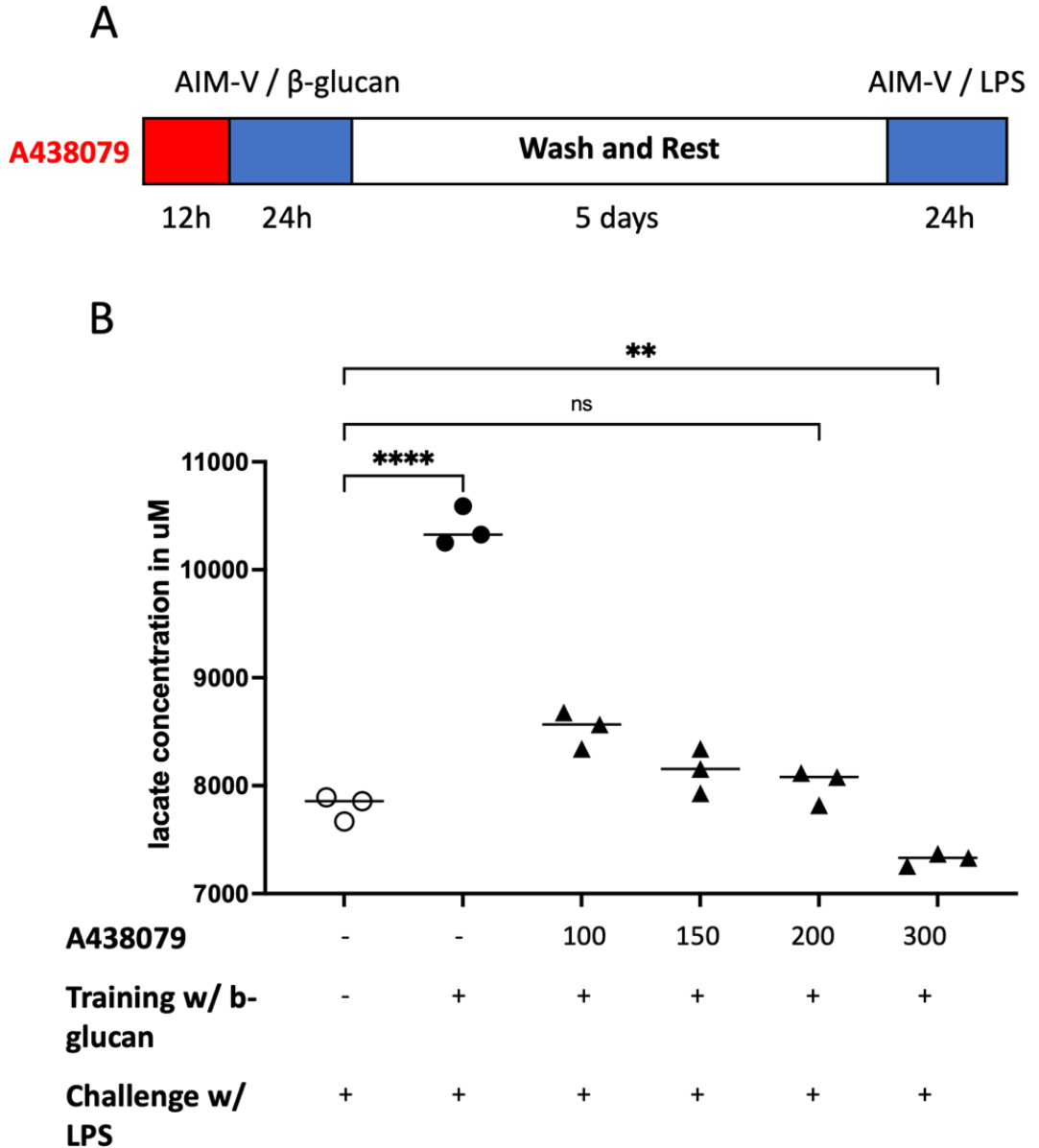
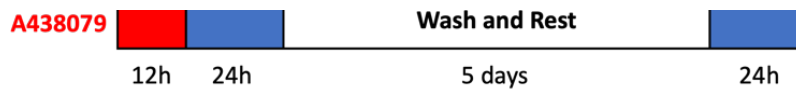


Figure 2. P2X7R inhibition prevents metabolic reprogramming of macrophages induced by β -glucan.

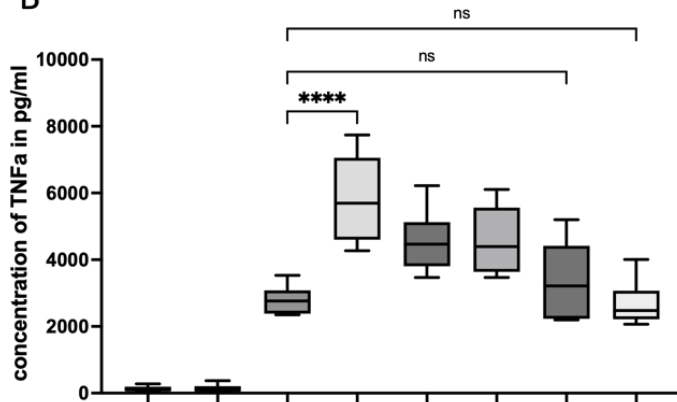
(A) Illustration of the in vitro training model. BMDM were pre-treated with A438079 and trained with β -glucan then rested for 5 days before being stimulated with LPS. Lactate concentration was evaluated in the supernatant (B) (results are representative of 3 independent experiments, ANOVA, **** $p < 0.0001$, ** $p < 0.005$).

Figure 3

A

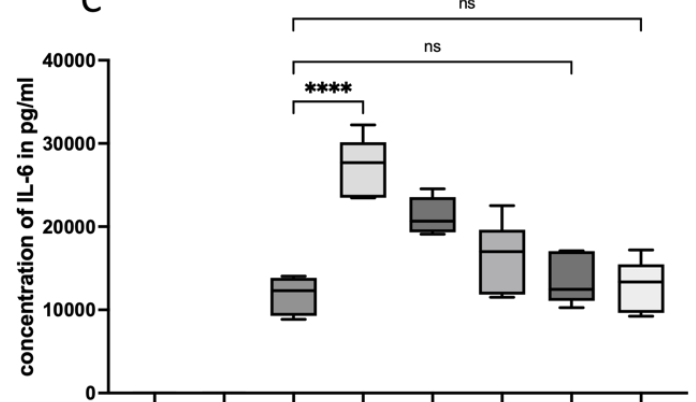


B



A438079	-	-	-	-	100	150	200	300
Training w/ b-glucan	-	+	-	+	+	+	+	+
Challenge w/ LPS	-	-	+	+	+	+	+	+

C



A438079	-	-	-	-	100	150	200	300
Training w/ b-glucan	-	+	-	+	+	+	+	+
Challenge w/ LPS	-	-	+	+	+	+	+	+

Figure 3. P2X7R inhibition prevents the enhanced ability to secrete inflammatory cytokines by macrophages treated with β -glucan.

(A) Illustration of the in vitro training model. (B-C) Inflammatory cytokines, TNF α (B), and IL-6 (C), released by BMDM pre-treated with A438079 and trained with β -glucan. Cytokine levels were evaluated in the supernatant after stimulation with LPS (the results are representative of 3 independent experiments, ANOVA, ****p<0.0001).

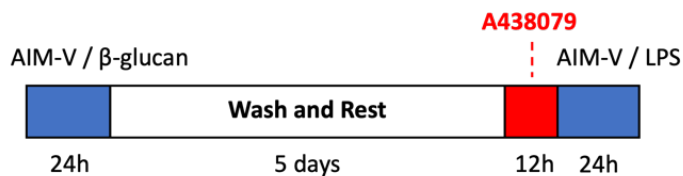
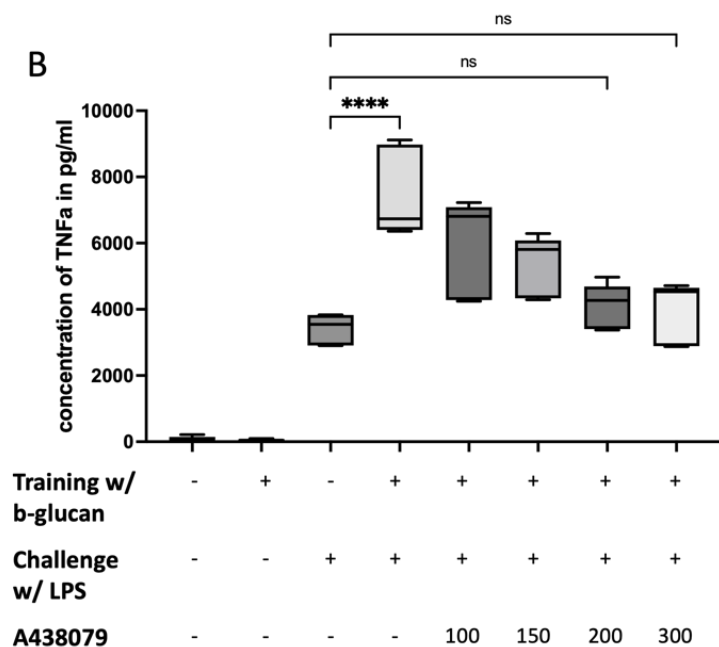
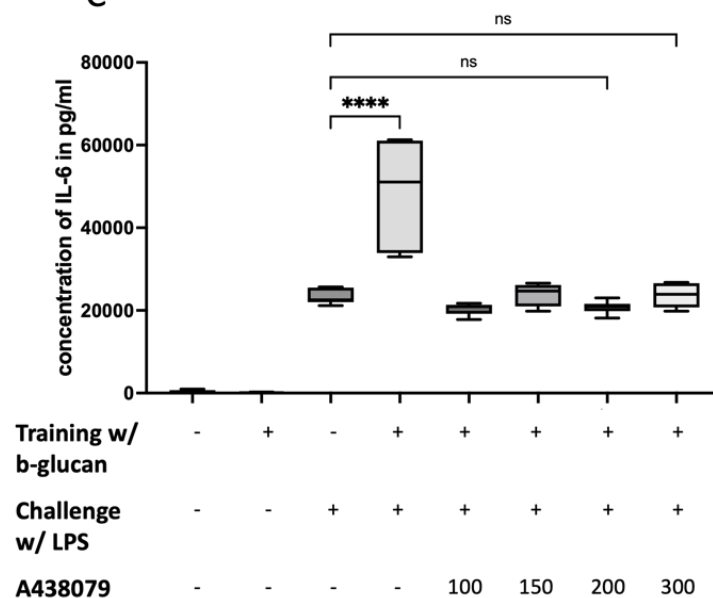
Figure 4**A****B****C**

Figure 4. P2X7R inhibition dampens inflammatory cytokine secretion by already trained macrophages.

(A) Illustration of the in vitro training model. (B-C) Inflammatory cytokines, TNF α (B), and IL-6 (C), released by BMDM trained with β -glucan and then treated with A438079. Cytokines were evaluated in the supernatant after stimulation with LPS (the results are representative of 3 independent experiments, ANOVA, ****p < 0.0001).

Figure 5

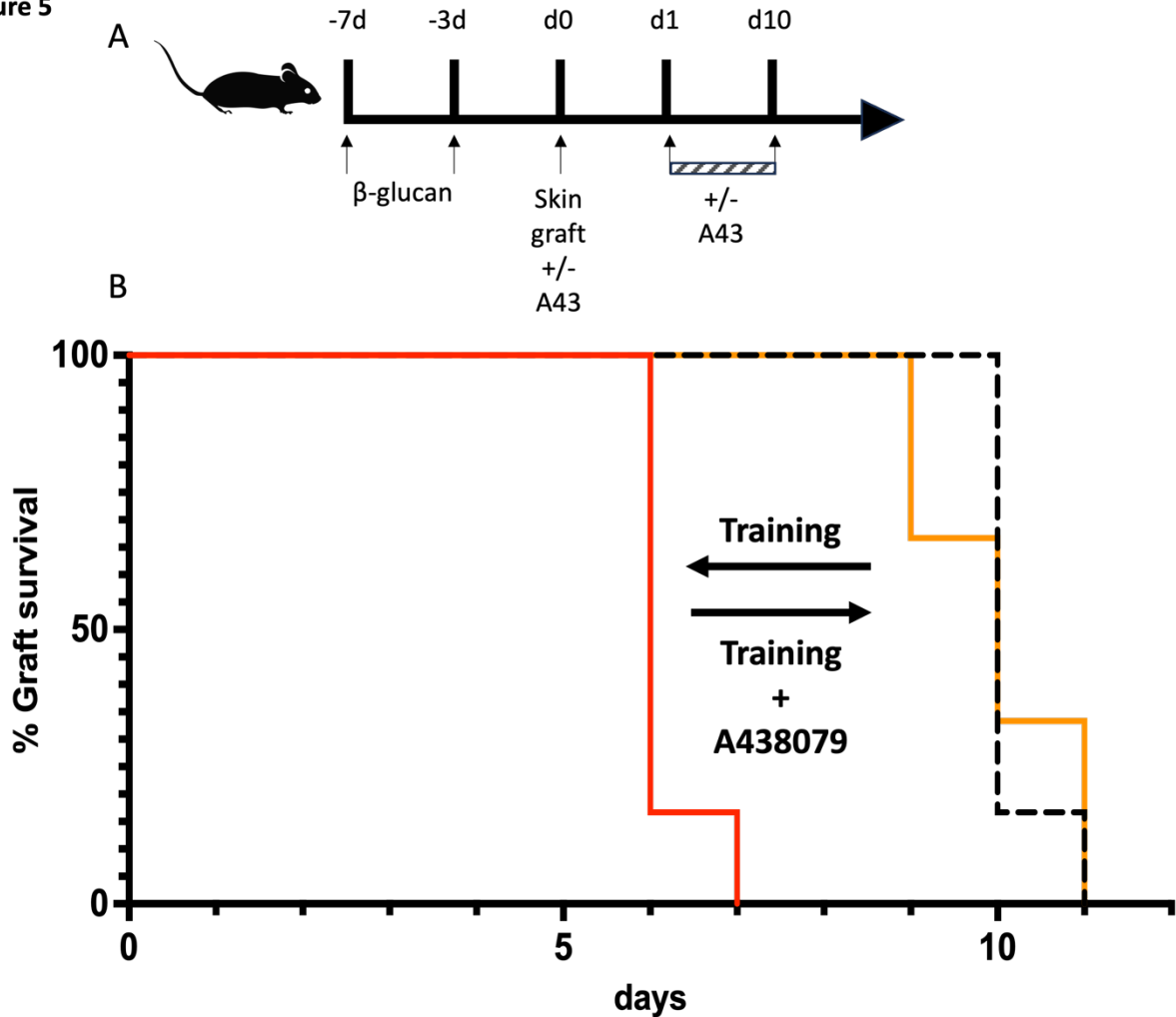


Figure 5: P2X7R inhibition abrogates the effect of trained immunity on skin allograft survival in mice.

(A) C57/BL6 mice were treated with β -glucan seven days prior to receiving a BALB/c fully allogeneic skin graft. Some mice were treated intraperitoneally with A438079. (B) Graft survival of mice treated or not with β -glucan and A438079. Dashed line: control untreated mice (n=6). Red line: mice injected with β -glucan (n=6). Orange line mice with β -glucan and A438079 P2X7R inhibitor (n=6).

Conclusion

This study demonstrates, for the first time, that P2X7R signaling controls the release of extracellular vesicles (EVs) by donor cells following transplantation. Inhibition of P2X7R signaling is linked to the absence of recipient antigen-presenting cells (APCs) cross-dressed with donor major histocompatibility complex (MHC) molecules in the spleen of mice transplanted with a skin allograft. This finding aligns with a recent study by Barrera-Avalos et al., which highlights the essential role of P2X7Rs in the cross-dressing of dendritic cells (Barrera-Avalos et al. 2021).

Given the absence of detected donor passenger leukocytes in the spleen of skin-grafted mice, it is likely that the deficiency in cross-dressed cells results from the abolished release of donor EVs post-transplantation. Consequently, this significantly extends the allograft survival (mean survival time: 19 days vs. 9 days, $p = 0.0016$). This outcome aligns with observations reported in a prior study by Amores-Iniesta et al., which demonstrated reduced T cell infiltration, diminished necrosis, and preserved epidermal and dermal structure at day 7 post-transplantation in skin allografts on mice treated with A438079 (100 $\mu\text{mol/kg/day}$) (Amores-Iniesta et al. 2017). In those mice, a profound suppression of the T cell alloresponse at days 3 and 7 post-transplantation and a significant, albeit modest, extension of skin graft survival (12 days vs. 8 days, $p < 0.01$) were noted (Amores-Iniesta et al. 2017).

Despite the absence of cross-dressed cells in the secondary lymphoid organs of skin-grafted mice, the rejection of skin grafts still occurred. It is plausible that, upon revascularization of the skin graft, some T cells infiltrate the allograft and become

activated in situ by recipient APCs cross-dressed within the graft itself. Supporting this hypothesis, our laboratory observed a substantial number of recipient APCs cross-dressed with donor MHC class I in the transplanted skin early after transplantation (data not shown). This observation is consistent with studies from the Lakkis' lab, which also reported a high number of recipient cells cross-dressed with donor MHC molecules in kidney and islet allografts (Hughes et al. 2020). It is conceivable that once cross-dressed with donor MHC class I proteins, these recipient cells, including vascular endothelial cells, may become targets for destruction by alloreactive CD8⁺ cytotoxic T cells, thereby contributing to local inflammation and acute rejection. Further studies are required to address this question.

Moreover, this study uncovers that inhibiting P2X7R not only prevents the initiation of metabolic and epigenetic changes in macrophages stimulated in vitro with β -glucan, a recognized inducer of trained immunity, but also confirms that macrophages co-treated with a P2X7R inhibitor and β -glucan lose their capacity to secrete elevated levels of inflammatory cytokines IL-6 and TNF α . The mechanisms through which P2X7R signaling inhibition hampers the training of macrophages by β -glucan remain unclear. Activation of the β -glucan/Dectin-1 axis is known to induce intracellular calcium influx (Goodridge, Simmons, and Underhill 2007) (Elloumi et al. 2012) (Xu et al. 2009). This influx triggers the translocation of the nuclear factor of activated T cells (NF-AT) into the nucleus, where it binds to DNA and initiates gene transcription (Elloumi et al. 2012). This process is linked to the deposition of epigenetic marks, specifically H3K27ac and H3K4me3, on the promoters of pro-inflammatory genes expressed during trained immunity (Akimzhanov et al. 2008) (Quintin et al. 2012) (Novakovic et al. 2016). These epigenetic modifications play

a crucial role in the long-term unfolding of chromatin structure, enhancing the transcriptional accessibility of pro-inflammatory genes (Netea et al. 2020). Simultaneously, P2X7R stimulation plays a key role in the intracellular increase of calcium ions (Liang et al. 2015). Given these principles, it is plausible that inhibiting P2X7R may impact trained immunity by limiting NF-AT translocation and subsequent epigenetic changes. Additionally, ATP may be an essential element in the process associated with trained immunity. Increased glucose metabolism likely leads to ATP production, activating P2X7R signaling, a process required for trained immunity (Sheedy and Divangahi 2021). This study also elucidates the connections between trained immunity, P2X7R signaling, and allograft rejection. Mice with trained immunity reject skin allografts at an accelerated rate. To our knowledge, this is the first report offering direct evidence that trained immunity can impact allograft rejection. We hypothesize that the pre-existing trained immunity in transplant recipients might expedite and intensify graft infiltration by the host's macrophages and dendritic cells, enhancing their ability to produce inflammatory cytokines. This potent and rapid innate inflammatory response may, in turn, boost subsequent adaptive alloresponses by T and B cells, thereby hastening allograft rejection. Further experiments will be necessary to test these hypotheses. Crucially, our study demonstrates that the detrimental effect of trained immunity on allograft rejection can be abolished by administering recipients with a P2X7R inhibitor at the time of transplantation. Finally, the combination of P2X7R inhibitors with other treatments, such as T cell costimulation blockade, could potentially prevent rejection or promote the induction of tolerance in allografts.

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Appendix

Pathways of Antigen Recognition by T Cells in Allograft Rejection

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Abstract. The adaptive immune response leading to the rejection of allogeneic transplants is initiated and orchestrated by recipient T cells recognizing donor antigens. T-cell allorecognition is mediated via 3 distinct mechanisms: the *direct pathway* in which T cells recognize allogeneic major histocompatibility complex (MHC) molecules on donor cells, the *indirect pathway* through which T cells interact with donor peptides bound with self-MHC molecules on recipient antigen-presenting cells, and the recently described *semidirect pathway* whereby T cells recognize donor MHC proteins on recipient antigen-presenting cells. In this article, we present a description of each of these allorecognition pathways and discuss their role in acute and chronic rejection of allogeneic transplants.

(*Transplantation* 2022;00: 00–00).

INTRODUCTION

Recognition of donor antigens by T lymphocytes initiates the adaptive immune response leading to rejection of allogeneic transplants. Understanding how T cells recognize alloantigens can bring new insights into the principles governing self versus non-self-discrimination by the immune system. Most importantly, elucidating the mechanisms involved in T-cell allorecognition is essential for understanding how allografts are rejected and subsequently designing selective immune therapies in transplantation. Initial immunogenetics studies in transplanted rodents and patients led to identification of highly polymorphic proteins encoded within the major histocompatibility complex (MHC) locus that serve as antigens in allorecognition.^{1,2} T cells can recognize either intact or processed forms of allogeneic MHC proteins through their T-cell receptors (TCRs) via 3 distinct pathways called *direct*, *indirect*, and *semidirect pathways* (Figure 1). In the direct pathway, T cells recognize intact allogeneic MHC proteins displayed on donor cells.^{3–5} Alternatively, the host's T cells can

become activated after recognition of recipient MHC molecules bound to donor peptides processed and presented by recipient antigen-presenting cells (APCs; indirect pathway).^{6–9} Finally, a third pathway was recently described in which recipient APCs acquire and display on their surface donor MHC proteins and thereby activate alloreactive T cells (semidirect pathway).^{10–13} In addition to MHC proteins, T cells can become activated after recognition of minor histocompatibility antigens (mHAs), which are peptides usually derived from housekeeping proteins displaying some degree of polymorphism.¹⁴ These mHA peptides can be presented to T cells in the groove of donor MHC molecules on donor APCs (direct pathway) or self-MHC molecules on recipient APCs (indirect pathway). The contribution of each of the aforementioned T-cell allorecognition pathways and alloantigens (MHC and mHAs) in acute and chronic allograft rejection depends on many factors both intrinsic and extrinsic to the transplant. This article provides an overview of some key findings regarding this question.

DESCRIPTION OF THE PATHWAYS OF T-CELL ALLORECOGNITION

T-cell Recognition of MHC Antigens

Direct Allorecognition

Placement of allogeneic tissues and organs triggers a potent inflammatory immune response leading to rapid destruction of the graft called acute rejection. Although innate immunity is an essential trigger in this process, activation of host's alloreactive T cells is necessary to mediate acute allograft rejection. Indeed, mice devoid of T cells or treated with drugs deleting or suppressing their T cells do not reject allografts in an acute manner. Original studies using skin allografts placed on mice established that after transplantation, intragraft donor bone marrow-derived MHC class II⁺ cells, traditionally called

Received 21 July 2022. Revision received 15 September 2022.

Accepted 29 September 2022.

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This study was supported by a grant from the National Institute of Health (NIH/NIDDK R01DK115618).

The authors declare no conflicts of interest.

All the authors have participated in the writing of the article.

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ISSN: 0041-1337/20/0000-00

DOI: 10.1097/TP.0000000000004420

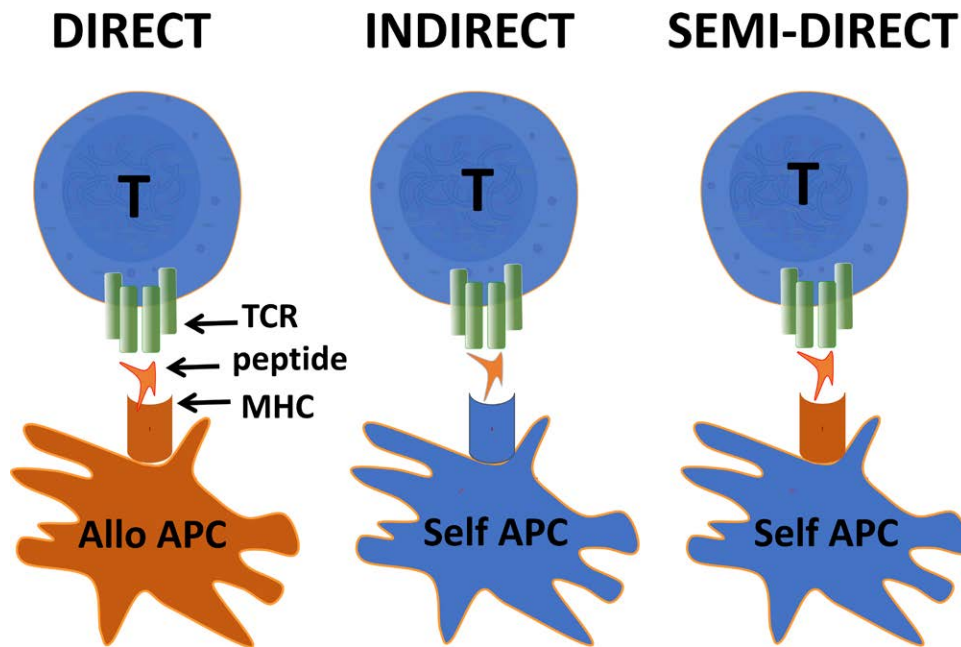


FIGURE 1. Pathways of allorecognition by T cells. T cells can interact with alloantigens via 3 distinct pathways: the direct pathway in which T cells recognize intact allogeneic MHC molecules displayed on donor APCs, the indirect pathway in which T cells recognize self-MHC molecules bound with donor peptides processed and presented by self-APCs, and the semidirect pathway in which T cells recognize intact donor MHC proteins displayed on recipient APCs (MHC cross-dressing). APC, antigen-presenting cell; MHC, major histocompatibility complex; TCR, T-cell receptor.

passenger leukocytes (essentially dendritic cells), migrate to the recipient's draining lymph nodes where they present donor MHC antigens to T cells.^{15,16} T-cell recognition of intact allogeneic MHC proteins on donor cells or *direct allorecognition* elicits a polyclonal inflammatory response engaging 1% to 10% of the T-cell repertoire.^{4,17-24} Because alloreactive T cells do not represent a distinct pool of T lymphocytes, T-cell allorecognition seems to violate the rules of self-MHC restriction.^{4,25} In addition, it is puzzling that so many T cells become stimulated when exposed to allogeneic MHC molecules compared with other protein antigens. Two mechanisms have been proposed to explain how and why so many T cells are activated through direct allorecognition: the *high determinant density* and the *multiple binary complex* models.^{24,26,27} The high determinant density model postulates that T cells recognize motifs present on allogeneic MHC molecules independently of the peptides bound to them.²⁸ In this scenario, the multiplicity of TCR-allo-MHC interactions leads to the activation of many T-cell clones including those whose TCRs display a low affinity for their cognate ligands (cross-reactivity). In the multiple binary complex model, individual alloreactive T-cell clones react with allogeneic MHC molecules associated with defined peptides.²⁴ This model implies that alloreactive T cells recognize complexes formed by allogeneic MHC molecules bound to endogenous peptides that mimic a variety of self-MHC + foreign peptide complexes, including microbial peptides²⁹⁻³² (molecular mimicry). In the latter scenario, the strength and polyclonality of the alloresponse observed in transplant recipients and in vitro mixed lymphocyte reactions may be explained by the reactivation of already antigen-experienced memory T cells. Altogether, these observations explain why some alloreactive T-cell clones are peptide specific, whereas others are not.^{33,34} The preeminence of mimicry over cross-reactivity

as a driving force behind direct allorecognition by T cells might depend on the degree of plasticity and promiscuity in TCR recognition of peptides and polymorphic residues on self- and allogeneic MHC molecules.^{35,36} For instance, it is remarkable that 3 mutations in the groove of MHC class II molecules A^b in C57Bl/6 bm12 (bm12) mutant mice is sufficient to elicit a polyclonal direct alloreactive CD4⁺ T-cell response leading to acute rejection of bm12 skin grafts by wild-type C57Bl/6 mice. In contrast, xenogeneic MHC molecules, which are highly different from self-MHC molecules, induce relatively poor direct responses by T cells.^{37,38} Therefore, as described by Archbold et al, T-cell allorecognition may be a combination of “mistaken identity” and “déjà vu.”²⁵

Semidirect Allorecognition

The passenger leukocyte theory originally proposed by Snell in 1957, and later supported by studies in skin-grafted mice, was based on the observation that depletion or replacement of graft leukocytes significantly delayed rejection.^{15,16,39-42} Supporting this view, Billingham and Silvers⁴³ showed that skin allografts undergo long-term survival when placed in vascularized but alymphatic sites (immune-privileged) such as the brain or cheek pouch. Moreover, Barker and Billingham⁴⁴ and Tilney and Gowans⁴⁵ documented that skin allografts placed on a piece of recipient's skin, which had been lifted while still connected to the recipient through blood vessels (pediculated skin flap), underwent longer survival. A more recent study by Celli et al⁴⁶ using 2-photon microscopy revealed that donor dermal dendritic cells (DCs) were rapidly eliminated (presumably by natural killer cells) after ear skin transplantation. In this study, although a few donor DCs were transiently detected in the graft-draining lymph nodes, they lacked membrane dynamics and had the appearance

of dead cells or cell debris.⁴⁶ Therefore, the evidence that passenger leukocytes are the main driving force behind the priming of alloreactive T cells in the host's lymph nodes of skin-grafted mice was indirect and mostly circumstantial. It is noteworthy that, in skin-grafted mice, lymphatic vessels are fully reconnected only 5 to 7 d posttransplantation.^{47,48} Therefore, it seems doubtful that skin donor leukocytes traffic to host lymph nodes shortly after skin grafting. Because anti-donor T-cell responses are detectable in these lymph nodes as early as 2 d posttransplantation,⁴⁹ it is plausible that another mechanism of antigen presentation is involved in initiation of T-cell alloimmunity. A few years ago, our laboratory revisited this question using a technique called imaging flow cytometry, which combines visualization of single cells through digital microscopy and flow cytometry. Unexpectedly, we did not detect any donor cells in lymph nodes of skin-grafted mice.¹⁰ The absence of donor-derived MHC class I gene expression among lymph node cells of these mice confirmed the lack of passenger leukocytes.¹⁰ In contrast, as early as 12 h after skin grafting, imaging flow cytometry revealed the presence of high numbers of recipient cells coated with vesicles exhibiting donor MHC molecules in recipient lymph nodes.¹⁰ By day 7, most of these recipient cells expressed donor MHC I and II molecules on their membranes alongside their own MHC proteins. Likewise, Smyth et al⁵⁰ documented continuous acquisition of donor MHC molecules by recipient APCs after skin transplantation in mice and showed the contribution of this process to the generation of alloimmunity by CD8⁺ T cells. Furthermore, studies from our and Morelli's group showed large numbers of recipient APCs bound to donor extracellular vesicles (EVs) in the spleens of heart-transplanted mice.^{10,11} This phenomenon, referred to hereafter as *donor MHC cross-dressing*, has now been documented in laboratory rodents and patients transplanted with various allogeneic solid organs, as well as pancreatic islets and bone marrow cells.^{11,51-56} Therefore, donor MHC cross-dressing of recipient APCs represents a general phenomenon in allotransplantation. The precise nature of the EVs released by allografts is not entirely clear. Evaluation of their size (50–150 nm diameter) and expression of MHC class II and tetraspanins suggest that these EVs correspond to exosomes derived from multivesicular bodies formed in the endosomes.^{11,57} However, it cannot be ruled out that some other EVs, such as microvesicles (also called ectosomes), which are assembled at and released from the plasma membrane, and apoptotic cell-derived vesicles could also participate in donor MHC cross-dressing after transplantation.

Intercellular transfer of proteins and micro RNA is a physiological process occurring regularly through cell-cell contact (trocytosis) and via EVs that are secreted or exchanged via nanotubes.⁵⁸ Intercellular communication via this mechanism is believed to play a pivotal role in the initiation and regulation of immunity to microbes and tumors.⁵⁸ Transfer of proteins between cells of the immune system was documented 40 y ago by Hudson and Sprent⁵⁹ and Bona et al,⁶⁰ who showed the acquisition by T cells of surface immunoglobulin molecules from B cells and antigens from macrophages. Furthermore, Frelinger et al⁶¹ reported in 1974 the transfer of MHC molecules between hematopoietic cells. In fact, transfer of MHC-peptide complexes from dermal DCs to lymph node DCs

and subsequent presentation to T cells is the normal route of naive T-cell sensitization in lymph nodes after a skin infection.⁶² A first demonstration that APCs cross-dressed in vitro through cell-cell contact with allogeneic MHC antigens could stimulate alloreactive T cells was provided by Lechler's laboratory.⁶³ Subsequently, we showed that APCs having acquired allogeneic MHC molecules after exposure to exosomes, but not free allogeneic exosomes, could mediate a potent inflammatory alloresponse both in vitro and in vivo and cause second-set allograft rejection in mice.⁶⁴ This type of alloresponse is now termed *semidirect alloresponse*.

Indirect Allorecognition

Seminal studies by Golding and Singer demonstrated that allogeneic MHC class I antigens could be presented by self-MHC class I on APCs and thereby stimulate some CD8⁺ cytotoxic T cells in vitro, a phenomenon then referred to as cross-presentation.⁶⁵ The first evidence suggesting that presentation of alloantigens by recipient APCs can contribute to allograft rejection was provided in 1982 by Lechler and Batchelor.⁶⁶ It was observed that kidneys, which had been "parked" in a first host and thus presumably devoid of passenger leukocytes, could sensitize alloreactive T cells upon retransplantation in a second host.⁶⁶ Based upon the assumption that activation of donor-reactive T cells could not result from alloantigen presentation by MHC class II-negative nonprofessional APCs of the graft, alloantigen presentation by recipient APCs represented an alternative route for CD4⁺ T-cell allorecognition. Ten years later, direct demonstration of the relevance of indirect T helper cell allorecognition as a mechanism of T-cell activation to allogeneic MHC proteins was provided in 3 studies in rodents and humans.^{67,67} First, lymph node and spleen T cells of skin-grafted mice were shown to become activated by recipient APCs presenting donor MHC peptides bound to self-MHC molecules.⁶ Second, immunization of rats with peptides derived from allogeneic MHC class I alpha-helices accelerated the rejection of allogeneic kidneys.^{7,68} Finally, studies from Liu et al documented the ability of allogeneic HLA-DR peptides presented by autologous APCs to stimulate human CD4⁺ T cells.⁶⁷ Unlike direct alloimmunity, the indirect T-cell response followed the rules of immunodominance in that it was mediated by a limited set of T-cell clones expressing selected TCR genes^{69,70} and recognizing a few dominant determinants usually located on polymorphic regions of donor MHC proteins.⁷⁰ The mechanisms by which donor antigens are taken up and processed by recipient APCs after transplantation are still unclear. Acquisition of donor antigens by recipient APCs may occur in the graft itself⁴⁶ or in the host lymphoid organs through pinocytosis of shed donor proteins, transfer of donor antigens through cell-cell contact, uptake of EVs, or phagocytosis of dead donor cells and apoptotic bodies.⁷¹

It is important to note that indirect alloreactivity is not restricted to CD4⁺ T cells. Indeed, proteins derived from viruses and tumor cells are regularly processed and presented in the form of peptides by MHC class I molecules to CD8⁺ T cells, a phenomenon referred to as cross-priming.⁷²⁻⁷⁴ Actually, the first observations supporting the existence of cross-priming were published in 1977 by Matzinger and Bevan⁷⁵ in skin transplantation. Further

studies by Ivaniy's group documented that a large fraction of alloreactive cytotoxic T lymphocyte (CTL) against class I HLA alloantigen generated *in vitro* during mixed lymphocyte reactions were self-class I HLA-restricted.⁷⁶⁻⁷⁸ The *in vivo* presentation of peptides derived from donor MHC class I molecules to alloreactive CD8⁺ T cells and its involvement in cytotoxic T-cell responses were first documented by Popov et al⁷⁹ in mouse recipients of MHC class I-mismatched skin allografts. Subsequent studies by Valujskikh et al,⁸⁰ showed that indirectly primed CD8⁺ T cells are a prominent component of the T-cell alloresponse in skin graft rejection.

T-cell Recognition of mHAs

It is firmly established that a variety of proteins called mHAs⁸¹ can trigger alloreactive T-cell responses after transplantation.⁸²⁻⁸⁴ Three distinct types of mHAs antigens have been described: mHAs derived from allelic variants of polymorphic proteins, proteins encoded by genes located on sex chromosome Y (in sex-mismatched transplantation), and proteins that are absent in the recipient but not the donor.⁸⁵ It is well known that a variety of peptides derived from mHAs proteins⁸¹ can serve as a source of determinants for recognition by alloreactive T cells after transplantation.⁸²⁻⁸⁴ Indeed, these proteins represent a large source of antigens for endogenous and exogenous processing by donor and recipient APCs, respectively, and subsequent direct/indirect presentation to recipient CD4⁺ and CD8⁺ T cells.⁸⁶⁻⁸⁸ We will see later in this article that the T-cell response to mHAs can play a key role in the rejection of certain transplants.

Allorecognition Pathways Involved in Acute Allograft Rejection

Role of Direct and Semidirect T-cell Allorecognition in Acute Rejection

Initiation of acute allograft rejection has been originally attributed to direct allorecognition by CD4⁺ T cells of donor MHC class II molecules displayed on passenger leukocytes in the host's lymph nodes. Directly activated alloreactive CD4⁺ T cells are considered to provide help for the differentiation of CD8⁺ cytotoxic T cells, which actively kill donor cells and cause graft dysfunction and failure. This model was originally based on the observation that injection of donor DCs to recipients of kidneys, which had been "parked" in a first host and thus presumably devoid of passenger leukocytes, restores rapid rejection.⁶⁶ Furthermore, reconstitution of the intermediate recipient with donor-derived bone marrow-induced mixed hematopoietic chimerism resulted in prompt rejection (presumably by replenishing DCs in the graft).⁸⁹ In contrast, kidney allografts from donors reconstituted with a bone marrow matched to the recipient showed prolonged survival.⁹⁰ Similar observations were made with cardiac allografts.^{91,92} At the same time, priming of the CD4⁺ T-cell alloresponse by donor leukocytes was believed to happen in graft-draining lymph nodes, based on Barker's studies showing extended survival of skin allografts placed on vascularized but alymphatic skin pedicles.^{44,93} However, these results provided only indirect evidence for the passenger leukocyte concept in skin transplantation because the presence of donor leukocytes in the lymph nodes of

skin-grafted mice was never formally demonstrated. Furthermore, because lymphatic vessels are severed at the time of skin transplantation and their reconnection (via neolymphangiogenesis and inosculation) requires 5 to 8 d to occur,^{47,48} it seems rather counterintuitive that graft leukocytes could traffic to the host's lymph nodes during this period. In 2016, the passenger leukocyte theory was challenged by Marino's study showing the lack of donor leukocytes in the lymph nodes of skin-grafted mice.¹⁰ In turn, as early as 12 h posttransplantation, substantial numbers of recipient dendritic cells and some B cells (>1500 cells per million) bound to vesicles expressing donor MHC class I and II molecules were detected in these lymph nodes.¹⁰ By day 7, although vesicles were no longer detected on recipient APCs, these cells were double positive in that they expressed their own MHC molecules along with donor MHC molecules.¹⁰ This implies that the source of antigen for T-cell allorecognition in lymph nodes of skin-grafted mice are donor vesicles and not passenger leukocytes. This corroborated a comment made in 1971 by Hall⁴¹ affirming that "debris rather than cells are found in the lymph after skin grafting." Because donor-specific T-cell activation was observed in the recipient's lymph nodes as early 2 d post-skin grafting,¹⁰ it is reasonable to assume that the alloresponse leading to acute rejection of skin allografts in mice is initiated by T cells recognizing recipient APCs cross-dressed with donor MHC antigens, that is, via semidirect rather than direct allorecognition.

In mice, CD4⁺ T cells recognizing donor but not recipient MHC class II antigens mediate acute rejection.^{92,94} In addition, replacement of intragraft donor bone marrow-derived leukocytes by recipient ones results in prolonged survival of cardiac allografts.^{91,95} This suggests that acute rejection of heart transplants is mediated by T cells activated via direct allorecognition. Examination of lymphoid organs from heart-transplanted mice using imaging flow cytometry revealed the presence of a few donor passenger leukocytes (100–200 cells per million cells).¹⁰ This confirmed seminal reports by Larsen et al⁹⁶ and more recent studies.⁹⁷ Although some donor leukocytes have also been detected in selected lymph nodes of these mice,⁹⁸ most passenger leukocytes migrate primarily to the spleen after cardiac transplantation, indicating that they traffic through blood after reverse transendothelial migration rather than via lymphatic vessels. This presumably reflects the fact that, unlike conventional skin grafts, cardiac transplants are fully vascularized at the time of their placement. Most importantly, as early as day posttransplantation, we and others showed the presence of high numbers of recipient APCs cross-dressed with donor MHC molecules (>25 000 per million cells).^{10,11} Moreover, the number of donor passenger leukocytes rapidly declined after transplantation, presumably because of their elimination by the recipient's immune system.¹⁰ In contrast, the frequency of recipient cells cross-dressed with donor MHC antigens rose to 90 000 per million spleen cells by day 7 after transplantation.¹⁰ Semidirect allorecognition might explain how indirectly activated CD4⁺ T cells can provide optimal help for the differentiation of CD8⁺ cytotoxic T cells and trigger acute rejection (3-cell model shown in Figure 2). Furthermore, recent studies from the Lakkis laboratory showed the presence of large numbers of recipient cells

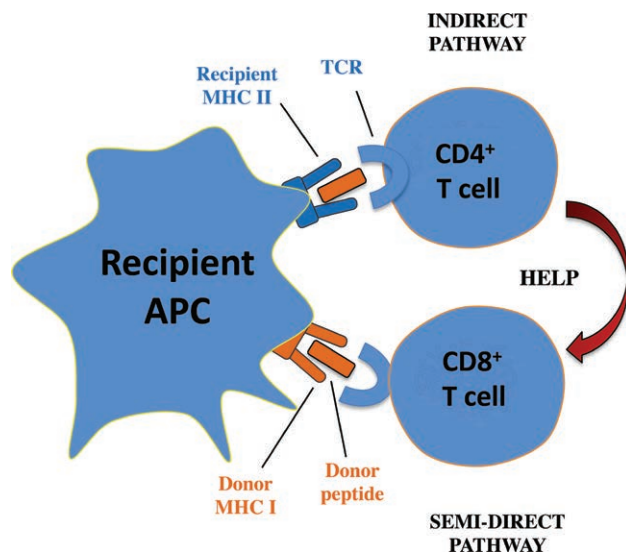


FIGURE 2. Cooperation between $CD4^+$ and $CD8^+$ T cells via semidirect allorecognition. This figure depicts how $CD4^+$ T cells specific for self-MHC class II molecules bound to donor peptides (indirect presentation) can provide help to $CD8^+$ T effector cells recognizing donor MHC class I proteins displayed on the same recipient APC (semidirect presentation). APC, antigen-presenting cell; MHC, major histocompatibility complex; TCR, T-cell receptor.

cross-dressed with donor MHC molecules in kidney and islet allografts early after transplantation.⁵⁶ It is possible that once cross-dressed with donor MHC class I antigens, these recipient cells, including vascular endothelial cells, could become targets for destruction by alloreactive $CD8^+$ cytotoxic T cells, a process contributing to local inflammation and acute rejection. Collectively, these studies suggest that semidirect rather than direct alloresponses by T cells initiate acute allograft rejection. Compelling evidence in support of this view was provided by a recent study from the Morelli's laboratory showing that *in vivo* inhibition of small EV biogenesis suppressed donor MHC cross-dressing and achieved long term cardiac allograft survival in mice.⁹⁹

Role of Indirect T-cell Allorecognition in Acute Rejection

In donor/recipient combinations mismatched only for MHC class I genes, no direct $CD4^+$ T-cell alloresponse is induced. In turn, MHC class I-disparate allografts elicit an indirect T-cell response by $CD4^+$ T cells directed to peptides derived from donor MHC class I proteins.^{71,100} This $CD4^+$ T cell-mediated indirect response is sufficient on its own to initiate the differentiation of $CD8^+$ cytotoxic T cells recognizing donor MHC class I antigens displayed on donor cells (direct pathway).¹⁰¹ These $CD8^+$ T cells can kill donor target cells and ensure the acute rejection of skin allografts.¹⁰¹⁻¹⁰⁴ Furthermore, proinflammatory indirect responses by $CD4^+$ T cells induce potent delayed-type hypersensitivity reactions and provide help for B-cell production of donor-specific antibodies, which contribute to the rejection process.¹⁰⁵⁻¹⁰⁸ This indicates that indirect $CD4^+$ T-cell responses are sufficient to cause acute rejection of skin allografts. This view was supported by studies from the Auchincloss' laboratory showing the rejection of MHC class II-deficient allogeneic skin transplants by $CD4^+$ T-cell activated indirectly.^{101,109} Finally, this conclusion was

further confirmed by experiments showing skin graft rejection in recipient mice adoptively transferred with $CD4^+$ T-cell clones recognizing donor antigens indirectly.¹¹⁰⁻¹¹²

The role of the $CD4^+$ indirect T-cell alloresponse in acute rejection of solid organ transplants is less clear. Acute rejection of cardiac allografts in mice is driven essentially by $CD4^+$ T cells recognizing donor but not recipient MHC class II molecules.^{92,94} In the absence of MHC class II mismatches between donors and recipients, it is expected that the $CD4^+$ T-cell alloresponse to MHC class I-disparate allografts is mediated exclusively via indirect allorecognition. The nature of the rejection of MHC class I-disparate cardiac grafts in mice varies with the donor/recipient strain combination tested. For instance, B10.A (K^k) mice acutely reject heart transplants from B10.AQR donors (K^q) (MST:16 d), which differ by a single allele in the K locus.¹¹³ In contrast, in the reverse combination, B10.A cardiac allografts survived for >100 d when transplanted in B10.AQR mice.¹¹⁴ In most MHC class I-disparate mouse combinations such as B10BR (D^k) into B10.A (D^d), which differ only at the MHC class I D locus, cardiac allografts enjoy long-term survival (>100 d).¹¹⁵ Furthermore, our laboratory showed that even fully MHC-disparate heart allografts fail to trigger significant $CD4^+$ T-cell indirect alloresponses.¹¹⁶ This was apparently because of the fact that these transplants were vascularized at the time of their placement.¹¹⁶ Therefore, unlike skin grafts, mouse heart allotransplants are unlikely to be acutely rejected by $CD4^+$ T-cell activated through indirect allorecognition. Whether this conclusion pertains to other vascularized organ allografts and to animals or patients with pretransplant alloreactive memory T cells is unclear.

Valujskikh and Heeger have demonstrated that indirectly activated $CD8^+$ T cells can reject skin allografts in an acute manner.^{80,117} In this study, anti-H-Y monospecific, H-2^b-restricted, Mata Hari $CD8^+$ T cells rejected H-2^k male skin grafts, whereas they could not interact directly with donor cells. Such rejection was interferon gamma (IFN- γ)-dependent and only occurred if the recipient vasculature (endothelium) expressed recipient MHC class I H-2^b. The observation that wild-type recipient mice reject skin grafts derived from MHC class I- and class II-deficient donors differing at multiple minor loci¹⁰² could also be explained by $CD8^+$ T-cell recognition of donor determinants presented by host vascular endothelial cells. The relevance of the indirect effector mechanism for $CD8^+$ T cells is not likely to be limited to rejection of transplanted skin but may also be important for rejection of other nonvascularized tissues, such as pancreatic islets. In contrast, vascularized organs retain most of their own blood vessel endothelial cells, which express donor (not recipient) MHC class I, thus limiting the cognate interactions between cross-primed T cells and donor vessels.

Indirect Responses to mHAs

In humans, mHAs are important drivers of immune responses triggered after HLA-identical hematopoietic stem cell transplantation.¹¹⁸ Likewise, mHAs-mismatched skin allotransplants are acutely rejected although usually in a delayed manner. As for mHAs disparate solid organ transplants, the strength and pace of the rejection process vary significantly upon the nature of the organ and

the donor/recipient combination.^{115,119-121} For instance, BALB/c (H-2^d) reject B10.D2 (H-2^d) hearts within 11 d, whereas they accept DBA/2 (H-2^d) cardiac allografts for >100 d.^{115,122} It is clear that mHAs represent a potential source of antigens for exogenous processing by recipient APCs and presentation to CD4⁺ T cells in the form of peptides bound to self-MHC class II molecules (indirect pathway). For instance, male H-Y mHAs preferentially activate CD4⁺ T cells via indirect allorecognition, that is, through antigen processing and presentation by female APCs in skin- and heart-grafted mice.⁸⁸ Likewise, certain immunodominant H-Y determinants can induce indirect CD8⁺ T cell-mediated alloresponses.^{86,87} Therefore, indirect allorecognition of mHAs peptides by T cells may represent an important element of the alloimmune response after transplantation. Finally, it is also possible that MHC molecules bound with endogenously processed mHAs peptides can be transferred from donor cells to recipient APCs (MHC cross-dressing) and thereby trigger a semidirect alloresponse leading to rejection.

Corneal transplantation represents a unique model in which rejection is mediated exclusively by CD4⁺ T cells recognizing donor mHAs indirectly.^{121,123,124} This is because of the fact that these allografts are devoid of MHC class II⁺ APCs at the time of transplantation and they are placed in the eye, which represents an immune-privileged site of the body.^{125,126} In the absence of MHC class II expression on their APCs, corneal grafts fail to trigger a CD4⁺ T-cell direct alloresponse.¹²⁷ Instead, transplanted corneas elicit potent CD4⁺ T-cell indirect responses directed exclusively to mHAs but not MHC antigens.^{123,128} In contrast, CD8⁺ T cells activated directly against donor MHC class I are regularly detected after corneal transplantation.^{124,127} However, these CD8⁺ T cells are not fully effective in that they release IFN- γ upon activation but they do not display cytotoxic functions^{124,127} and do not contribute to graft rejection.^{121,124} Therefore, unlike skin and organ transplants, corneal allograft rejection is driven only by CD4⁺ T cells recognizing mHAs indirectly. Interestingly, although no MHC class II⁺ cells were originally detected in the cornea, R. Dana's group has documented the presence of donor professional APCs in the cervical lymph nodes draining corneal allografts.¹²⁹ These cells are presumably derived from CD11c⁺ DCs and CD11b⁺ macrophages present in the corneal epithelium.¹³⁰ Interestingly, in "high-risk" recipients whose corneal transplants were placed in an inflamed eye bed environment,¹³¹ corneal DCs expressed MHC class II molecules as well as CD40, CD80, and CD86 costimulatory receptors at the time of transplantation.¹³¹ Consequently, these corneal allografts elicited potent direct alloresponses by host CD4⁺ T cells recognizing intact donor MHC class II molecules and they were acutely rejected in a few days like skin grafts.¹³¹ This suggested that lack of immunogenicity of corneal DCs was not an intrinsic property of these cells, but it is because of the microenvironment of the eye. This view is supported by Niederkon's studies showing that heterotopic corneal allografts elicit bona fide CTL responses.¹³² Likewise, we have documented that corneal allograft placed subcutaneously in mice triggers CD4⁺ T-cell direct alloresponses to donor MHC antigens.¹²⁷ Therefore, both intrinsic (APC contents) and extrinsic (site of placement) factors control the allorecognition pathway and the nature of

alloantigens involved in the T-cell response after corneal transplantation.

Intragraft Versus Lymphoid Organ Sensitization of T Cells

It is traditionally accepted that alloreactive T cells initiating acute allograft rejection become activated through donor MHC recognition occurring in the recipient's secondary lymphoid organs. As mentioned earlier, allogeneic MHC antigens can be carried by donor passenger leukocytes or vesicles trafficking through lymphatic or blood vessels. However, the possibility that some T cells are sensitized to alloantigens within the graft cannot be ruled out. This concept is referred to as "peripheral sensitization" in which the graft antigens stimulate circulating lymphocytes because they pass through the vascular bed of the organ itself and was originally proposed in the 1960s by Medawar and Strober.^{133,134} In 1971, Tilney and Gowans¹³⁵ provided some evidence supporting this concept in a model of rat allografts transplanted to alymphatic pedicles of skin. More recently, we and others showed that mice devoid of secondary lymphoid organs rejected skin allografts in an acute manner.^{136,137} Although naive T cells reside essentially in lymph nodes and do not leave these sites unless they have become sensitized to their antigens, memory T cells (Tmems) do traffic through peripheral tissues.¹³⁸ Because naive mice display some preexisting endogenous alloreactive T cells, it is possible that these T cells could infiltrate the graft as a result of inflammation and become activated in situ. In fact, Fairchild's studies showed that heart allografts placed in mice were infiltrated within 12 to 24 h by endogenous Tmems, which proliferated and produced IFN- γ in situ but failed to cause rejection.¹³⁹ This was confirmed by a more recent study by Ganchiku et al¹⁴⁰ showing that graft infiltrating memory T cells collected 72 h after heart transplantation showed no donor-specific reactivity and failed to reject the allograft. In contrast, Tmems infiltrating the graft at 120 h and displaying donor-specific reactivity, due presumably to alloantigen priming in secondary lymphoid organs, were able to mediate acute rejection.¹⁴⁰ In contrast, after cold ischemia, early graft infiltration by endogenous Tmems was sufficient to induce acute cardiac allograft rejection in mice.¹⁴¹ This suggests that in patients whose heterologous immunity is more vigorous than in laboratory mice that are raised in sterile environments, some endogenous alloreactive Tmems could infiltrate vascularized solid organ allografts early after transplantation, become activated in situ, and cause acute rejection.

T-cell Allorecognition Pathways in Chronic Allograft Rejection

Chronic rejection (CR) occurs within months to years after transplantation.¹⁴²⁻¹⁴⁵ The key feature of CR is arteriosclerosis or progressive luminal narrowing of graft vessels (vasculopathy) often accompanied by graft tissue (parenchymal) fibrosis. This leads to progressive loss of function that culminates in graft failure. CD4⁺ T cells are known to play an important role in CR.¹⁴⁶ However, the mechanisms by which they contribute to this condition are still unclear. It has been proposed that indirect T-cell alloreactivity is the driving force behind CR. This was

based on the assumption that once passenger leukocytes have been eliminated, only indirect alloresponses can be elicited. Indeed, several studies have provided evidence in transplanted mice and patients supporting the contribution of indirect alloresponses by CD4⁺ T cells, including TH2 cells, in CR.^{103,144,147-154} First, some studies showed that injection of mice and swine with donor MHC peptides could accelerate CR after heart transplantation.^{155,156} Yamada et al¹⁵⁷ showed that upon elimination of the CD4⁺ T cell-mediated direct pathway and depletion of CD8⁺ T cells, heart allografts underwent long-term survival (>100 d) but succumbed to CR (cardiac allograft vasculopathy [CAV]). Furthermore, several studies have documented the presence of T cells reactive to donor MHC peptides in recipients of kidney, heart, and lung allografts displaying histological features of CR.^{147,148,151,158,159} Therefore, the involvement of indirect T-cell alloreactivity in CR seems plausible. In addition, it is conceivable that indirectly activated CD4⁺ T cells being self-MHC class II-restricted are the only cells involved in the T- and B-cell cooperation required for alloantibody production, which is often associated with CR.^{107,160,161} However, a previous study performed on monkeys challenged these views. We evaluated T- and B-cell alloresponses in cynomolgus monkeys that had received combined kidney/bone marrow allografts and myeloablative immunosuppressive treatments.¹⁶² We measured donor-specific direct and indirect T-cell responses and alloantibody production in monkeys that did not reject their transplant acutely but developed CR.¹⁶² All CR recipients exhibited high levels of antidonor antibodies and mounted potent T-cell alloresponses to donor APCs in vitro (mixed lymphocyte reaction). Such apparent direct alloreactivity could be detected for >1 y after transplantation. In contrast, only 30% of monkeys with CR had a detectable indirect alloresponse.¹⁶² This contradicts the view that an indirect T-cell pathway is absolutely required for antibody production. In the absence of donor professional APCs (macrophages and DCs), it is possible that direct alloreactivity may be maintained by memory alloreactive T cells recognizing allogeneic MHC molecules on vascular endothelial cells. Alternatively, T cells may be activated within the allograft in a semidirect manner by recipient APCs cross-dressed with donor MHC molecules. In this scenario, CD4⁺ T cells recognizing donor MHC class II (direct pathway) could provide help to recipient B cells having acquired donor MHC class II molecules via cross-dressing. The detection of large numbers of cross-dressed cells in kidney and islet allografts supports this hypothesis.⁵⁶ Whether semidirect alloreactivity by T cells persists within transplanted organs and contributes to alloantibody production and CR remains to be investigated.

In addition to donor-reactive T cells, some studies support the view that CR may be mediated by T cells recognizing tissue-specific autoantigens, such as cardiac myosin and collagen type V in heart and lung transplantation, respectively.^{113,163-165} Indeed, organ allografts undergoing CR often share histological and pathological features associated with chronic autoimmune inflammatory diseases such as autoimmune myocarditis for the heart.¹¹³ Therefore, it is plausible that CR could result from an autoimmune process. Finally, a recent TCR repertoire study of T cells isolated from heart transplants of patients with CAV suggests that CR could be induced by activated

bystander T cells, which are not activated via TCR-MHC-peptide interactions.¹⁶⁶ It is possible that these bystander T cells contribute to CR through stimulation of natural killer cells and macrophages. Altogether, these observations underscore that the nature of the T cells involved in CR and their antigen specificity and mechanism of action remain elusive. Gaining insight into this question will be crucial to the design of therapies to prevent or suppress CR in clinical transplantation settings.

CONCLUDING REMARKS

Following allotransplantation, T cells can recognize donor antigens via 3 distinct mechanisms: the direct, indirect, and semidirect pathways. The nature of the T cells (CD4⁺ versus CD8⁺), their antigen specificity (MHC versus mHAs), and the pathway by which they become activated are governed by various factors both intrinsic and extrinsic to the allograft. Consequently, the pace and magnitude of the alloresponse and acute rejection vary dramatically depending upon the nature of the organ or tissue transplanted, its anatomical site of placement, and the immune status of the host. Recent studies suggest that semidirect alloresponses by T cells recognizing recipient APCs cross-dressed with donor MHC molecules play a key role in allograft rejection. However, this has not been formally demonstrated. In addition, although there is ample evidence showing that T cells play a key role in chronic allograft rejection, the nature of these T cells, their target antigens, and their mechanisms of action remain elusive. There is strong circumstantial evidence suggesting that CD4⁺ T cells activated through recognition of donor MHC peptides presented by recipient APCs mediate CR. This is based on the assumptions that direct alloresponses can no longer be present in the absence of donor professional APCs and that indirectly activated T cells are the unique driving force behind alloantibody production. However, this remains to be formally demonstrated. Actually, one cannot exclude that T cells activated via semidirect allorecognition or autoreactive T cells recognizing tissue-specific autoantigens elicit CR. Finally, a recent study showing a lack of clonally expanded T cells in the heart of patients with CAV suggests that CR could be mediated by bystander T cells. Gaining more insights into the mechanisms by which T cells recognize donor antigens after transplantation will help design more selective immunosuppressive therapies and tolerance procedures to prevent acute and chronic allograft rejection.

ACKNOWLEDGMENTS

The authors would like to thank Dylan Muldoon for proofing the article.

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