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Eosinophils acquire immune checkpoint molecules through trogocytosis: implications in cancer immunity.

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1. INTRODUCTION.

1.1 Trogocytosis.

Trogocytosis is an active process characterized by the transfer of membrane fragments between living cells in a contact dependent way. This process was also known as "cell cannibalism", "stripping", "cross dressing", or "nibbling". Since 2002 this process is termed and recognized as "trogocytosis" that derived from the Greek word "*trogo", w*hich means "biting*"* (Harshyne, 2001), (Hudrisier, 2002), (Joly, 2003), (Abdu, 2016).

This phenomenon was described for the first time in 1970 by C.G. Culbertson as a process regarding Amoebae and microbes' behaviour (Culbertson, 1970; Brown, 1979). Now it is known that trogocytosis is a diffused mechanism also involved in immune system response, in the central nervous system and during the embryonic development. Eukaryotic microbes perform trogocytosis for cell killing. In fact, *Entamoeba histolytica "*bites" and internalizes fragments from human Jurkat cells in order to kill them (Ralston, 2014). *Naegleria Fowleri,* acquiring host cell material in own cytoplasm, exerts its cytopathic effect against secondary mouse-embryo cells (Brown, 1979). Thus, the term "trogocytosis" was coined. Moreover, *E. histolytica* employs trogocytosis to escape immune system. In particular, by stealing and displaying human cell proteins on its membrane, *E. histolytica* finds protection from cell lysis operated by serum complement system (Miller, 2019).

Trogocytosis is a widespread process also during the remodeling and the development of nervous system. It has been shown that microglia

and astrocytes remodel synapses and axons, respectively, through trogocytosis. Using correlative light and electron microscopy techniques, Weinhardand and collaborators demonstrated that microglia participate to the maturation of neuronal circuits performing trogocytosis versus presynaptic component. The microglia ability to exert trogocytosis was observed also *in vivo* in the developing *Xenopus laevis* retinotectal circuit. The microglial acquisition of axonal materials from retinal ganglion cell leads to the remodeling of these structures and to the proper neural wiring during development (Lim, 2021).

In post laminar compartments of the optic nerve head there are galectin-3^{+} astrocytes that constitutively internalize large axonal extensions (Nguyen, 2011).

Trogocytosis plays a key role also during the embryonic development. In *Caenorhabditis elegans* has been demonstrated that endodermal cells can ingest lobes rich in mitochondria and other contents that derived from primordial germ cells, leaving the remaining cells alive (Abdu, 2016). In Xenopus, during the stage of gastrula, the trogocytosis of EphB/ephrinB complex leads to a correct assembling and efficient cell rearrangements (Gong, 2019). Interestingly, Valle et al. discovered that vaginal neutrophils could eliminate sperm in a trogocytosis-dependent but not-dependent neutrophil extracellular traps (NETosis) manner (Olivera-Valle, 2020).

1.1.1 Trogocytosis in immune cells.

NK cells.

Trogocytosis is a central process in natural killer (NK) cell function. It is reported that the acquisition of non-self molecules could lead NKs to gain new characteristics. For example, acquisition of CCR7 from mature dendritic cells (DCs) endows NK cells with the ability to reach the secondary lymphoid compartments in response to CCL19 and CCL21 (Marcenaro, 2009). This process may include the increased expression of molecules adhesion such as LFA-1 and CD2 and it may be reflected also in haploidentical hematopoietic stem cell transplantation. The capture of inner membrane protein H-Ras from tumor cells induced activation of human NK cells increasing ERK phosphorylation, IFN-γ and TNF-α secretion, enhancing lymphocyte proliferation and NK-mediated target cell killing (Rechavi, 2007). Tabiasco and colleagues demonstrated that during preliminary states of Epsten Bar virus (EBV) infection, activated NK cells are able to acquire CD21 (the primary receptor for EBV) from B cells. Thus, the foreign receptor renders NK cells susceptible to infection and allows greater spread to the virus (Tabiasco, 2003).

On the other hand, trogocytosis may produce a loss of function for NK cells. The acquisition of the major histocompatibility complex -I (MHC class I) molecules (H-2Dd) operated by NK cells expressing Ly49 inhibitory receptors decrease the killing capacity versus tumor cells (Sjöström, 2001). This was shown also in an ovarian cancer setting. Gonzalez and colleagues reported the ability of NK cells to capture CD9 tetraspanin from ovarian cancer cells by trogocytosis.

This process resulted in a more immune tolerant phenotype and an impaired tumor cytotoxicity in NK cells, leading to an ineffective antitumoral response (Gonzalez, 2021). Of interest, Hasim et al. demonstrated that NK cells acquire PD-1 from leukemia cells *in vitro* and *in vivo*, through trogocytosis, *via* signaling lymphocytic activation molecule (SLAM) receptors. Despite PD-1^+ NK cells possessed a more activated phenotype producing more IFN-γ and displaying increased levels of activating receptors, they exhibited suppressed antitumor immunity (Hasim, 2022).

T and B Lymphocytes.

Trogocytosis shapes T cell functions and it is a common mechanism in all T cell subsets. For example, it has been well reported that both $CD4^+$ and $CD8^+$ T cells acquire MHC complexes from tumor cells or from other immune cells by trogocytosis. In fact, Martinez-Martin et al. have shown that the T cell receptor (TCR) is necessary for the acquisition of MHC class I from antigen presenting cells (APCs), together with the action of small GTPases such as RhoG and TC21 (Martínez-Martín, 2011).

There are several studies indicating that naïve $CD4^+$ T cells can capture antigen-MHC complexes from DCs upon the immunological synapse (SI) (Wetzel, 2005) (Game, 2005), (Tsang, 2003). After the interaction with DCs, $CD4^+$ T cells can acquire other molecules such as CD80, OX40L, CD31, CD49d, CD54, CD61 and CD62E (Tatari-Calderone, 2002) (Baba, 2001) (Brezinschek, 1999). Therefore, after the acquisition of these costimulatory and adhesion molecules, $CD4^+$ T cells gain APC function (Zhou, 2005) (Helft, 2008). Moreover, the

acquisition of MHC class II molecules induces prolonged TCR signaling (Osborne, 2012), which influence $CD4^+$ T cell survival and activation promoting a Th2 phenotype, supported by an increased production of IL-4 and IL-5 (Reed, 2019).

Trogocytosis is also observed in the effector phase of cytotoxic T lymphocytes (CTLs). It has been reported that when CTLs attack tumor cells, they are able to acquire MHC class I from their targets in an antigen dose–dependent manner. Notably, these pMHC class I molecules are internalized by CTLs and presented on own surface membrane, rendering CTLs susceptible to lysis operated by neighboring CTLs of the same clone (fratricide cell death) (Huang, 1999) (Hwang, 2000). Moreover, it seems that TCR-mediated trogocytosis can strip tumor antigens from target tumor cells, leading to antigen loss and tumor escape (Hamieh, 2019) (Chung, 2014).

Despite the role of trogocytosis in boosting or repressing CTL activity is still under debate, it has been reported that there is a positive correlation between CTLs cytotoxic activity and CTLs trogocytosis ability (Daubeuf, 2006) (Machlenkin, 2008).

Regulatory T (Treg) cells are able to acquire CD80 and CD86 from DCs through CTLA-4 *via* trogocytosis with consequent impairment of DC-mediated co-stimulation signals (Qureshi O. S., 2011). A recent study also reported that inducible Tregs (iTregs) have higher trogocytic activity than naïve or effector T cells in removing the antigen-MHC class II complex from DCs (Akkaya, 2019).

Also, double negative T (DNT) cells (TCR^+ $CD3^+$ $CD4^-$ $CD8^-$ T cells) can carry out trogocytosis. In a mouse model of OVA-induced

allergic asthma, DNT acquired pMHC class II molecules from DCs *via* LAG-3/CD223, thus reducing the antigen-presenting activity of DCs (Tian, 2019).

With regards to B cells, Hudrisier et al. identified MHC and B-cell receptor (BCR) as molecules that can trigger trogocytosis in B cells (Hudrisier D. A., 2007). During infection with avian H5N1 influenza virus, B cells acquire 2,3 sialic acid molecules (receptor for avian flu) from monocytes *via* trogocytosis, thus facilitating the spread of influenza virus (Lersritwimanmaen, 2015) (Kongsomros, 2020).

Xu et al. found that the BCR could mediate the trogocytosis of different epitopes from red blood cells in order to suppress the immune response to erythrocyte antigens. Of note, this process seems to be mediated by an antigen-IgG complex (Xu H. &., 2020).

A particular role of trogocytosis has been described for marginal zone (MZ) B cells. MZ-B cells can acquire pMHC class II molecules from conventional DCs and present the associated antigens to T cells. Interestingly, the acquisition of pMHC class II occurs through interaction between CR2 (complement receptor 2) on B cells and C3 that binds MHC class II on DCs. Moreover, Schriek et al. demonstrated that this kind of trogocytosis process is highly regulated by ubiquitin ligase MARCH1, which avoids that DCs undergo cell death due to prolonged trogocytic events (Schriek, 2022).

Dendritic cells.

DCs are the professional antigen presenting cells (APC) of immune system. They process cytosolic or extracellular antigens and load the resulting peptides on MHC class I and MHC class II complex in order to activate T cells response. On the other hand, DCs are able to acquire peptide-MHC (pMHC) complexes from adjacent cells presenting new epitopes without antigens processing (Nakayama, 2015). This mechanism was originally called cross-dressing but it is now recognized as trogocytosis (Harshyne, 2001) (Campana, 2015). Cross-dressing is a process described both *in vivo* and *in vitro* for virus and tumors antigens. MacNabb and collaborators demonstrated that tumor associated DCs are able to acquire pMHC class I complexes from C1498 leukemia cancer cells and to activate $CD8^+$ T cells both *in vivo* and *in vitro*. Moreover, the activity of T cells is directly proportional to the ability of DCs to perform pMHC class I trogocytosis and to have access to antigens. The more DCs acquire pMHC class I complexes the more T cells are primed and activated. This particular correspondence was demonstrated for a model of Vaccinia virus and also for tumor antigens of CMT.64 lung carcinoma (Zhang, 2008). Trogocytosis was described also for plasmacytoid dendritic cells (pDC). These cells are inefficient in the internalization of tumor cells by phagocytosis. However, pDCs can acquire tumor antigens through trogocytosis and present them to tumor restricted $CD8^+$ T cells, thus actively participating to antitumor immunity (Bonaccorsi, 2014).

The transfer of pMHC complexes between adjacent cells is a process that was observed also in adoptive transfer settings. It has been shown that mouse DCs, when co-cultured with allogenic endothelial cells, can acquire both class I and class II pMHC complexes in ATP- and temperature-dependent manner. *In vivo*, following adoptive transfer, DCs acquire allogeneic pMHC class II complexes and induce proliferation of Ag-specific T cells whose Ag specificity is restricted by the captured MHC allele (Herrera, 2004).

The transfer of MHC class II has been also implicated in the development of peripheral tolerance. It has been reported that $CD4^+$T cells can acquire MHC class II complexes from DCs during a primary immune response *in vivo*. In this context, given the absence of costimulatory molecules on T cells, MHC class II dressed $CD4^+$T cells induced tolerance in surrounding $CD4^+$T cells, mitigating their response (Helft, 2008) (Tsang, 2003). It is important to observe that trogocytosis in DCs can also result in loss of T cell stimulatory function. For example, transfer of MHC class II complexes from DCs to NK cells limits the DC induced $CD4^+$ T cells response. (Nakayama M. T., 2011). As described above, Tregs can "steal" co-stimulatory molecules, such as CD80 and CD86 from the membrane of DCs, gaining increased suppressive ability while resulting in insufficient antigen presenting activity by the DCs (Gu, 2012).

Monocytes and macrophages.

Trogocytosis performed by macrophages has a known role in interfering with antibody therapy, since the bite may remove from target cells both therapeutic antibodies and antigens. This is the case

of leukemia patients treated with rituximab (anti-CD20) where macrophages and monocytes through FcγR can steal ("shaving" process) the αCD20/CD20 complex from cancer cell surface, allowing for tumor immune-evasion (Beum, 2011) (Beum P. V., 2006). This mechanism was demonstrated also for other immunotherapeutic antibodies targeting tumor cells (Beum P. V., 2008) (Taylor, 2015). In contrast, persistent trogocytosis of tumor cells may lead to tumor cell death, in a process termed "trogoptosis" (Van Rees, 2022). Trogoptosis is a mechanical process ensuing trogocytosis that involves the disruption of the plasma membrane of target cancer cells leading to lysis and necrotic death. As demonstrated Velmurugan et al, macrophages perform trogocytosis mediated cell death versus $HER2^+$ breast tumor cells and, interestingly, this process is increased by opsonized breast tumor cells, underlining the role of FcγR in this context (Velmurugan, 2016). In non-small cell lung cancer, administration of a bispecific antibody targeting epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (HGFR) caused FcγR-mediated trogocytosis of both receptors from cancer cells, leading to downregulation of these proteins and subsequent reduction in cell proliferation (Vijayaraghavan, 2020).

Monocytes and macrophages can also exert trogocytosis of inhibitory molecules. Monocytes co-cultured with Hodgkin lymphoma cells can capture membrane fragments containing PD-L1/L2 molecules and, subsequently $PD\text{-}L1^+$ monocytes can limit the IFN-γ release of T cells (Kawashima, 2020)

Neutrophils.

Neutrophils are the most abundant granulocyte population that plays prominent inflammatory and anti-microbial functions (Mayadas, 2014) (Nauseef, 2014). To kill their targets, neutrophils usually use different process such as phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), and release of reactive oxygen species (ROS) or cytotoxic granules (Ustyanovska Avtenyuk N. V., 2020). Recently, it has been reported that neutrophils employ trogocytosis and subsequent trogoptosis to kill antibody-opsonized cancer cells (Matlung, 2018). Van der berg group discovered that interruption of the CD47–SIRPa signal induces trogoptosis of opsonized tumor cells in a mechanism dependent on the CD11b/CD18 complex. In fact, studies of trogocytosis in neutrophils from patients affected with leukocyte adhesion deficiency type III (LADIII), which specifically lack the integrin-associated protein kindlin3, demonstrated the importance of CD11b/CD18 stabilization in trogoptosis process (Matlung, 2018), (Bouti, 2021), (Zhao, 2011). Moreover, Bremer group demonstrated that neutrophils were able to perform trogocytosis versus six different carcinoma cell lines through Galectin-9 (Gal-9). In this study, Gal-9 treatment triggered trogocytosis *via* the induction of the "eat me" signal phosphatidylserine, expressed on tumor cells, and the increase of adhesion molecules on neutrophils membrane (Ustyanovska Avtenyuk N. C., 2021), probably leading to the trogoptosis of tumor cells.

Trogocytosis in neutrophils not always leads to trogoptosis and target cell death. In fact, Horner group observed a simply mutual membrane fragments exchange between neutrophils and Raji^{H} HER-2/neu^{+} cells, amplified by trastuzumab treatment. (Horner, 2007). Masuda et al. demonstrated that peripheral blood neutrophils acquire T-cell derived membrane molecules in the presence of serum and cell-cell contact as a consequence of FcγR-dependent trogocytosis, explaining the odd event of false positives resulting in flow cytometry analyses (Masuda, 2012). Moreover, trogocytosis may be involved in neutrophils migration to inflamed sites. Damage-associated molecular patterns (DAMP) molecules can induce an increased trans-endothelial migration operated through trogocytosis process. In particular, pro-inflammatory treatment with extracellular cold-inducible RNA-binding protein (eCIRP) leads to an enhanced expression of adhesion molecules (such as CD11b and ICAM-1) and to the acquisition of junctional epithelial proteins such as VE-cadherin and JAM-C from endothelial cells. All these data suggest that the continuous bites operated by recipient cells could mediate also an epithelial barrier crossing (Takizawa, 2022). Interestingly, neutrophils are able to discriminate between trogocytosis and phagocytosis, although how they do so still need to be completely understood. In human neutrophils it has been observed a preferential engagement of trogocytosis versus anti-CD20 opsonized chronic lymphocytic leukemia (CLL) B cells, which does not culminate in trogoptosis but it may result in a reduction of CD20 expression on tumor cells, with relevant implications for CLL patients undergoing

treatment. In this study, the authors propose that the major size of tumor cells respect neutrophils may skew neutrophils towards trogocytosis, rather than phagocytosis (Valgardsdottir, 2017).

Basophils.

Basophils are the rarest population of granulocytes, representing <1% of the blood circulating cells. They are implicated in Th2 immunity, in particular in responses against parasites and in allergies (Marone, 2000). Evidence that basophils can carry out trogocytosis comes from a single study from Miyake and collaborators, which demonstrated that basophils can acquire peptide–MHC class II complexes from DCs both *in vivo* and *in vitro* (Miyake, 2017). This process occurred within 15 minutes after the initiation of basophils and DCs co-culture. Transfer of MHC class II molecules from DCs to basophil membranes was contact-dependent, since it was abrogated when the two cell populations were separated by a 0.4-μm pore membrane. Blocking of either CD11a or ICAM-1 (but not CD11b) abrogated MHC class II transfer from DCs to basophils, indicating a role for these adhesion molecules in basophil trogocytosis. Moreover, in lymph nodes of atopic dermatitis mice the authors demonstrated that basophils could acquire MHC class II complexes exclusively from DCs. Finally, acquisition of pMHC class II molecules conferred basophils APC properties, gaining a new function and driving the T cells response towards a Th-2 orientation.

1.1.2 Role of trogocytosis in immune regulation.

An interesting characteristic of trogocytosis is the outcomes that result from the transfer of membrane proteins. The process begins with the engagement of two living cells, termed respectively "recipient cell" (the active one) and "donor cell" (the target one). The recipient cell bites the donor cell, it acquires membrane fragments and relative molecules gaining new cellular functions. As contrast, the donor cell, losing some of its surface molecules, is forced to reduce its cellular functions.

For example, NKs can acquire CCR7 from different $CCR7^+$ target cells and gain the ability to migrate to lymph nodes, providing crucial effects to antitumoral response and to allogeneic hematopoietic stem cell transplantation (Marcenaro, 2009).

R. Gary and collaborators demonstrated the new fratricide role of those $CD8^+$ T cells that have acquired programmed death ligand – 1 (PD-L1) molecules from APCs. In contact dependent manner, human $CD8^+$ T cells can acquire PD-L1 from mature dendritic cells and tumor cells target, and then induce apoptosis in target PD-1 expressing $CD8^+$ T cells. This function is gained *via* trogocytosis and could offer a new regulation mechanism to immune response. (Gary, 2012). Moreover, $CD8^+$ T cells can acquire specific TCR from neighbor virus-specific cytotoxic $CD8^+$ T lymphocytes (CTL), gaining the function to eliminate the specific cells targeted before by donor $CD8^+$ T cells (Chaudhri, 2009).

A demonstration of loss of function for donor cells mediated by trogocytosis was given by a study from Qureshi and collaborators.

They demonstrated that CTLA-4 expressing cells may mediate the trogocytosis of CD86 molecules from target donor cells, thus hindering and inhibiting the possible co-stimulation *via* CD28 receptor (Qureshi, 2011). However, it is still unclear how donor-derived molecules are located on recipient cells. The identification of donor proteins on recipient cells by flow cytometry raises the following possibilities. Donor membrane fragments may be merely attached or fused to recipient cells or alternatively, donor-derived proteins may be re-expressed on recipient cells after being absorbed and recycled. Interestingly electron microscopy showed that a human NK cell line acquired APC plasma membrane fragments, which were loosely attached to the plasma membranes of these NK cells (Williams, 2007). Similar morphology was observed on $CD4^+$ T cells that acquired APC plasma membrane fragments (Hudrisier D. C., 2009).

For several years, the process now recognized with the term "trogocytosis", has been named "incomplete phagocytosis" (Uribe-Querol, 2021), indicating a close correlation between these two mechanisms. Now it is clear that they are different processes. Phagocytosis includes the entire ingestion of target death cells. In contrast, trogocytosis requires cell-cell contact, it is performed in a short time versus live cells and it is characterized by the acquisition of little membrane fragments and the associated molecules from target cells (Batista, 2001) (Wetzel, 2005) (Vanherberghen, 2004). Trogocytosis requires proteins involved in membrane scission such as Gulp1 (Gong J. G., 2019), (Abdu, 2016) and a small GTPase

(Martínez-Martín, 2011), none of which normally have the role in engulfment and internalization of target cells during phagocytosis.
On the other hand, trogocytosis and phagocytosis share a lot of molecules and activation pathways such as: PI3K, RhoG, Src and Syc, but it is not clear what mechanism can trigger one or the other process. Probably, the engagement of particular receptors through the binding to specific target ligands are important for the induction of trogocytosis or phagocytosis. These interactions may lead to distinguish between live or death cells or to grasp the size of target cells. For example, the exposure of phosphatidylserine on the outer surface of a membrane leads immune cells to activate phagocytosis (and not trogocytosis), because it is a signal of apoptotic cell. (Penberthy, 2016).
It is now clear that trogocytosis is a process involved in different mechanisms, between different species and in different state of life. It is engaged in cell killing, in organism development, in cell-cell communication and in immune cells response and is now fundamental to understand each aspect of this process.

1.1.3 Mechanisms of trogocytosis.

Trogocytosis in immune system requires cell-cell contact and the establishment of an immunological synapse, cytoskeleton rearrangements and specific activated pathways.
The first step to let trogocytosis process happen is the recognizing between the two involved cells and the establishment of cell-cell contact. Usually for immune cells it occurs with the formation of the immunological synapse (Ahmed, 2008) and the engagement of TCR, BCR and FcγR (Martínez-Martín, 2011) (Xu H. &., 2020) (Masuda, 2012) or by the contact of ligands and receptors (Hudrisier D. A., 2007) (Tabiasco, 2003) (Li, 2016) (Matlung, 2018).
When recipient cell receptors recognize corresponding ligands on the target cells, they undergo an energy-consuming process, including actin cytoskeletal remodeling and signal transmission, and then occur membrane scission of recipient cell and cell engulfment (Aucher, 2008), (Gong J. G., 2019), (Nakada-Tsukui, 2017).
As mentioned above, the molecules involved in this process are TC21, RhoG, Src, and Syk (Martínez-Martín, 2011) (Hudrisier D. A., 2007). In fact, trogocytosis of membrane fragments and MHC class II molecules performed by T cells was dependent on PI3K, TC21, and RhoG (Martínez-Martín, 2011). Actin along with Src, Syk, and PI3K have all been implicated also in B cell trogocytosis (Hudrisier D. A., 2007). Furthermore, Src, actin, and Syk were involved in MHC class II transfer from dendritic cells to basophils (Miyake K. S., 2017). All these proteins above are also involved in phagocytosis except TC21.

Interestingly several studies have shown that inhibitors of trogocytosis mainly include ATPase inhibitors, actin skeleton blockers, Src, Syk, PIK3 pathway kinase inhibitors, and acidification inhibitors. In fact, an ATPase inhibitor such as Concanamycin A can abolish Ag-specific trogocytosis (Gary, 2012). Pham et al. demonstrated that the restriction of actin polymerization and lack of energy would interrupt the process of trogocytosis and blocking PI3K activity delayed trogocytosis mechanism. Alternatively, the inhibition of Src kinases activity slowed the process and reduced the amount of trogocytosis (Pham, 2011). Wortmannin (PI3K inhibitor) and PP2 (tyrosine-protein kinase Src inhibitor) significantly inhibited the trogocytosis exerted by $CD4^+$ and $CD8^+$ T cells (Pham, 2011), (Aucher, 2008). Picetanannol, a tyrosine-protein kinase Syk inhibitor, limited the trogocytosis performed by T cells and neutrophils (Leka, 2020). Ammonium chloride, an acidification inhibitor, reduced amoebic trogocytosis and cell killing but did not weaken initiation while inhibiting the process of receptor dependence (Gilmartin, 2017). Finally, it has been reported both for NK and T cells that trogocytosis process is inhibited by low temperatures (Alari-Pahissa, 2021) (Aucher, 2008).

1.2 Eosinophils and cancer.

Eosinophils are a subset of granulocytes that, in physiological conditions, count for about 1-3% of total circulating leukocytes (Mattei, 2020). Eosinophil differentiation is mediated by several cytokines, such as IL-5, granulocyte/monocyte colony stimulating factor (GM-CSF) and IL-3. In particular, IL-5 is essential to promote the terminal differentiation, growth and survival of eosinophils (Kandikattu, 2019). Following differentiation and maturation in the bone marrow, eosinophils are released into the bloodstream, as terminally differentiated cells, where under normal conditions they remain in circulation for 8 to 18 hours and until 3-4 days in tissues (Sastre, 2018). Eosinophils are able to migrate from the bloodstream to the sites of inflammation and to the tumor microenvironment (TME) of solid tumors. This condition of tumor-associated tissue eosinophilia (TATE) has been observed in many types of cancer, including breast cancer (Chouliaras, 2021), oral squamous cell carcinoma (OSCC) (Choudhary, 2021), colorectal cancer (Harbaum, 2015), and cervical cancer (Kurose, 2019). There are several studies that highlight the anti-tumor activity of eosinophils, considering their presence in the peripheral blood or in the TME as a favorable prognostic factor. In opposition, other studies attribute to eosinophils a pro-tumoral function and the presence of these cells negatively correlates with the survival of patients (Mattei, 2020), (Grisaru-Tal S. I., 2020),(Varricchi G, 2017). It is worth noting that treatment of melanoma patients with pembrolizumab (PD-1 antibody) or with the combination of nivolumab and ipilimumab (anti-PD-1 and anti-

CTLA-4, respectively) promote an increase in the number of eosinophils both in peripheral blood and in the TME, coupled with an increment of tumor-infiltrating $CD8^+$ T cells, improving the effectiveness of immunotherapy (Simon, 2020). On the other hand, a pro-tumoral role of eosinophils was observed in cervical carcinoma patients. In fact, the presence of a high percentage of eosinophils in the infiltrate might reflect a less effective antitumor response, resulting in a worse overall survival (van Driel, 1996) (Xie, 2015). Moreover, in hematological tumors such as Hodgkin's lymphoma and T-cell leukemia/lymphoma the presence of eosinophils in the tumor or in blood respectively reflected unfavorable prognostic factors for overall survival (OS) (Von Wasielewski, 2000) (Enblad, 1993), (Utsunomiya, 2007).

Eosinophils in the TME can perform direct anti-tumor activity, mediated by the release of cationic proteins. It has recently been shown that bone marrow derived eosinophils activated with IL-33 exert tumor-killing activity against several tumor cell lines, mediated by CD11b/CD18 integrin complex and cytotoxic proteins such as ECP (Eosinophil Cationic Protein), EPX (Eosinophil-peroxidase) and Granzyme B through synapse polarized degranulation (Andreone, 2019). Moreover, ECP inhibits the viability of human squamous oral carcinoma cells (SCC-4 and SCC-25) in a dose-dependent way (De Lima, 2015). In experimental mouse models of colorectal cancer, eosinophils have been shown to exert cytotoxic activity, independent of $CD8^+$ T cells, against cancer cells. In fact,

depletion of $CD8^+$ T cells with specific antibodies did not affect the anti-tumor activity of eosinophils (Reichman, 2019).

Several studies show an indirect anti-tumor activity of eosinophils, mediated by interaction with other cells of the immune system in the TME. In mice transplanted with breast cancer cells (4T1 and PyMT), eosinophils infiltrating lung metastases promote the recruitment of $CD8^+$ and $CD4^+$ T cells. In addition, eosinophils activated with TNF-α and IFN-γ secrete chemokines such as CCL5, CXCL9 and CXCL16, involved in chemotaxis of T cells (Grisaru-Tal, 2021). In a mouse model of melanoma, where mice were inoculated with a cell line of ovalbumin-expressing B16 (MO4) cells, eosinophils played a key role in the recruitment of $CD8^+$ T lymphocytes through the production of CCL5, CXCL9 and CXCL10 chemokines, with the final effect of tumor eradication (Carretero, 2015). In addition, eosinophils and T cells cooperate to normalize blood vessels, promoting up-regulation of the expression of adhesion molecules, including Vascular Cell Adhesion Molecule 1 (VCAM-1), decreasing hypoxia, increasing vascular perfusion and polarization of tumor-associated macrophages towards an M1 phenotype expressing low levels of pro-angiogenic factors, such as vascular-endothelial growth factor (VEGF) (Carretero, 2015).

Overall, these studies indicate that eosinophils may promote anti-tumor immunity through interaction with T cells or by causing direct tumor cell killing.

1.3 IL-33 and cancer.

IL-33 is a nuclear protein associated with chromatin, since it binds histones H2A and H2B through the pattern that lies within its nuclear N-terminal domain (Roussel, 2008). In non-pathological conditions IL-33 is constitutively expressed in several human and murine cells, including fibroblasts, epithelial and endothelial cells. Its expression may be strongly increased during inflammatory states that may determine its release in the extracellular space, where IL-33 acts as an alarmin for the immune system (Cayrol, 2018). IL-33 performs its activity through the binding to its primary specific receptor ST2, a member of the super family of Toll-like/IL-1 receptors.

ST2 is constitutively expressed by many hematopoietic cells, especially those involved in Th2 immune responses, including ILC2, Th2 cells, Tregs, mast cells, eosinophils, basophils, myeloid-derived suppressor cells (MDSCs), and finally some subsets of DCs. However, recent studies have revealed that IL-33 is also able to act on cells involved in type 1 immune response, such as Th1 cells, NK cells, $CD8^+$ T lymphocytes, neutrophils, macrophages, and B lymphocytes. Furthermore, it is known that even non-hematopoietic cells, including epithelial cells, endothelial cells and fibroblasts, express ST2 receptor and therefore respond to the signals induced by IL-33 (Afferni, 2018).

The IL-33/ST2 axis not only plays a role in pathologies associated with the Th2-type of immune responses, but it is also involved in Th1 immunity, including tumor immune response. In cancer, both pro-tumor and anti-tumor functions of IL-33 have been reported, and

evidences suggest that this duality may be correlated with the tumor histotype, the particular TME and the immune cells on which this cytokine may act (Afferni, 2018).

IL-33/ST2 signaling is involved in the remodeling of TME through the recruitment of different immune cells, and it is capable to regulate the anti-tumor response producing several mediators. For example, mast cells, macrophages, MDSCs, neutrophils and Treg cells support the formation of a TME favorable the tumor growth and progression, while cytotoxic NK cells and T lymphocytes appear to be involved in the anti-tumor response, promoting a suppressive-related TME (Larsen, 2018).

Various studies reveal that IL-33 recruits and activates $CD8^+$ T lymphocytes and NK cells in TME. One report, for example, has shown that the administration of IL-33 in mice inoculated with lung adenocarcinoma cells is able to promote $CD8^+$ T and NK cell-mediated anti-tumor immunity with consequent decrease of tumor growth (Xu, 2020). Furthermore, the transgenic expression of IL-33 in melanoma (B16) and breast cancer (4T1) cells is able to increase the number of $CD8^+$ T lymphocytes and NK cells that infiltrate the tumor and produce IFN-γ, promoting the development of a TME prone to tumor eradication (Gao, 2015). In a mouse model of hepatocarcinoma, injection of IL-33 expressing tumor cells resulted in slower tumor growth due to the increased percentage of $CD4^+$ and $CD8^+$ T cells in liver and to enhanced production of IFN-γ (Jin, 2018). However, IL-33 is also able to recruit $CD4^+$ $Foxp3^+$ Tregs, promoting evasion of the immune response and thus promoting tumor growth.

In a study using Treg cells that do not express IL-33 (Treg $^{IL\text{-}33\text{-}/\text{-}}$), it was shown that these cells have an attenuated immunosuppressive function and, following activation, they undergo epigenetic modifications that promote the expression and production of IFN-γ, thus exerting anti-tumor function (Hatzioannou, 2020). These studies highlight the role of IL-33 in the modulation of the adaptive immune response, resulting in an anti- or pro-tumor function depending on the type of lymphocytes that it activates.

IL-33/ST2 axis is also able to recruit and activate innate immune cells. Tumor associated macrophages (TAMs) represent a population of immune cells infiltrating solid tumors. Based on specific factors present in TME, TAMs can differentiate into type 1 (M1) macrophages, with anti-tumor properties, or type 2 (M2) macrophages, which promote tumor proliferation and progression (Pan, 2020). In an immunodeficient mouse model, in which tumor tissues from patients with non-small cell lung cancer (NSCLC) were transplanted, blocking of IL-33 using inhibitory antibodies, produced a decrease in tumor growth and an inhibition of the polarization in the M2 direction of the TAMs, as well as a reduced accumulation of Tregs in the TME (Wang, 2017).

Furthermore, IL-33 activates basophils by increasing the expression of granzyme B and the CD63 degranulation marker. The co-culture between IL-33-activated murine basophils and B16.F10 metastatic melanoma cells decreased tumor cell growth (Marone G. G., 2020).

IL-33 can directly activate human (Cherry, 2008); (Suzukawa M. K., 2008) and mouse (Lucarini, 2017); (Andreone, 2019) (Kienzl, 2020)

eosinophils, increasing expression of CD69 (marker of early activation of hematopoietic cells), of adhesion molecules (ICAM-1, CD11b/CD18), and of degranulation markers (CD63 and CD107a) resulting in the killing of several tumor cell types. In mice implanted with subcutaneous melanoma B16.F10 cells, intra-peritoneal treatment with IL-33 induced an increased migration of eosinophils and $CD8^+$ T cells on tumor site, promoting a reduction of tumor growth. Moreover, in a melanoma metastasis model, intra-nasal injections of IL-33 prevents metastasis formation, as a consequence of eosinophil recruitment to the lung. Of note, selective depletion of eosinophils by *in vivo* treatment with a Siglec-F antibody, abrogated the ability of IL-33 to both restrict primary tumor growth and metastasis formation, indicating that the anti-tumor activity of IL-33 is eosinophils-dependent (Lucarini, 2017). Similar results were obtained in models of transplantable and colitis-associated colorectal cancer. Here, tumor growth reduction induced by IL-33 was abrogated in eosinophil-deficient DdblGATA-1 mice, but was restored by adoptive transfer of eosinophils activated with IL-33 *ex vivo* (Kienzl, 2020).

We recently demonstrated that IL-33 can activate murine eosinophils promoting tumor cell killing in a contact dependent way. In particular, IL-33 is able to increase CD11b/CD18 integrin complex expression on eosinophils, stimulating cell-cell adhesion and consequent release of cytotoxic granules at the level of the immunological synapse (Andreone, 2019).

2. AIM OF PROJECT.

Trogocytosis is an active process whereby a live cell bites a target cell in a contact-dependent manner, resulting in the acquisition of novel functions in recipient cells and in the loss of cellular functions in donor cells (Uribe-Querol, 2021). With the exception of eosinophils, this process has been described in virtually all immune cells (Bettadapur, 2020) and it represents an important mechanism of cell-cell communication and immune regulation in infection and in cancer. Eosinophils are emerging as important components of the tumor microenvironment (Varricchi G, 2017). Our previous studies showed that activation of eosinophils with the alarmin IL-33, promotes their tumoricidal functions resulting in tumor growth inhibition *in vitro* and *in vivo* (Lucarini, 2017) (Andreone, 2019).
In the present study, we evaluated trogocytosis in eosinophils and the possible implications in tumor immunity. By using murine bone-marrow derived resting (CTR EO) or IL-33 activated eosinophils (EO-33) we investigated whether these cells may perform trogocytosis upon contact with different tumor cells (B16.F10 melanoma, EG.7-OVA lymphoma, TC-1 lung carcinoma, MC38 colon carcinoma and MCA205 fibrosarcoma). We explored the ability of eosinophils to acquire specific molecules from target cells and focused on immune checkpoints (i.e PD-1 and TIGIT), exploring the mechanisms underlying this particular process. We investigated the engagement of integrin complex CD11b/CD18 and Programmed Cell Death 1 Ligand 1 (PD-L1) during the acquisition of PD-1 on eosinophils membrane. Of note, since the molecular mechanisms of

trogocytosis are still unclear, we proposed a hypothetical molecular model for the stabilization of the immunological synapse that may occur in eosinophils, and possibly in other innate immune cells, enabling trogocytosis.

Trogocytosis of immune checkpoints by eosinophils may not only confer a new role in immune response to these granulocytes but also impact cancer immunotherapies.

3. MATHERIAL AND METHODS.

3.1 Generation of bone marrow-derived eosinophils.

Bone marrow-derived murine eosinophils were obtained following a protocol already described (Andreone, 2019), but with some modifications. Briefly, bone marrow cells from naïve C57Bl/6 mice were cultured in RPMI 1640 containing 20% FBS, 1% glutamine, 25 mM Hepes, 1X NEAA, 1 mM sodium pyruvate, supplemented with 100 ng/mL SCF and 100 ng/mL FLT3-L (all from Cell Guidance Systems). rmIL-5 (10 ng/mL; Peprotech) was added to the culture at day 4, 8, 10 and 12 with fresh medium. At day 14, eosinophils were harvested and re-plated at the concentration of $1{,}5x10^6$/ml in the presence of 100 ng/mL rmIL-33 (Biolegend; EO-33) or 10 ng/ml rmIL-5 (control EO). In some experiments, eosinophils were exposed to the following stimuli: 100 ng/ml rmCCL11 (Biolegend); 2 ug/ml SEB (Sigma-Aldrich); 100 ng/ml IL-25 (R&D Systems, Minneapolis, MN, USA) 100 ng/ml TSLP (R&D Systems, Minneapolis, MN, USA); 25 ng/mL IFN-γ (Sigma-Aldrich); 0,5 μg/ml LPS (Sigma-Aldrich); 100 μg/ml Poly I:C (Sigma-Aldrich). GM-CSF (10 ng/mL; Cell Guidance Systems) was added to the cultures 24 h prior to use (day 15 or 16). Eosinophil purity (>80%) was determined by flow cytometry ($CD11b^+Siglec\text{-}F^+Ly6G^-CD11c^-$).

3.2 Tumor cell lines.

Murine EG.7-OVA lymphoma cells (CRL-2113; American Type Culture Collection), B16.F10 metastatic melanoma cells (ATCC, CRL-6475), MC38 colon carcinoma cells (kindly provided by Dr. Carlos Alfaro, University of Navarra, Pamplona, Spain), TC-1 lung carcinoma cells (kindly provided by Dr. Guido Kroemer, Gustave Roussy Cancer Institute, Villejuif, France) and MCA205 fibrosarcoma cells (Merck Millipore, Burlington, MA, USA, SCC173) were used.

3.3 Trogocytosis assays.

Tumor cells were labelled with either PKH67 Green or PKH26 Red fluorescent Cell Linker (Sigma) according to the manufacturer instructions and then co-cultured with control or stimulated eosinophils at 4:1 EO:tumor cells ratio for 1 hour for confocal microscopy analyses and at 2:1 for up 2 hours for flow cytometry analyses. In experiments with CD18 or PD-L1 antagonist, IL-33 activated eosinophils were pre-incubated with 10 µg/mL CD18 monoclonal antibody (mAb) (clone M18/2, Biolegend) or PD-L1 mAb (Clone 10F.9G2, Biolegend) for 25 min at 4°C prior the co-culture with PKH-labelled or not tumor cells. For trogocytosis assay by confocal laser scanning microscopy (CLSM), cells were transferred onto poly-L-lysine-coated coverslips and the cover

glasses were mounted on microscope slides with Vectashield antifade mounting medium containing DAPI (Vector Laboratories). To detect trogocytosis of PD-1 and TIGIT, the cells were stained with TIGIT (clone 1G9, Biolegend) and PD-1 (clone J43, eBioscience, Invitrogen). DAPI was used to distinguish cells nuclei and in some experiment, we added the anti-Siglec-F (clone E50-2440, BD Bioscience) label to better distinguish eosinophils. When needed cells were labelled with PD-L1 mAb (clone 10F.9G2, Biolegend). CLSM observations were performed with a Leica TCS SP2 AOBS apparatus, using a 63X/1.40 NA oil objective and excitation spectral laser lines at 405, 488 and 594 nm. Image acquisition and processing were carried out using the Leica Confocal Software 2.6 rel 1537 (Leica Microsystems) and Adobe Photoshop CS5 software programs. Signals from different fluorescent probes were taken in sequential scan settings. Several cells for each condition were analyzed for quantitative analyses and representative images of PKH^+ or $PD\text{-}1^+$ or $TIGIT^+$ eosinophils.

For trogocytosis assays by flow cytometry, at the end of the co-culture, cells were labelled with BV421 Siglec-F mAb (BD Biosciences) in the presence of 0.5 mM EDTA to disrupt cell conjugates and analysed by flow cytometry. The percentage of trogocytosis in eosinophils was evaluated as percentage of PKH^+/ $PD\text{-}1^+$/$TIGIT^+$ cells in a population gated on Siglec-F^+ and doublets exclusion.

3.4 Time-lapse video microscopy.

For analyses of trogocytosis of eosinophils by time-lapse video microscopy, MC38 and TC-1 cells were labelled with PKH26 Red fluorescent Cell Linker and 24h prior the co-culture plated on Cellvis 6 Well glass bottom plate with high performance #1.5 cover glass (Fisher scientific). Control or activated eosinophils were added to the culture at a 2:1 EOS: tumor cell ratio. Time lapse recordings were performed over a period of 1 h at a 30 seconds interval with a 20X objective in a Zeiss LSM 900 confocal microscope.

3.5 Ultrastructural analysis by transmission electron microscopy (TEM).

Control and IL-33 activated eosinophils were co-cultured with B16.F10 melanoma cells (10:1 ratio) for 60 min at 37°C and then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Following washes in cacodylate buffer, cells were post-fixed in 1% OsO4 in the same buffer and further washed with 0.1 M cacodylate. Cells were then dehydrated in ethanol gradient from 50% to 100% (v/v) and embedded in Agar 100 resin (Agar Scientific, Essex, UK) at 65°C for 48 h. Ultrathin sections were obtained using an ultra-microtome and collected on 200-mesh grids, counterstained with uranyl acetate for 10 min and lead citrate for further 10 min. Samples

were observed in a Philips 208s transmission electron microscope at 100 kW (Philips, Amsterdam, The Netherlands).

3.6 Immune checkpoint expression by qPCR.

Control and IL-33 activated eosinophils were sorted in a MoFlo Astrios EQ (Beckman Coulter, Pasadena, CA, USA) as population $CD45^{+}$, $Ly6G^{-}$ and $Siglec\text{-}F^{+}$ at day 15 or 16. Total RNA was extracted from cells by using TRIsure reagent (Bioline, London, UK). mRNA was reverse transcribed by means of Tetro cDNA Synthesis Kit (Bioline). Quantitative reverse transcription-PCR (qPCR) with forward and reverse primers for TIM-3, LAG-3, TIGIT, PD-1, PD-L1 and HPRT (Biomers.net the biopolymer factory) was performed using Sensimix Plus SYBR Kit containing the fluorescent dye SYBR Green (Bioline) and by means of an ABI 7500 Real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, Ma, USA). The conditions of real-time PCR reaction were given as follows: 15 sec at 95 °C, 30 sec at 60 °C, and 45 sec at 72 °C (45 cycles). Triplicates were performed for each experimental point. Data were normalized to HPRT with 2^{-DCt} method (Schmittgen, 2008).

3.7 Flow cytometry.

For phenotypic analyses, bone marrow-derived eosinophils and tumor cells when occurred were stained with the following fluorescently labeled mAbs from BD Biosciences, BD Pharmigen, Biolegend or Thermo Fisher: anti-PD-1 (clone J43), anti-CD11b (clone M1/70), anti-PD-L1 (clone 10F.9G2), anti-PD-L2 (clone TY25), anti-TIGIT (clone 1G9), anti-CD69 (clone H1.2F3), anti-CD45 (clone E50-2440), anti-Ly6G (clone 1A8), anti-Siglec-F (clone E50-2440), anti-TIM-3 (RMT3-23) and anti-LAG-3 (C9B7W). Samples were run on a Gallios flow cytometer and analysed with the Kaluza Analysis Software (both from Beckman Coulter).

3.8 Statistical Analysis.

Statistical analyses were performed using GraphPad Prism Software (GraphPad, La Jolla, CA). One-way ANOVA analysis of variance was performed to compare means among multiple groups, followed by post hoc testing (Tukey). Mann-Whitney test was used for the nonparametric analysis of differences between two groups. Values were considered significant when the probability was below the 5% confidence level ($p < 0.05$).

3.9 Protein docking analysis and generation of 3D in silico protein models.

Whenever required, protein structure database codes from the Universal Protein Resource (UniProt, https://www.uniprot.org) were transformed into the equivalent for the U.S. Data Center for the Global Protein Data Bank (RCSB-PDB, https://www.rcsb.org). This procedure allows to convert a protein code and 3D structure from UniProt to the exact RCSB-PDB structural equivalent. To perform our *in-silico* studies, we employed the protein-protein docking webtool ClusPro (https://cluspro.org/), hosted by the Boston University (Boston, MA, USA) and Stony Brooks University (New York, NY, USA) with the aid of the 3D protein viewer PyMol (downloadable at https://pymol.org/). ClusPro (Kozakov D. H., 2017) is an automated and versatile protein docking server, based on the Fast Fourier Transform (FFT)-based software PIPER (Kozakov D. B., 2006). In some cases, the Uniprot-derived models were obtained by using the Swiss-Model NGL protein viewer from EXPASY website (https://swissmodel.expasy.org/). This protein viewer is equipped with adjunctive visual options to show some particular features of the 3D model.

4. RESULTS.

4.1 IL-33 activated eosinophils acquire membrane fragments from tumor cells.

In order to investigate whether eosinophils are able to perform trogocytosis, we used as model murine control (CTR EO) or IL-33 activated bone-marrow derived eosinophils (EO-33), obtained through a differentiation protocol already described by our group (Andreone, 2019) (Lucarini, 2017), with some modifications (see Methods and **Figure 1**).

Figure 1. Schematic representation of generation of eosinophils from mouse bone marrow. Bone marrow was extracted from tibiae and femurs of C57BL/6 mice. Bone marrow cells were cultured with Flt3-L (100 ng/ml) and SCF (100 ng/ml) for 4 days followed by IL-5 (10 ng/ml). At day 14, cells were further grown with IL-5 (control EO) or with 100 ng/ml IL-33 (EO-33) till day 16. GM-CSF was added to eosinophil cultures 24 h before cell harvesting.

In a first set of experiments, we sought to explore whether eosinophils were able to acquire any membrane fragments from target tumor cells. To this end, we set up a co-culture between eosinophils and tumor cells labelled with the green fluorescent membrane dye PKH67, according to the scheme reported in **Figure 2**.

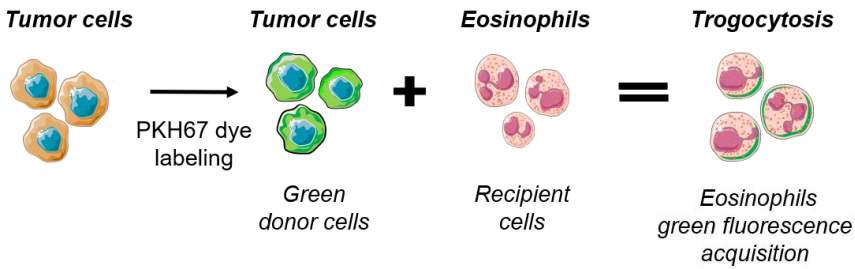


Figure 2. Schematic representation of trogocytosis assay. Tumor cells are labelled with the green fluorescent membrane dye PKH67 and then co-cultured at various ratios with eosinophils, either control or IL-33 activated, for up to 2 hours at 37°C. Trogocytosis is then evaluated by flow cytometry or confocal microscopy as acquisition of green fluorescence by eosinophils.

After 1 hour, by Confocal Scanning Laser Microscopy (CSLM) analysis we observed that EO-33 acquired green membrane fragments that directly derived from EG.7-OVA lymphoma cells, with higher extent than control eosinophils (**Figure 3A**).

To confirm these results, we analysed eosinophils trogocytosis with other target tumor cells and through flow cytometry. The indicated tumor cells (B16.F10, TC-1, MCA205, and MC38) were labelled with PKH26 red dye and co-cultured with control eosinophils or EO-33. After 2 hours incubation, cells were then stained with BV421 Siglec-F mAb and the eosinophils trogocytosis was evaluated by gating on Siglec-F^+ and $PKH26^+$ red cells, in order to detect eosinophils that had acquired red membrane fragments from target tumor cells. Flow cytometry analysis confirmed that EO-33 are far more efficient at acquiring membrane fragments through trogocytosis from different tumor cells, as compared to resting eosinophils (**Figure 3B**).

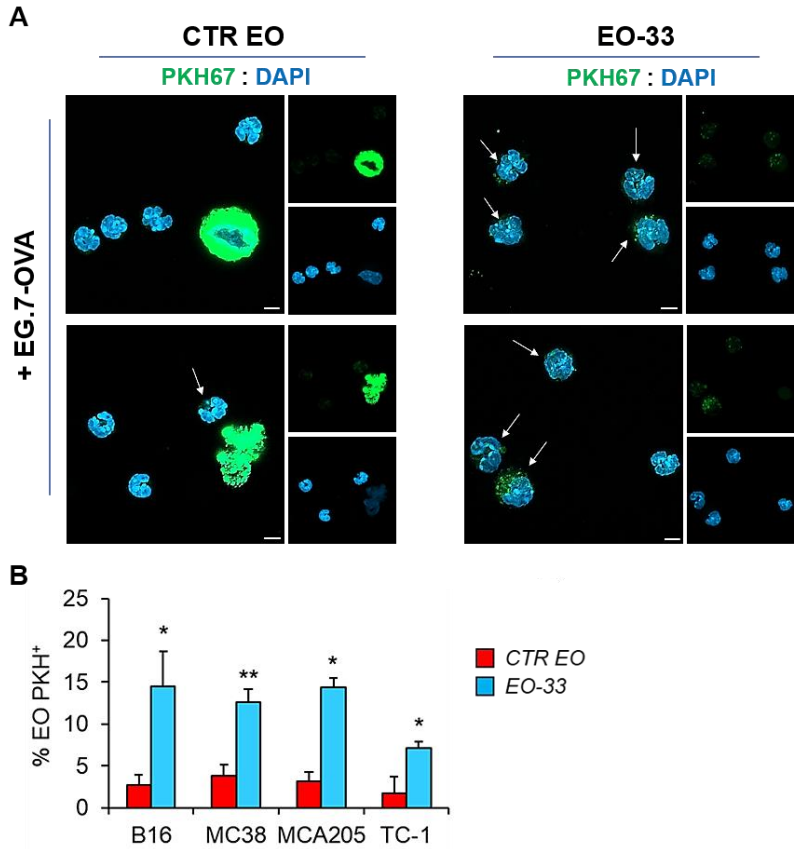


Figure 3. Increased trogocytosis in IL-33 activated eosinophils. (**A**) CLSM images of control (CTR EO) or IL-33 activated (EO-33) bone marrow-derived murine eosinophils co-cultured with PKH67-labeled EG7.OVA lymphoma cells (4:1 ratio).

DAPI stain allowed to distinguish multilobate eosinophil nuclei. Arrows indicate the capture of PKH67 membrane dye by several EO-33. Rare events of trogocytosis operated by CTR EO are indicated. Inserts represent separate channel images. Images are representative of two independent experiments. Bar, 5 micron. (**B**) Flow cytometry analysis of trogocytosis in control (CTR EO) *vs* IL-33 activated eosinophils (EO-33) co-cultured (4:1 ratio) with the indicated tumor cell lines labelled with PKH26 red dye. Trogocytosis in eosinophils was evaluated through flow cytometry by detecting Siglec-F^+ $PKH26^+$ cells following doublets exclusion. Mean ±SD of culture triplicates is shown. One representative experiment out of two is shown. *P<0.05; **P<0,001.

4.2 Trogocytosis in IL-33 activated eosinophils (EO-33) requires cell adhesion *via* CD11b/CD18.

It has been shown that cell-cell contact is a necessary condition for trogocytosis (Matlung, 2018) (Treffers, 2019) (Ustyanovska Avtenyuk N. C., 2021). In our lab we have already demonstrated that cell-cell adhesion through the CD11b/CD18 integrin complex is mandatory for EO-33 degranulation and tumor cell killing (Andreone, 2019).

Through transmission electron microscopy (TEM) we observed that IL-33 activated eosinophils that take contact with B16.F10 melanoma cells form typical protrusions, termed “trogocytic invaginations” (Matlung, 2018), that surround membrane portions of

melanoma target cell and help in the trogocytosis process (**Figure 4A-B**).

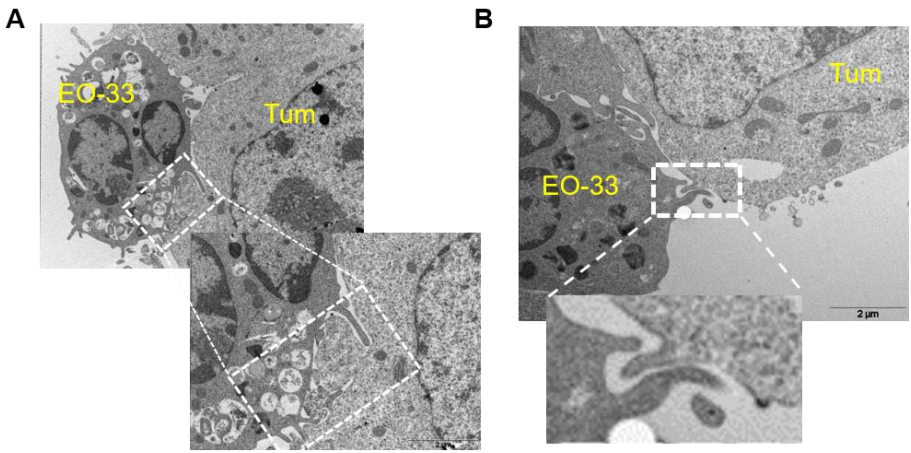


Figure 4. Ultrastructural analysis of trogocytosis in IL-33 activated eosinophils. IL-33 activated eosinophils were co-cultured at 2:1 ratio with B16.F10 tumor cells. Transmission electron (TEM) analysis shows (**A**) eosinophil embracing a tumor cell and (**B**) a trogocytic invagination at the interface of eosinophils with the tumor cell. The lower rectangles delineate a magnified image of the white dashed contour. Representative images of two independent experiments are shown.

Furthermore, time-lapse fluorescence microscopy revealed that trogocytosis by IL-33 activated eosinophils occurs rapidly (within 2 minutes) over MC38 (**Figure 5A**) and TC-1 (**Figure 5B**) tumor cells. In particular, these time-lapse videos show that the acquisition of tumor cell-derived red fluorescent membrane fragments operated by EO-33 occurs after several consecutive cell-cell contact and trogocytic events.

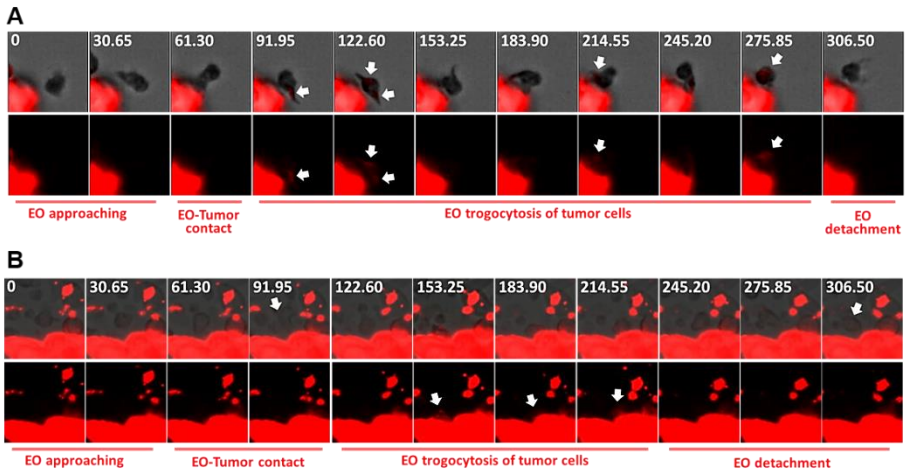


Figure 5. Trogocytosis in IL-33 activated eosinophils occurs early after contact with cancer cells. IL-33 activated eosinophil (EO) trogocytosis following contact with PKH26-labelled MC38 (**A**) and TC-1 (**B**) tumor cells. Representative time-lapse showing an activated eosinophil approaching tumor cells (red fluorescence) and acquiring tumor cell membrane fragments by multiple trogocytic events (arrows).

Numbers depict the elapsed time (in seconds) from the first acquired image frame. Time-lapse from one out of three independent experiments are shown.

We previously showed that IL-33 can increase the expression of CD11b/CD18 complex on eosinophils (Andreone, 2019) and we confirmed this data in our IL-33 activated bone marrow-derived eosinophils (**Figure 6A**). In order to explore whether cell-cell contact and whether CD11b/CD18 integrin complex are necessary for eosinophils to carry out trogocytosis, we co-cultured EO-33 and tumor cells in presence or absence of CD18 mAb, an antibody that blocks the CD11b/CD18 integrin complex without activating degranulation that would otherwise be triggered by direct CD11b ligation (Kato, 1998). EO-33 were pre-incubated with CD18 blocking antibody prior the co-culture with indicated $PKH67^{+}$ tumor target cells. Through CSLM quantification analysis, we observed a marked reduction of trogocytosis in presence of CD18 antibody blockade (**Figure 6B**).
Overall, these data demonstrate the importance of cell-cell contact and CD11b/CD18 adhesion in trogocytosis performed by eosinophils.

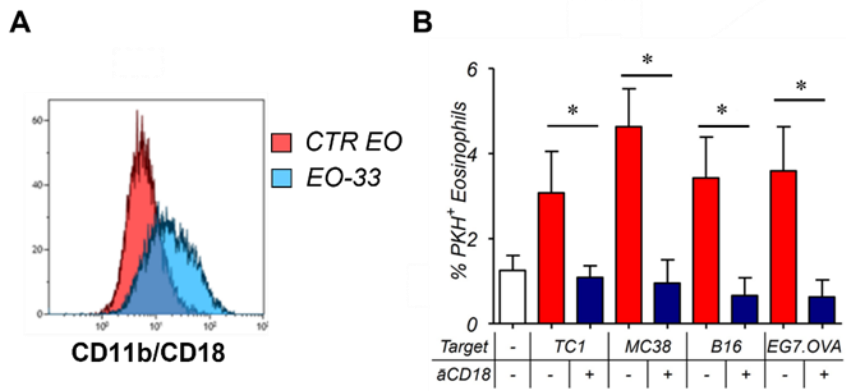


Figure 6. Role of CD11b/CD18 in trogocytosis by IL-33 activated eosinophils. (**A**) Flow cytometry analysis of CD11b/CD18 (Mac-1) surface membrane expression in control eosinophils (CTR EO) and IL-33 activated eosinophils (EO-33). Histogram plots are representative of one out of five independent experiments. (**B**) EO-33 were incubated with 10 µg/ml CD18 blocking antibody prior the co-culture with the indicated PKH67-labelled tumor target cells. The graph represents the effect of blocking CD18 mAb on eosinophil trogocytosis thought CSLM quantification. Mean of several fields ±SD from one out of two independent experiments is shown. *P<0.05.

4.3 Expression of immune checkpoint on bone marrow derived eosinophils and EG.7-OVA.

To evaluate whether eosinophils may acquire specific molecules from target tumor cells through trogocytosis we focused on immune checkpoint molecules. It has been already reported that eosinophils do not intrinsically express certain immune checkpoints (such as, TIM-3, LAG-3, TIGIT and PD-1), while they express myeloid related molecules, such as PD-L1 (Bouffi, 2013). Through quantitative real-time PCR (qPCR) and flow cytometry analysis we confirmed the lack of TIM-3, LAG-3, TIGIT and PD-1 mRNA (**Figure 7A**) and surface (**Figure 7B**) expression in highly purified control or IL-33 activated eosinophils. By contrast, eosinophils expressed PD-L1, which was further up-regulated by IL-33 as shown by both qPCR (**Figure 7A**) and flow cytometry analyses (**Figure 7B**).
We next screened the expression of immune checkpoints molecules not expressed by eosinophils (i.e., TIM-3, LAG-3, TIGIT and PD-1) in all tumor cell lines employed in our studies and we found weak expression in most of our tumor cell lines except for EG.7-OVA lymphoma cells, which expressed high levels of PD-1 and TIGIT (**Figure 8**). Based on these analyses, we selected EG.7-OVA cells as target tumor cells to study trogocytosis of immune checkpoints (PD-1 and TIGIT) in eosinophils.

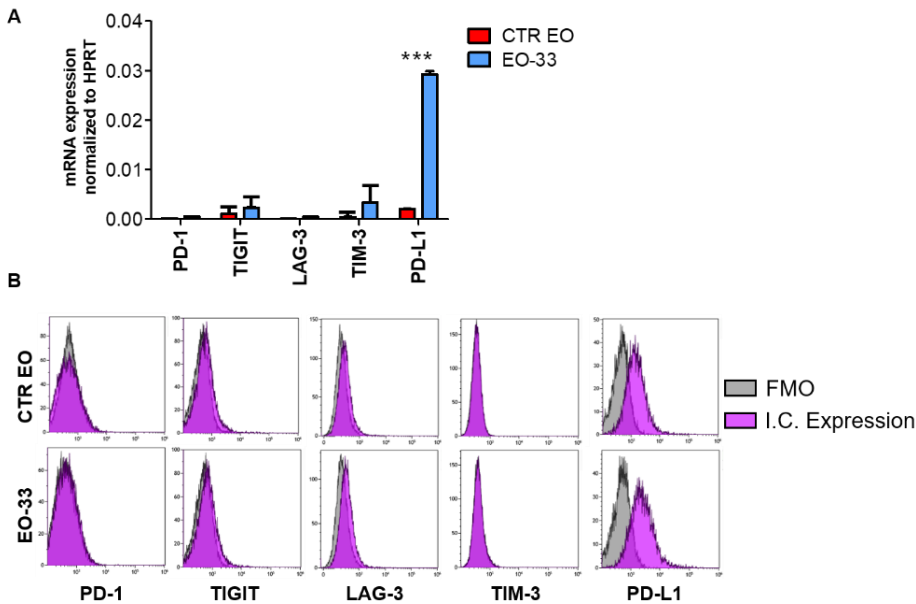


Figure 7. Expression of immune checkpoints in control and IL-33 activated eosinophils. (**A**) Quantitative PCR analysis of expression of indicated immune checkpoints in sorted control vs IL-33 activated bone marrow derived eosinophils. Data are expressed as mRNA expression normalized to HPRT. Histogram plots are representative of one out of three independent experiments. *P<0.05; **P<0,001; ***P<0,001. (**B**) Flow cytometry analysis of immune checkpoints on control and IL-33 activated bone marrow-derived eosinophils. Histogram plots are representative of one out of three independent experiments.

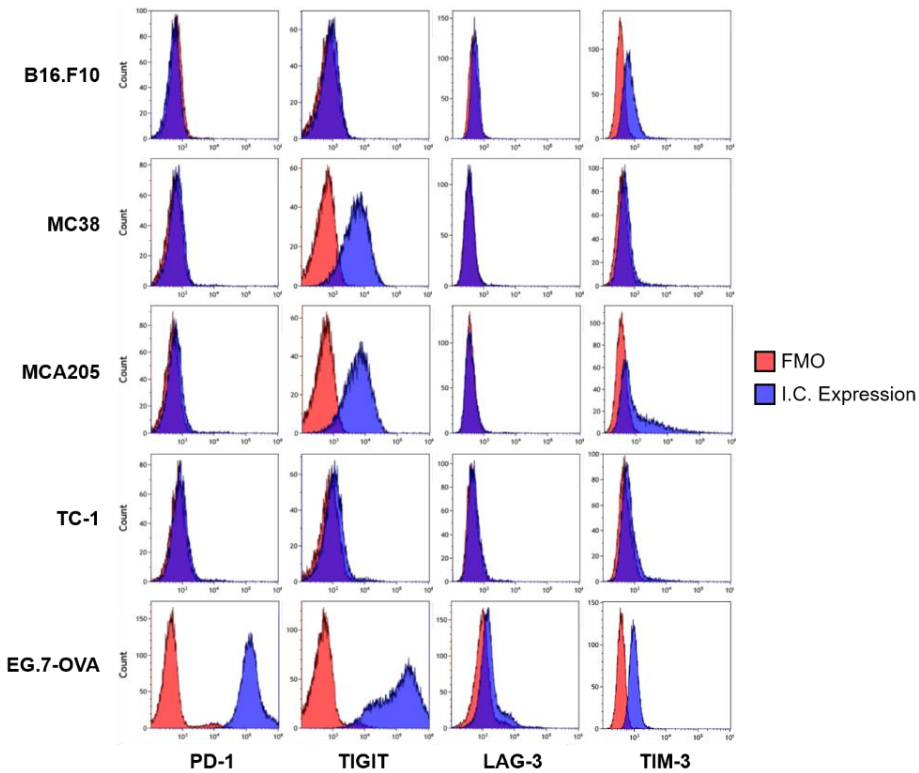


Figure 8. Expression of immune checkpoints on tumor cell lines. The indicated tumor cell lines were labelled with fluorescent antibodies directed to the indicated immune checkpoint molecules and analysed by flow cytometry. Histogram plots are representative of one out of three independent experiments.

4.4 IL-33 activated eosinophils acquire PD-1 and TIGIT through trogocytosis from EG.7-OVA cells.

The transfer of specific molecules during trogocytosis is a key point of this process. The gain or the loss of function subsequent the exchanges between recipient and donor cells are a new frontier in immunology studies (Hasim, 2022) (Marcenaro, 2009).
To evaluate the immune checkpoint trogocytosis operated by eosinophils we co-cultured EG.7-OVA lymphoma cells with control or IL-33 activated eosinophils, following the scheme reported in **Figure 9**.

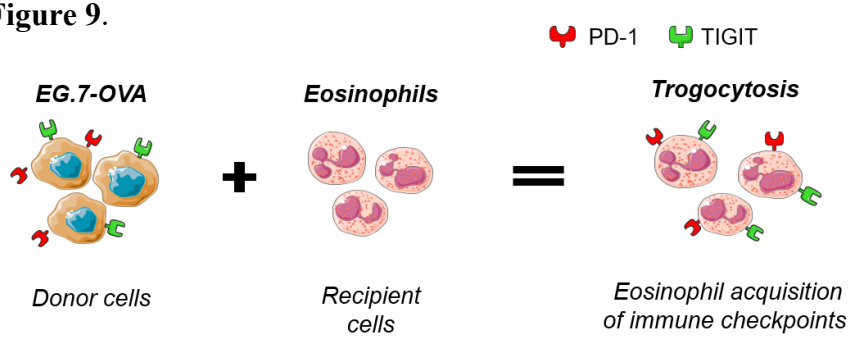
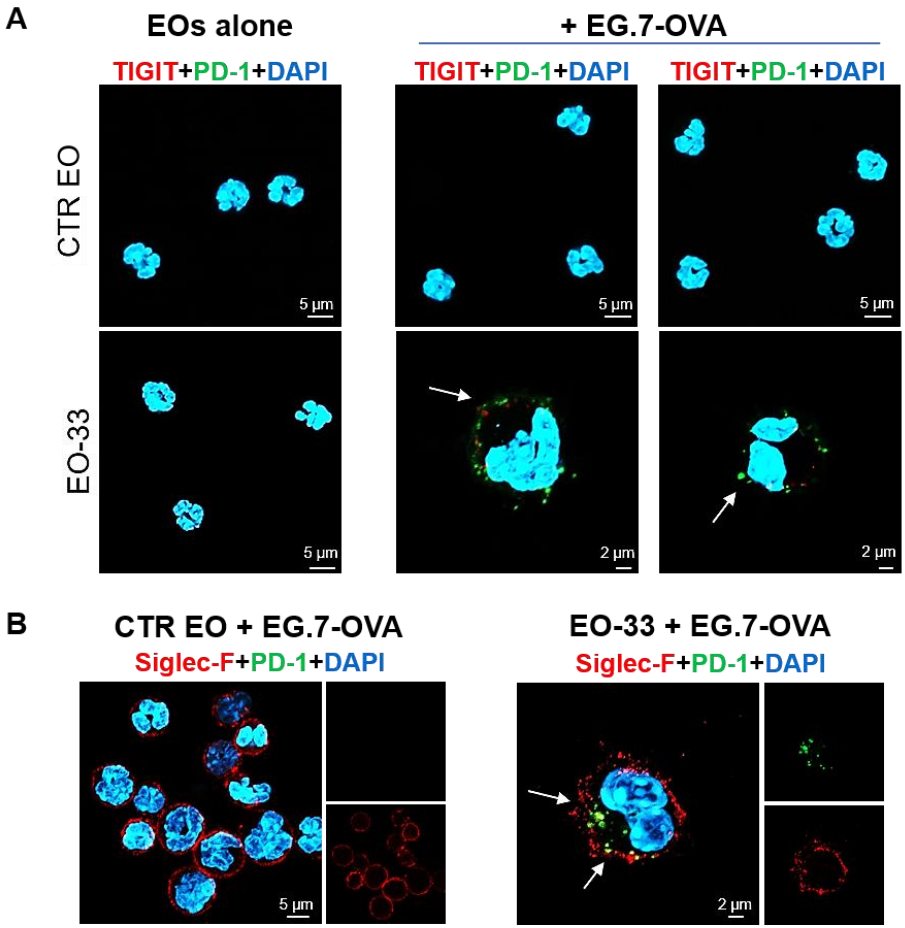


Figure 9. Representative scheme of immune checkpoint trogocytosis by eosinophils. EG.7-OVA cells expressing high levels of TIGIT and PD-1 (donor cells) are co-cultured at various ratios with eosinophils (recipient cells), either control or IL-33 activated, for up to 2 hours at 37°C. Trogocytosis of immune checkpoint molecules in recipient eosinophils is evaluated by flow cytometry or confocal microscopy by acquisition of TIGIT and/or PD-1 positivity.

CSLM analyses confirmed that both control eosinophils and EO-33 cultured alone do not express either PD-1 (green) or TIGIT (red) (**Figure 10A**). Remarkably, after 1-hour co-culture with EG.7-OVA lymphoma cells, EO-33 became positive for PD-1 (red) and TIGIT (green), strongly suggesting that these molecules were acquired from EG.7-OVA cells (**Figure 10A**). In contrast, control eosinophils rarely displayed PD-1 or TIGIT expression following co-culture with EG.7-OVA cells (**Figure 10A**). We further confirmed the acquisition of immune checkpoints by eosinophils by labelling the cells with the eosinophil-specific Siglec-F (red) antibody. **Figure 10B** shows that EO-33, but not control eosinophils, acquired PD-1 (green) after the co-culture with EG7.OVA.

To confirm these results, we analysed immune checkpoint trogocytosis also through flow cytometry. EG.7-OVA cells labelled with PKH26 red dye were co-cultured with eosinophils, and after two hours we analysed the percentage of cells that were Siglec-F^+, PKH^+ and PD-1^+ or $TIGIT^+$, thus extrapolating the percentage of eosinophils who had performed trogocytosis. As shown in **Figure 10C**, EO-33 acquiring the PKH dye (PKH^+) also acquired more TIGIT and PD-1 molecules from EG.7-OVA compared to control eosinophils. Moreover, this experiment reveals that this process is effector:target ratio dependent. These data indicate that IL-33 activated eosinophils succeed to acquire immune checkpoints from target tumor cells and express these molecules on their membrane.


A
EOs alone
+ EG.7-OVA
TIGIT+PD-1+DAPI
TIGIT+PD-1+DAPI
TIGIT+PD-1+DAPI
CTR EO
EO-33
5 µm
5 µm
5 µm
5 µm
2 µm
2 µm
B
CTR EO + EG.7-OVA
EO-33 + EG.7-OVA
Siglec-F+PD-1+DAPI
Siglec-F+PD-1+DAPI
5 µm
2 µm

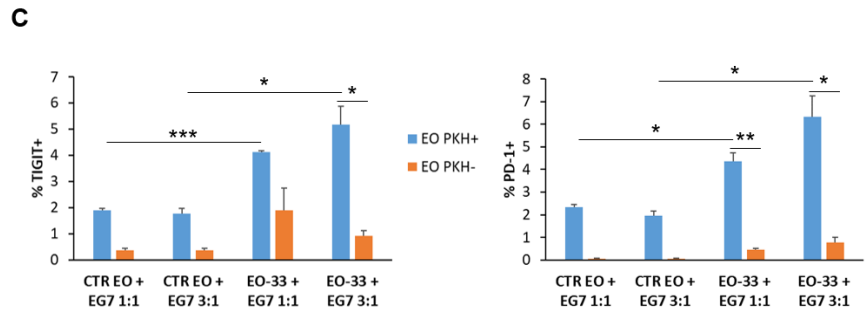


Figure 10. IL-33 activated eosinophils acquire immune checkpoint molecules *via* trogocytosis. (**A, B**) CLSM images of control (CTR EO) or IL-33 activated eosinophils (EO-33) co-cultured with EG.7-OVA lymphoma cells (4:1 ratio). (**A**) Arrows indicate the capture of PD-1 (green) and TIGIT (red) operated by EO-33. DAPI stain allowed to distinguish multilobate eosinophilic nuclei. Images are representative of two independent experiments (**B**) DAPI (blue) and Siglec-F (red) labelling allowed to further distinguish eosinophils. Arrows indicate the capture of PD-1 (green) by EO-33. Images are representative of two independent experiments. (**C**) Flow cytometry analysis of trogocytosis in CTR EO and EO-33 co-cultured with PKH26-labelled EG.7-OVA lymphoma cells at the indicated ratios at 37°C. Trogocytosis of immune checkpoint molecules by eosinophils was evaluated through flow cytometry by detection of TIGIT or PD-1 positivity in trogocytic ($PKH26^+$) vs non trogocytic ($PKH26^-$) Siglec-F^+ eosinophils. Mean ±SD of two experiments. *P<0.05; **P<0,001; ***P<0,0001.

4.5 Trogocytosis of PD-1 is receptor-dependent *via* eosinophil PD-L1.

Trogocytosis of specific molecules and the new function acquired by the recipient cell are subject of interest for cell biologists. Also important is understanding when this kind of process is receptor-mediated and when it occurs in trans-endocytosis (Anton, 2020), (Uribe-Querol, 2021), (Bettadapur, 2020).

PD-1 has two known ligands, namely PD-L1 and PD-L2, whose binding triggers potent inhibitory signals for immune response (Bardhan, 2016). As reported above, eosinophils express PD-L1, which is further up-regulated by IL-33. We confirm that the expression of PD-L1 is increased by IL-33 at the membrane level through flow cytometry (**Figure 11A**) and CSLM (**Figure 11B**). In contrast, PD-L2 is neither expressed by control nor in IL-33 activated eosinophils (**Figure 11A**). This finding excluded PD-L2 as a possible ligand for eosinophils PD-1 trogocytosis and therefore we focused on PD-L1.

To investigate receptor-mediated trogocytosis of PD-1, we first analysed the intracellular localization of PD-L1 molecule on eosinophils membrane after PD-1 trogocytosis. CLSM revealed that after co-culture between EO-33 and EG.7-OVA, PD-L1 (green) and PD-1 (red) co-localized on the membrane of eosinophils (**Figure 11C**).

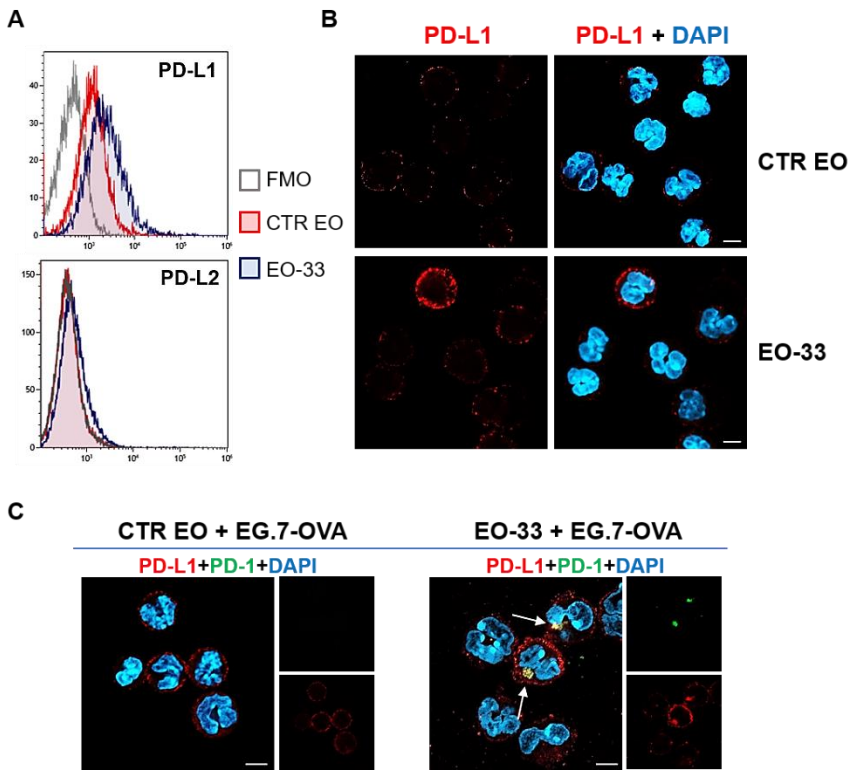


Figure 11. Co-localization of PD-1/PD-L1. (**A**) Flow cytometry analyses of PD-L1 and PD-L2 surface expression in control (CTR EO) and activated with IL-33 eosinophils (EO-33). Histogram plots are from one out of three independent experiments.

(**B**) CSLM images of PD-L1 membrane expression on control (CTR EO) and IL-33 activated eosinophils (EO-33). DAPI (blue) stain allowed to distinguish nuclei. Images are representative of two independent experiments. Bar, 5 micron. (**C**) CSLM images showing PD-1 and PD-L1 staining in control (CTR EO) and IL-33 activated eosinophils (EO-33) after co-culture with EG.7-OVA lymphoma cells. PD-L1 and PD-1 co-localization (yellow), selectively on EO-33 membrane, is shown in merged images and indicated by arrows. DAPI (blue) stain allowed to distinguish nuclei. Inserts represent separate channel images. Images are representative of two independent experiments. Bar, 5 micron.

This result led us to hypothesize that PD-1 trogocytosis may be mediated by PD-L1 engagement. To confirm this idea, we co-cultured EO-33 and EG.7-OVA in the presence of PD-L1 blocking mAb, to disrupt PD-L1/PD-1 interaction and we observed a marked reduction of PD-1 trogocytosis as revealed by CSLM (**Figure 12A-B**). These data indicate that trogocytosis of PD-1 operated by EO-33 is receptor-dependent through eosinophil PD-L1.

As mentioned above, cell-cell adhesion is crucial for trogocytosis (Treffers, 2019) (Matlung, 2018) (Li K. J., 2021). Moreover, CD11b/CD18 integrin complex is significant for eosinophils to exert their tumoricidal functions (Andreone, 2019). For these reasons, we wondered whether CD11b/CD18 blockade could also affect the trogocytosis of PD-1. Thus, EO-33 were incubated with CD18 blocking antibody prior the co-culture with EG.7-OVA lymphoma cells. Remarkably, we observed a clear reduction of PD-1

trogocytosis operated by EO-33 in the presence of CD18/CD11b blocking antibody, indicating that the cell-cell contact mediated by this integrin complex is necessary for trogocytosis of PD-1 (**Figure 12C, D**).

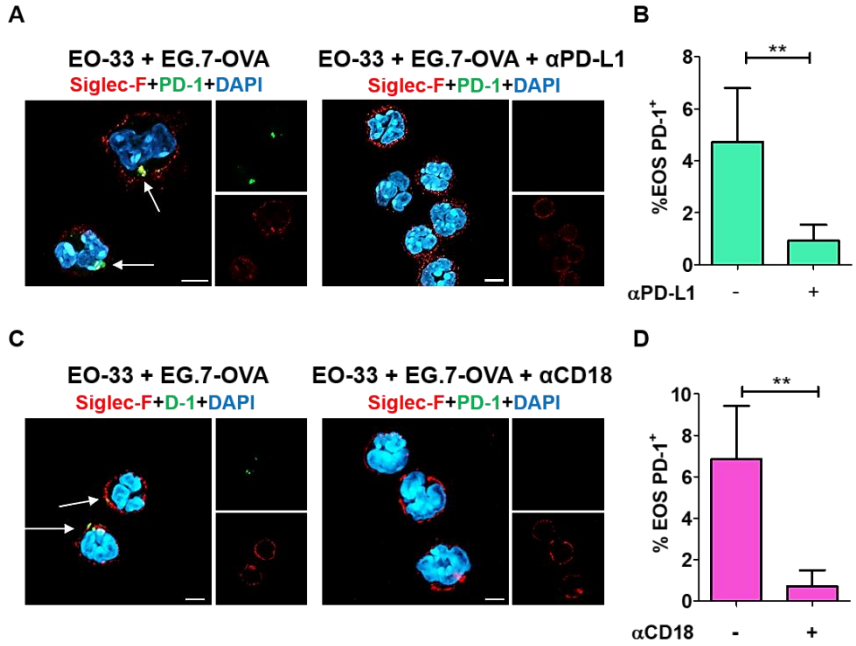


Figure 12. Effect of PD-L1 and CD11b/CD18 blockade on PD-1 trogocytosis of EO-33. (**A, B**) IL-33 activated eosinophils (EO-33) were incubated (or not) with 10 µg/ml PD-L1 blocking antibody prior co-culture with EG.7-OVA (4:1 ratio).

(**A**) CSLM images show PD-1 acquisition (green) by Siglec-F^+ eosinophils (red). Inserts represent separate channel images. DAPI (blue) stain allowed to distinguish nuclei. Images are representative of two independent experiments. Bar, 5 micron. (**B**) Quantitative analysis of PD-1 trogocytosis by EO-33. Mean of several fields ±SD from 2 replicates. **P<0.01. (**C, D**) IL-33 activated eosinophils (EO-33) were incubated (or not) with 10 µg/ml CD18 blocking antibody prior the co-culture with EG.7-OVA (4:1 ratio). (**C**) CSLM images show PD-1 acquisition (green) by Siglec-F^+ eosinophils (red). Inserts represent separate channel images. DAPI (blue) stain allowed to distinguish nuclei. Images are representative of two independent experiments. Bar, 5 micron. (**D**) Quantitative analysis of PD-1 trogocytosis by EO-33. Mean of several fields ±SD from 2 replicates. **P<0.01.

4.6 Increased activation in eosinophils following trogocytosis.

Since it is well reported that newly gained proteins after trogocytosis are functional molecules and that they usually conduct to new functions for recipient cells (Marcenaro, 2009), we questioned about the effect of trogocytosis on eosinophils biology. We co-cultured CTR EO and EO-33 with EG.7-OVA lymphoma cells labelled with PKH26 dye, and after two hours we analysed the activation state of eosinophils, by quantifying the expression of two eosinophils activation markers (i.e., CD11b and Siglec-F) (Suzukawa, 2008) (Johansson, 2017) (Johansson, 2014) on eosinophils that had performed trogocytosis (i.e., trogocytic eosinophils, PKH^+) as compared to those are not trogocytic (PKH^-). **Figure 13** shows an increment of both Siglec-F and CD11b on PKH^+ EO-33 compared with PKH^- eosinophils. This observation may indicate an activation role for trogocytosis on eosinophils**.**

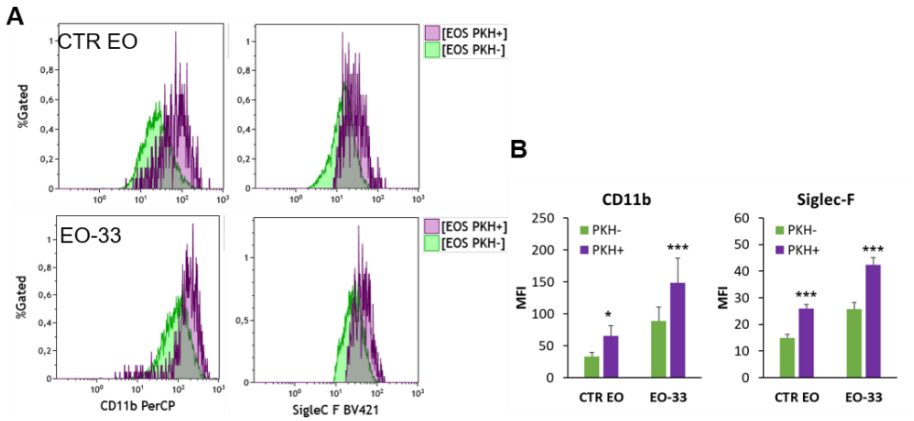


Figure 13. Phenotypic analysis of eosinophils following trogocytosis. Flow cytometry analysis of CD11b and Siglec-F expression in control (CTR EO) *vs* IL-33 activated eosinophils (EO-33) following trogocytosis of membrane fragments from PKH26-labelled EG7.OVA lymphoma cells. (**A**) Histogram plots represent the expression of Siglec-F and CD11b in trogocytic (PKH^+) *vs* non trogocytic (PKH^-) eosinophils. (**B**) Quantitative analysis of indicated markers expression (expressed as mean fluorescence intensity) in the two gated populations. Mean ±SD of two experiments. *P<0.05; ***P<0,001.

4.7 Studying eosinophils trogocytosis through other activation stimuli.

Next, we investigated whether, besides IL-33, other stimuli could also activate eosinophils and promote trogocytosis. For this purpose, we stimulated bone marrow-derived eosinophils (at day 14) with CCL11, Staphylococcal Enterotoxin B (SEB), IL-25, Thymic stromal lymphopoietin (TSLP), interferon-γ (IFN-γ), Lipopolysaccharide (LPS) or Poly I:C.

Through flow cytometry, we evaluated eosinophils activation markers, such as CD11b and CD69 as well as PD-L1. **Figure 14A-C** shows that, among all stimuli tested, only the chemokine CCL11 and LPS moderately affected eosinophil phenotype, by up-regulating CD11b or PD-L1, respectively. Expectedly, IL-33 up-regulated both the activation markers (CD11b and CD69) and PD-L1 (**Figure 14A-C**). To test whether stimulation with CCL11 or LPS was sufficient to trigger PD-1 trogocytosis in eosinophils, we co-cultured EG.7-OVA cells with eosinophils activated with CCL11 (EO-CCL11) or LPS (EO-LPS) and performed CLSM. While IL-33 activated eosinophils acquired PD-1 from EG.7-OVA cells, we found no trogocytosis of PD-1 in either EO-CCL11 or EO-LPS (**Figure 14D-F**).

These data may indicate that up-regulation of both CD11b and PD-L1 is necessary for eosinophils to be able to engage in PD-1 trogocytosis.

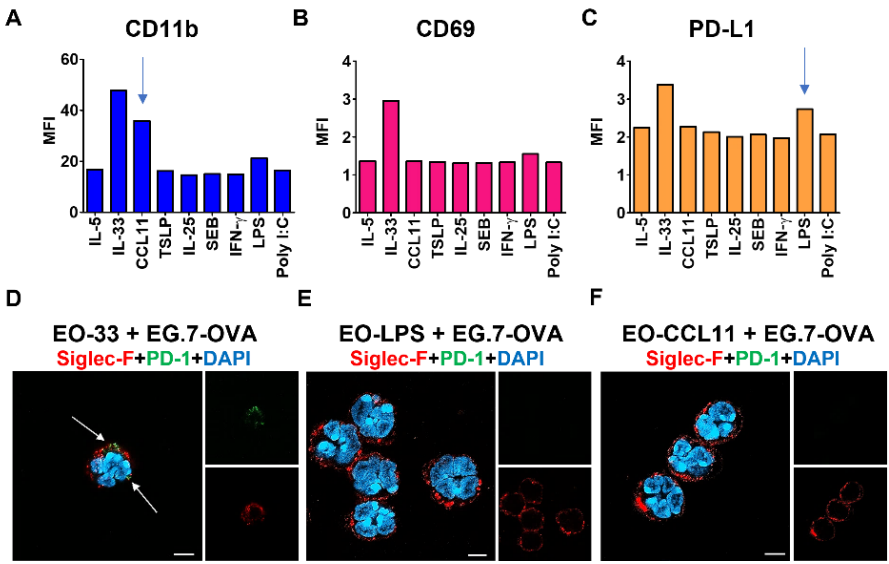


Figure 14. LPS and CCL11 do not induce trogocytosis in eosinophils. Flow cytometry analysis of CD11b (**A**), CD69 (**B**) and PD-L1 (**C**) membrane expression in murine bone marrow-derived eosinophils activated with the indicated stimuli. Concentrations used for stimuli is reported below: IL-5, 10 ng/ml; IL-33, 100 ng/ml; CCL11, 100 ng/ml; SEB, 2 ug/ml; IL-25, 100 ng/ml; TSLP, 100 ng/ml; IFN-γ, 25 ng/mL; LPS, 0.5 μg/ml; Poly I:C, 100 μg/ml.

(**D**,**E**,**F**) CLSM analyses of PD-1 trogocytosis in eosinophils activated with IL-33 (**D**) LPS (EO-LPS) (**E**) and CCL11 (EO-CCL11) (**F**) co-cultured with EG.7-OVA lymphoma cells. DAPI (blue) and Siglec-F (red) labelling allowed to distinguish eosinophils. PD-1 (green) is detected only in the co-culture between eosinophils activated with IL-33 (EO-33) and EG.7-OVA. Inserts represent separate channel images. Images are representative of two independent experiments. Bar, 5 micron.

4.8 In silico model of immunological synapse occurred in eosinophils trogocytosis.

Cell-cell contact is an essential condition for trogocytosis process and in immune cells it is mediated by different receptor molecules, depending on the cell type. In neutrophils, trogocytosis is dependent on both FcγR engagement and cell-cell interactions via the CD11b/CD18 integrin complex that mediates the formation of conjugates with cancer cells (Martinez Sanz, 2021) (Matlung, 2018) (van Spriel, 2001) (van Spriel A. B., 2003). Based on these premises and on our results, we hypothesized that CD11b/CD18 integrin complex could mediate the trogocytosis process in eosinophils. To test this, we evaluated a possible association of the CD11b/CD18 complex to two focal adhesion molecules, such as talin-2 and kindlin-3. Of note, kindlin-3 and talin-2 are necessary for the binding to and activation of integrins, including CD11b/CD18 (Bouti, 2021); (Critchley, 1999) and these integrins are able to bind specific focal adhesion molecules, including talins and kindlins, as reported previously (Sun, 2020) and as represented in our *in silico* model (**Figure 15A**). Strikingly, kindlin-3, but not kindlin-1 is selectively expressed in hematopoietic cells and has a key role for the long-term stability of CD11b/CD18 integrin complex (Fagerholm, 2014). These considerations reinforce the role of talins and kindlins (kindlin-3 for granulocytes in general) as key adapter molecules permitting integrins association with F-actin. Moreover, our computational model processed by the protein-protein docking platform ClusPro (Kozakov, 2017); (Kozakov D. B., 2006); (Comeau, 2004) suggests

that talin-2 and kindlin-3 not only bind to actin homotrimers but also combine to form a stable talin/kindlin complex (**Figure 15A**). These observations, summarized in our hypothetical model **(Figure 15B)**, confirm that the CD11b/CD18 complex can bind to actin through a stable association to talin-2/kindlin-3 dimers, which allows the clusterization of integrins onto the granulocyte membrane and a simultaneous local accumulation of F-actin filaments. The F-actin architecture inside the granulocyte membrane is pivotal for the generation of membrane tubules around the target cell membrane. On the other hand, this stable complex allows ligand on the target cancer cell to firmly bind the CD11b/CD18 complex in the immunological synapse and dictates the starting of the eosinophil trogocytosis process.

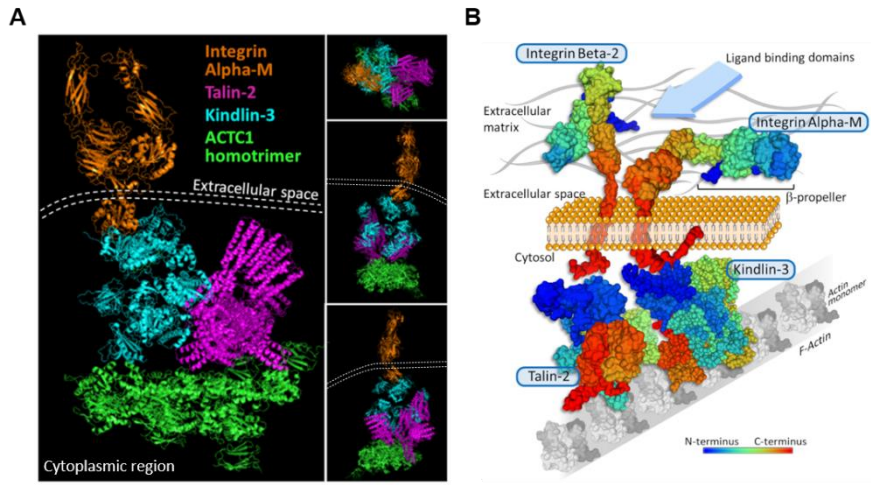


Figure 15. Association of CD11b/CD18 integrins to F-actin through talin-2 and kindlin-3 in eosinophils. (**A**) 3D *in silico* model (generated by ClusPro via sequential docking) of integrin Alpha-M (CD11b) showing its C-terminal sequence into the cytoplasmic region (part of integrin alpha-M binding the talin-2/kindlin-3/actin homotrimer multicomplex). Talin-2 and kindlin-3, two focal adhesion molecules, that contribute to the stability of the actin homotrimer. Dashed lines represent the cell membrane separating the two cell compartments (extacellular space and cytoplasmic region). Upper right boxes describe three different side views of the 3D *in silico* model. (**B**) Hypothetical surface model layout of human integrins CD18 (integrin Beta-2) and CD11b (integrin Alpha-M) associated to F-actin through talin-2 and kindlin-3 as an early signal for integrin/ligand stabilization and clusterization within the granulocyte cell membrane. The focal adhesion molecule talin-2 markedly activates Integrin-beta-2 thus increasing the affinity binding of the CD18/CD11b integrins to its ligand on cancer cells in the indicated cavity (arrow).

4.9 Role of the actin network in trogocytosis *via* talin/kindlin switchers.

To further elucidate the central role of F-actin in trogocytosis, we performed a set of *in silico* experiments (**Figure 16**) and demonstrated that actin homotrimers are capable to bind the complex Arp2/3 (**Figure 16A**), which allows diffuse ramification of F-actin fibers. Moreover, these experiments also show that ACTC1 homotrimers are able to strictly bind actin elongation elements (i.e., VASP and Profilin, **Figure 16B**), actin-stabilizing elements (i.e., Fascin, **Figure 16C**) and factors that dictate the curvature of the membrane surface (i.e., BAIAP2, **Figure 16D**).

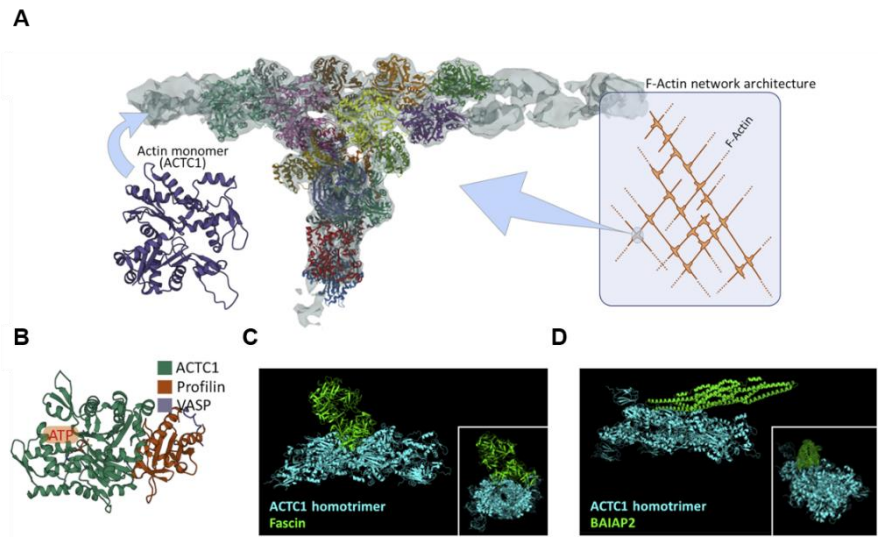


Figure 16. Models of relevant actin-binding molecules mediating the actin network architecture dynamics. (**A**) Arp2/3 subunit components (colored protein subunits) complexed to F-actin filaments (grey). These filaments are generated via ACTC1 monomers. Box shows the role of the Arp2/3 complex (light cyan circle) in the ramification of F-actin network architecture. (**B**) Actin (ACTC1) monomer associated with profilin and VASP, two elongation elements needed for the formation and extension of the F-actin network. Orange box depicts the ATP/GTP binding cavity in the ACTC1 monomer. (**C**) 3D *in silico* model obtained by ClusPro and showing fascin complexed to an ACTC1 homotrimer. Fascin binds ACTC1 monomers to maintain the stability of F-actin fibers. Box depicts a front view of the model. (**D**) 3D *in silico* model computed by ClusPro and showing a membrane deformation element which elicits membrane ruffling (BAIAP2) in complex with an ACTC1 trimer. Box illustrates a front view of the model.

5. DISCUSSION.

Eosinophils are granulocytes canonically associated with a detrimental role in allergic disorders (Massey, 2022). On the other hand, they may produce a protective immune response against helminths, bacterial, viral and fungal pathogens (Gaur, 2022). Moreover, eosinophils are implicated in the normal tissue homeostasis (Jackson, 2022). Eosinophils are found in the infiltrate of several human cancers, however their role in tumor progression is still under debate. In some cancers (i.e., melanoma, gastric, colorectal, oral and prostate cancer) eosinophils have been reported to exert anti-tumoral functions, while in others (Hodgkin's lymphoma, cervical carcinoma) they seem to play a pro-tumoral role (Varricchi G. G., 2018). This dichotomy may depend on the tissue type where the neoplasm forms, which may dictate the composition of the tumor microenvironment and, thus, the kind of soluble mediators released by eosinophils. In fact, eosinophils can release granule-associated cationic proteins (i.e., ECP, EPX) and other cytotoxic molecules (i.e., Granzyme, TNF-α), which may induce direct tumor cell killing, but they also produce pro-angiogenic factors that may promote tumor survival and progression (Mattei, 2020). Moreover, they can release chemokines (i.e.,CCL5, CXCL9 and CXCL10) that promote $CD8^+$ T cells recruitment to the tumor site, thus indirectly contributing to anti-tumor immunity (Carretero, 2015) (Lucarini, 2017). Our group has demonstrated that following activation with IL-33, eosinophils acquire the ability to kill tumor cells in a contact dependent way through the CD11b/CD18 integrin

complex that allows for synapse-polarized degranulation (Andreone, 2019). Here, we demonstrate that eosinophils employ this CD18/CD11b-guided immunological synapse to engage in trogocytosis with cancer cells.

Trogocytosis in IL-33 activated eosinophils was demonstrated by multiple approaches. First, flow cytometry and CLSM clearly demonstrated that after co-culture with various tumor cells labeled with PKH membrane dye, IL-33 activated eosinophils acquired the dye to a greater extent compared to resting eosinophils. Second, time lapse fluorescence microscopy revealed that acquisition of tumor cell-derived membrane fragments by eosinophils required multiple contact events and occurred within few minutes, as described for other immune cells (Miyake K. &., 2021). Third, ultrastructural analysis of eosinophil and tumor cell conjugates showed the formation of typical trogocytic invaginations resembling the tubular structures found in neutrophils during their trogocytic engagement with cancer cells (Matlung, 2018). Finally, our data demonstrated that blockade of CD11b/CD18, an integrin complex that is required for formation of eosinophil and tumor cell conjugates (Andreone, 2019), prevented eosinophils from trogocytosis of tumor-derived PKH. Although the transfer of different molecules between live cells could be mediated also through other mechanisms, such as extracellular vesicles (McCoy-Simandle, 2016), our data underline the fact that cell-cell contact and that CD11b/CD18 are necessary for trogocytosis performed by eosinophils.

Therapeutic targeting of T cells through neutralization of immune checkpoints has become the most promising direction in cancer immunotherapy. Despite eosinophils are not directly implicated in immune checkpoint therapies, some studies identify these granulocytes as biomarkers to develop better target therapies. For example, the absolute eosinophil count was positively correlated with the overall survival in patients with stage IV melanoma treated with at least one dose of ipilimumab (anti-CTLA-4) (Martens, 2016). Anti-CTLA-4 treatment in models of breast cancer leads to intra-tumoral eosinophil migration that is dependent on CCL11 and CCL5 secreted respectively from $CD4^+$ and $CD8^+$ T cells (Zheng, 2020). In another study, increased eosinophil counts during ipilimumab treatment correlated with longer survival rates of patients with melanoma (Lang, 2018). In head and neck squamous cell carcinoma patients, the treatment with Nivolumab (anti-PD-1) increases the relative eosinophil count and the ratio of eosinophil increase, which represented a prognostic score correlating with better overall survival (Nishikawa, 2021).

Here, we show that eosinophils acquire immune checkpoint molecules (i.e., PD-1 and TIGIT) from $PD\text{-}1^+$ and $TIGIT^+$ cells through trogocytosis. In fact, we selected EG.7-OVA lymphoma cells as donor tumor cells for their elevated expression of PD-1 and TIGIT and analysed the transfer by trogocytosis to resting and IL-33 activated eosinophils. Our CSLM and flow cytometry results clearly indicate that IL-33 activated eosinophils expressed PD-1 and TIGIT only after contact with target tumor cells. Although a definitive

demonstration of PD-1 and TIGIT trogocytosis requires genetic knock-outs of tumor and immune cells, it is worth pointing out that eosinophils do not intrinsically express these immune checkpoint molecules, as reported previously (Bouffi, 2013) and confirmed here by qPCR and flow cytometry analyses. Our finding adds to other reported studies showing that immune cells may acquire immune checkpoints through trogocytosis. For example, human $CD8^+$ T cells can take up functionally active PD-L1 from DCs in an Ag-specific manner, leading to killing of neighboring PD-1-expressing T cells (Gary, 2012). Furthermore, NK cells were shown to acquire PD-1 from leukemia cells via trogocytosis, which resulted in suppression of NK cell-mediated anti-tumor immunity (Hasim, 2022). Although the functional consequences of immune checkpoint acquisition by eosinophils are unknown, we observed increased expression of CD11b and Siglec-F molecules in the population of trogocytic eosinophils, indicating a major activation state, as observed for NK cells (Hasim, 2022). Whether these molecules are up-regulated as a result of trogocytic events or simply represent a population with higher expression of CD11b/CD18 integrin complex and, thus, with a higher chance to bind to target cells and engage in trogocytosis remains to be established. In any case, the increased expression of CD11b integrin on trogocytic eosinophils may further boost their adhesion activity to target cells, and it may induce tumor cell killing triggered by contact-dependent granules release (Andreone, 2019). This aspect still needs to be investigated, also because the effects of trogocytosis process on recipient cells could strongly depend on

which molecules are trogocytosed. On the other hand, also the donor cell deserves particular attention. In fact, certain immune checkpoint molecules, such as PD-1 and TIGIT, have been studied mainly on immune cells, while tumor cells are reported to usually express PD-L1. Only in recent years it has been shown that cancer cells occasionally express PD-1, even though the underlying mechanism of tumor cell-expressing PD-1 have not been deeply studied. In fact, tumor cell-intrinsic PD-1 have both an oncogenic and a tumor-suppressive role (Kim, 2021). In melanoma cells, intrinsic PD-1 expression increased the phosphorylation level of kinase S6 and eIF4E, promoting tumor growth (Kleffel, 2015) (Schatton, 2010). Instead, in lung cancer the expression of PD-1 inhibits tumor growth limiting the activation of AKT and ERK signaling (Wang X. Y., 2020). Our preliminary observations suggest that PD-1 loss does not induce any effect on tumor donor target (data not shown), but this aspect needs further investigation.

In most cases, trogocytosis in immune cells is triggered by ligand–receptor interactions (Joly, 2003). For example, TCR/MHC interactions mediate trogocytosis in T cells while the Fcγ receptor/antibody ligation is important for monocytes, macrophages, neutrophils and NK cells to carry out trogocytosis (Miyake K. &., 2021). We demonstrate here that eosinophils PD-1 trogocytosis is also dependent on receptor/ligand interaction and requires PD-L1. In fact, we found no expression of the PD-1 ligand PD-L2 on eosinophils, while PD-L1 was expressed and further up-regulated by IL-33 activation. CSLM revealed that the PD-1 molecules acquired

from EG.7-OVA donor cells co-localized with PD-L1 on the membrane of IL-33 activated eosinophils, selectively. Furthermore, blocking the interaction between PD-1 and PD-L1 with a PD-L1 antibody blockade prevented the trogocytosis of PD-1 by IL-33 activated eosinophils. Of note, PD-1 trogocytosis was also abrogated by blockade of the CD11b/CD18 integrin complex on eosinophils, indicating that both signals were required. This assumption is supported by the observation that trogocytosis could be triggered in eosinophils only upon IL-33 activation, which could increase the expression of both PD-L1 and CD11b/CD18. Instead, other stimuli increasing either PD-L1 (i.e., LPS) or CD11b (i.e., CCL11) failed to activate trogocytosis in eosinophils.

Trogocytosis occurs when recipient and donor cells take contact and establish an immunological synapse engaging different ligands and receptors depending on which cell types are involved (Mattei, 2020). We have already demonstrated that cell-cell contact through the CD11b/CD18 integrin complex is mandatory for IL-33 induced eosinophil degranulation and tumor cell killing (Andreone, 2019). Ligation of CD11b/CD18 with a CD11b antibody activates human eosinophils causing degranulation (Kato M. A., 1998) and CD11b/CD18 is also essential for eosinophils antibody-dependent cellular cytotoxicity (ADCC) against parasites (Capron, 1987). Interestingly, Matlung and colleagues demonstrated that neutrophil trogocytosis occurs through the engagement of CD11b/CD18 integrin complex that allows for the formation of conjugates with cancer cells (Matlung, 2018). Furthermore, they show that the

integrin-associated protein kindlin-3, through the binding to talin-2 and the β chain of the integrin, stabilizes both the interaction of CD11b/CD18 with the cancer target and the anchorage to the cytoskeleton (Bouti, 2021) (Matlung, 2018). Since eosinophils and neutrophils share the expression of the same adhesion molecules and integrins, it may be speculated that eosinophils employ a neutrophil-like mechanism (Matlung, 2018) to establish the immunological synapse over the target cells and to carry out trogocytosis, as corroborated by our *in silico* protein-protein docking analyses. Therefore, we propose a model highlighting key dynamical processes that dictate the entire trogocytic event, and that in eosinophils the CD11b/CD18-based immunological synapse is pivotal for the success of trogocytosis (**Figure 17**). First, the accumulation of F-actin fibers (*via* talin-2/kindlin-3 focal adhesion molecules) close to the CD11b/CD18 complex allows their clusterization on eosinophils membrane surface and then the association of the ligand on the cancer cell. Second, the Rho-guided actin polymerization generates the tubular membrane structures that in turn surround the cancer cell near the clusterization zone. Third, when the tubular structures fuse together, this terminates the eosinophil trogocytosis and then actin fibers depolymerize via the guanine nucleotide exchange factors (GEFs)-induced GTP formation, with a subsequent declusterization of the CD11b/CD18 units, dissociation of focal adhesion molecules and acquisition of ligand molecules on the eosinophil membrane surface. In summary, actin network (F-actin fibers), actin elongation and stabilizing factors (VASP, Profilin, Fascin, BAIAP2),

granulocyte-specific focal adhesion molecules (talin-2 and kindlin-3) represent central determinants for the stabilization of the CD11b/CD18-dependent immunological synapse and for the successful completion of eosinophil trogocytosis (**Figure 17**).

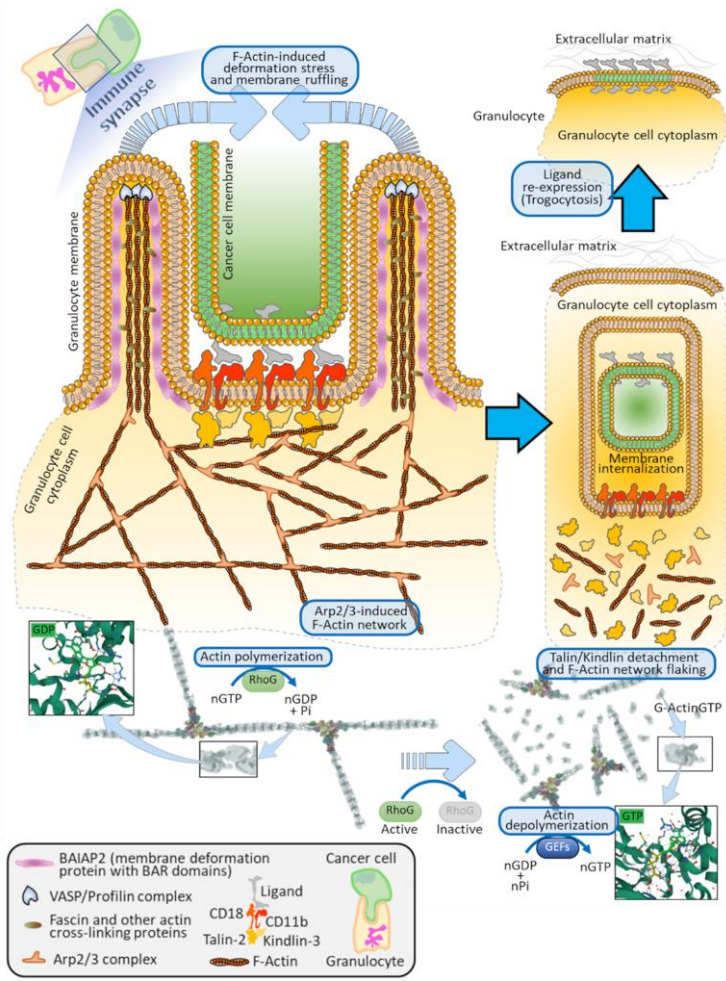

Immune synapse
F-Actin-induced deformation stress and membrane ruffling
Granulocyte membrane
Cancer cell membrane
Granulocyte cell cytoplasm
Arp2/3-induced F-Actin network
GDP
Actin polymerization
RhoG
nGTP
nGDP + Pi
Extracellular matrix
Granulocyte
Granulocyte cell cytoplasm
Ligand re-expression (Trogocytosis)
Extracellular matrix
Granulocyte cell cytoplasm
Membrane internalization
Talin/Kindlin detachment and F-Actin network flaking
G-ActinGTP
RhoG Active
RhoG Inactive
Actin depolymerization
GEFs
nGDP + nPi
nGTP
GTP
BAIAP2 (membrane deformation protein with BAR domains)
VASP/Profilin complex
Fascin and other actin cross-linking proteins
Arp2/3 complex
Ligand
CD18
CD11b
Talin-2
Kindlin-3
F-Actin
Cancer cell
Granulocyte

Figure 17. Role of actin network in CD11b/CD18-dependent trogocytosis through talin-2/kindlin-3 switchers. F-actin network reorganization acts as a key biomechanical driver for granulocyte trogocytosis and is strictly modulated by the CD11b/CD18 complexation to the ligand on cancer cell, in one side, and on the focal adhesion molecules, in the other side, to form the immune synapse. This model is specifically proposed for granulocytes since kindlin-3 is exclusively expressed on these cells. CD18/CD11b integrin-ligand complex clusterizes inside the granulocyte membrane surface (orange). This elicits the recruitment of talin-2 and kindlin-3 to bind the cytosolic regions of integrins. These two focal adhesion molecules also firmly bind the actin fibers, and this activates the accumulation of F-actin filaments close to the integrin/focal adhesion molecule clusters. Arp2/3 complexes allow the F-actin network to ramify locally, leading to the extesion of F-actin architecture. VASP, Fascins, and BAIAP2 permit the formation of peculiar tubular membrane structures, also sustained by actin polymerization via active RhoG, which in turn allows the local extension of the actin network through the degradation of GTP into GDP and phosphate (Pi). Free GDP then interacts with actin monomers (upper left box). Then, the tubular architectures on the granulocyte membrane, guided by the actin network driven biomechanical force, entrap the cancer cell membrane (green) and its ligands. The ligands on cancer cell membrane then detach from the integrins located on the granulocyte membrane. This leads to a dynamically spontaneous dissociation of talin-2 and kindlin-3 from the cytoplasmic region of the integrins and in turn to F-actin decomplexation, with subsequent actin network disaggregation. Concomitantly, the prevalence of inactive RhoG stimulates the GEFs-dependent F-Actin depolymerization and GEFs-induced GTP formation (lower right box), culminating with the exposure of ligands onto granulocyte membrane by sequential membrane fusion events. This event terminates the process of granulocyte trogocytosis of cancer cell membrane fragments with the re-expression of ligand molecules on the granulocyte membrane.

6. FUTURE PERSPECTIVES AND CONCLUSION.

Trogocytosis allows cells to expose on their membrane surface molecules they do not genetically express, acquiring specific signaling features provided by the captured molecules. With the exception for eosinophils, this process has been described in all immune cells and plays an important role in the regulation of immune responses to pathogens and cancer. Our findings demonstrate for the first time that eosinophils can carry out trogocytosis of tumor-derived membrane proteins and that this process is strongly enhanced by IL-33, an activating stimulus that promotes cell adhesion through CD11b/CD18. This integrin complex through focal adhesion molecules and association of F-actin fibers plays a pivotal role in the stabilization of the immune synapse that enables trogocytosis. Our experiments showing the eosinophils capture of immune checkpoint molecules (i.e., PD-1 and TIGIT) denote the role of receptor/ligand in driving trogocytosis. The finding that activated eosinophils can acquire immune checkpoints from tumor cells, also within the tumor microenvironment, may potentially affect immunotherapy response strategies in cancer patients. Future studies will be addressed to better understand the implications of eosinophil-driven immune checkpoint trogocytosis *in vitro*, by in-depth characterization of eosinophils that have captured immune checkpoint molecules and *in vivo*, in tumor-bearing mice undergoing immunotherapy. Moreover, we will evaluate eosinophils trogocytosis in a human setting on blood eosinophils from healthy donors.

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