ABSTRACT

Title of Thesis: CTL-DERIVED EXOSOMES ENHANCE THE ACTIVATION OF CTLS STIMULATED BY LOW AFFINITY PEPTIDES

Shu-Wei Wu, Master of Science, 2018

Thesis Directed By: Associate Professor, Dr. Zhengguo Xiao, Department of Animal and Avian Sciences

Cytotoxic T cell (CTL) is the key to induce an effective immune response against infections caused by intracellular pathogens, such as virus and cancer. CTLs with low affinity can induce and maintain a substantial population during an adaptive immune response, although CTLs with a highest-affinity receive competitive activation signals. Low-affinity CTLs are important to induce effector response and maintain a diverse memory repertoire. However, the mechanism of generating and maintaining the expansion of lower affinity CTLs is still unknown. Here, we showed that the presence of exosome (Exo) enhanced the CTL survival and increased the cell proliferation especially in CTLs treated with the low-affinity peptide. Exo together with peptides also improved the production of IFN-γ and GZB. The exosomal stimulation in CTLs was relative to up-regulation of CD25 expression, which enhanced the IL-2 survival signals. Moreover, Exo derived from an early stage of activation had a similar but weaker function comparing with Exo derived from a late stage of activation in activating CTLs. This study identified the important role of the exosome derived from CTLs stimulated by the highest-affinity peptide in activating the naïve T cells stimulated by the low-affinity peptide.

CTL-DERIVED EXOSOMES ENHANCE THE ACTIVATION OF CTLS STIMULATED BY LOW AFFINITY PEPTIDES

by

Shu-Wei Wu

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Advisory Committee:

Professor Zhengguo Xiao, Chair

Professor Debabrata Biswas

Professor Meiqing Shi

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Chapter 1: Introduction

Introduction of Cytotoxic T Lymphocytes (CTLs)

T cells develop in the thymus where they start to proliferate, differentiation and undergo a series of selection processes to obtain mature single positive (CD4+ or CD8+) T cells [1]. Mature T cells play a leading role in adaptive immunity against viral infection and cancer. CD8+ T cells, or so-called CTLs, are the key player in recognizing through TCR ligation, and killing infected or malignant cells through direct lysis, secreting antiviral cytokines and killing-related molecules, like perforin and granzymes (GZB) [1]. CTLs can also trigger cell apoptosis in a target cell through secretion of Fas and APO2L. As a result, CTLs are responsible for the destruction of cells infected by intracellular pathogens or malignant tumor cells via cytotoxic activity and antitumor immunity.

Once the pathogen is eliminated, a dramatic contraction of cell number happens and most of CTLs (around 90%) will undergo programmed cell death. Only a small population of CTLs will survive for a long-term as memory T cells. When encountering the same or similar antigen, memory T cells can react against secondary infection by providing faster, more robust and more effective immune response [1]. It is the fundamental principle for the vaccination development.

Due to the contribution to our immunity, CTLs are thought as a promising target for disease treatment and vaccine development, and many types of research are aimed at the identification of CTL activation.

T-cell activation and three signals

Naïve T cells need to be activated to undergo clonal expansion, develop functions and generate a long-term memory population after the clearance of antigen. Therefore, three kinds of signals are critical to activating naïve CTLs.

Traditionally, the full activation first requires T-cell receptors (TCRs) to recognize an antigen presented by antigen presenting cells (APCs), often from activated dendritic cells. Each T cell possesses a unique TCR to specifically recognize a foreign antigen. TCRs are expressed on the surface of a T cell and they are highly variable and unable to bind to antigen directly like the antibody. Therefore, TCRs need APCs to present broken-down peptides of antigens from pathogens through binding to major histocompatibility complexes I (MHC I). In addition, the pMHC/TCR interaction is very sensitive and the optimal kinetic is an important factor of T cell activation. For example, short time (less than 6hrs) exposure to antigen is enough to induce naïve CD8+ T cells to undergo multiple rounds of cell division [2, 3]. However, Mescher and his team have shown that T cells only exposed to antigen for a short-term did not survive and limited the colony expansion and impaired the effector function [4]. In order to further enforce the interaction, several adhesion molecules help to promote cell-cell adhesion and to respond to signaling via TCR [5]. In short, the formation of conjugation between a TCR on T cell and a peptide held in the MHC on APCs provide the first signal to initial activation of T cells and the interaction between TCR and antigen/MHC is very important.

Next, a second signal referred as co-stimulation and several cytokine signals are needed for T cell activation [6]. The co-stimulators and the third signals are usually

provided by dendric cells [7, 8]. Notable costimulatory molecules such as CD28, lymphocyte function-associated antigen 1 (LFA-1), and intercellular adhesion molecule 1 (I-CAM) are essential for T cell activation [9]. CD28 is a glycoprotein expressing constitutively on the surface of human T cells and on most of the murine T cells and can significantly enhance the downstream signaling from the triggered TCRs by simultaneously engaging with the cognate ligands B7.1 and B7.2 expressed on APCs [10]. Some see interleukin-2 (IL-2), which signals through Janus kinase (JAK) [11], as the T cell growth factor that help T cells to undergo several rounds of cell proliferation and enhance the naïve T-cell survival in non-lymphoid tissues.[12]. The other helping cytokine signals, like IL-12 and Type 1 IFNs, are serving as the third signal [13-16]. Previous studies have shown that the third signal is required for optimal activation, induction of effector function, improvement of colony expansion and priming into memory T cell [15, 16].

After an encounter with the appropriate antigen, co-stimulation and inflammation cytokines, T cells undergo vigorous clonal expansion, transcriptional, epigenetic and metabolic reprogramming and acquire effector functions. This activation, or ‘priming’, will determine the fate of naïve CD8+ T cells, and whether they will initiate the differentiation and the T cell function [1].

After activation, the effector T cells become cytotoxic and can produce multiple cytokines like interferon-γ (IFN-γ) [17], tumor necrosis factor (TNF) and IL-2. Cytokines produced by T cells or other immune cells can mediate direct effects on cytotoxic effects or assist in activating T cell. After activation, T cells will perform cell-mediated cytotoxicity against a viral infection through secretion of pore-forming

perforin and proteases, granzyme B, to trigger programmed cell death in the target cell. Another secreted substance, Fas ligand, can also activate certain caspases to induce cell apoptosis [18]. Once antigen and/or inflammation are cleared and return to homeostasis, a large-scale apoptotic episode occurs, and most activated T cells die. During this transition state from the effector expansion phase into the contraction phase, a small population of T cell subsets escapes death and remains as a stable population of memory T cells. The functional memory CD8+ T cells persist and maintain the ability to recall rapidly activated effector functions upon secondary infection.

Several parameters determine the fate and magnitude of T cell activation. Insufficient activation will restrict proliferation and colony expansion, deplete cytotoxic effector function and impair cytokine production. This T cell dysfunction, including exhaustion, tolerance, anergy, and senescence, impedes the immune protection and fails to clean the infection. Moreover, inadequate exposure to the third signal will result in a tolerance state which often develops into a chronic disease [4]. On the other hand, over-activation will lead to autoimmune disease. Therefore, achieving optimal activation of CD8+ T cells is a critical issue in defending infection and disease.

pMHC/TCR affinity in the CTL activation

How to achieve the optimal activation of CTLs cell is puzzling. However, naïve T cell activation is strictly dependent on the affinity of antigen recognition via pMHC I/TCR interaction which further triggers the signal transduction. CTLs will only

recognize short peptide fragments of pathogen protein antigens which are bound to MHC molecules on the surfaced of antigen presenting cells (APC). MHC molecules have a cleft running across their surface where a various peptide can bound. Each MHC molecules can carry up to 12 of the possible variants allowing a wide range of peptides that bond to MHC [19]. CTLs recognize the peptide/MHC complex through TCR which expressed at the cell membrane as a complex with CD3 chains. TCR is membrane-bound complexes and consists of a disulfide-linked heterodimer with an amino-terminal variable (V) region and a carboxy-terminal constant (C) region [20]. A binding groove will form during the TCR recognition where TCR directly interact with both MHC helices and peptide [1]. The binding of TCR to peptide/MHC complex with high affinity results in transduction of activation signals and lead to CTL immune response like proliferation and differentiation.

The strength and the binding ability of the TCR/pMHC interaction can be determined by the affinity. As a result, the affinity of pMHC/TCR interaction is the primary determinant of T cell response and the strength of activation [21, 22]. The specificity of peptide-dependent TCR recognition triggers signal transduction and the highest affinity peptide will receive the competitive advantage of signaling which stimulates vigorous proliferation [23, 24]. There are many factors will influence the pMHC/TCR affinity measuring through surface plasmon resonance (SPR) measurement, micropipette adhesion assay and tetramer binding assay [25, 26]. Research has found that peptide sequence plays an important role in pMHC-TCR affinity [27]. When the peptide sequence of TCR ligand is altered, it will result in various modification of biological effects including cytokine production and

proliferation [28, 29] Any change of pMHC/TCR sequence would greatly alter the binding ability[30] by reducing the binding energy [26, 31], and consequently change the T cell response and T cell functional capacity [24, 32]. Also, if the peptide sequence of MHC anchor residue was altered, the binding ability was changed, and T cell would lose the ability to recognize the foreign peptide. Therefore, the amino acid position is highly specific to T cell recognition. When the antigen was altered in sequence to increase the affinity toward TCR, this stronger binding resulted in stronger T cell responses including activation of nuclear factor of activated T-cells (NFAT) and expression of IL-2 [33]. Many studies have shown high-affinity of pMHC/TCR interaction were important to maintain continued CTL response [30] since it will result in enhanced survival during infection [21, 34]. Also, there is a strong co-relation between affinity and proliferation [35, 36]. Therefore, many studies are aiming to design the highest affinity molecules to optimize the therapeutic efficacy by improving T cell function and proliferation [37].

However, both high-affinity and low-affinity CTLs share the naive repertoire in the real word. During an immune response, low-affinity CTLs can still response and sustain a substantial population [30, 38] even though they receive defective activation signals comparing with high-affinity CTLs in polyclonal T-cell responses [30]. Although the function of low-affinity CTLs and their frequencies in polyclonal T cell response is still unclear, many studies have shown that low-affinity CTLs are important to induce the effector function of CTLs and play an important role against infection[30, 38, 39]. For example, low-affinity CTLs also develop the effector function while the magnitude of the immune responses is reduced [27]. T cells with higher affinity

developed into asymmetric cell division while T cells with lower affinity underwent symmetric division. Moreover, T cells with higher affinity displayed higher surface expression of CD25, CD69 and IFN-γ and vigorous proliferative response and differentiate into tissue-infiltrating effector T cells but T cells with lower affinity fail to do this [27]. Mice in vivo stimulated with lower-affinity peptide induced CTLs response that was relative to the development of autoimmunity upon infection [40]. This indicated the potentially different roles and pathways of activating the low and high-affinity T cells [38]. Although low-affinity peptide stimulation could induce the CTL proliferation, the effector function of T cell was limited. Low-affinity CTLs reached the division limitation earlier than high-affinity CTL and stronger activation signals must be delivered in order to prevent the impaired effector function like a production of cytokine and cytotoxic molecules [30]. Therefore, identifying the potential assistance to help the activation of CTL is needed.

Transcription factors of CTLs

To maximize the T cell expansion and effector function, activation of T cell induces several transcription factors like T-bet. They play an important role in effector function and regulating the formation of long-term memory in murine T lymphocyte[41, 42]. T-bet, a T-box transcription factor in T cell, T-bet was first found in the murine Brachyury gene. The following research showed T-bet is closely associated with promoting T-cell development into effector T cell and maintenance of memory T cell repertoire. The T-bet expression level determined the differentiation of CTLs into short-lived effector T cells or long-term memory T cells [43]. High level of

T-bet expression results in increased secretion of perforin and granzyme B [44]. Moreover, T-bet modulated cell signaling in IL-12 and IL-2 [45]; and positively influenced the IFN-γ production [46]. T-bet also help the T cell mobilization through CRCR3 signaling. Lastly, evidence has emerged that T-bet helped the adaptive cell-mediated immunity against tumor by improving the infiltration and migration of CD8+ T cells to the tumor sites [47]. In conclusion, T-bet is a key transcriptional drivers of lymphocyte differentiation, proliferation and development effector function.

Introduction of Exosome

Exosomes are a subset of extracellular vesicles (EVs). They are best defined as small round-shaped membrane-bound vesicles of 30–150 nm in diameter, appearing with a lipid bilayer structure [48-50].

Extracellular vesicles were discovered more than 50 years ago, from the study on the maturation of rat erythrocytes [51]. The Further demonstration showed consistent results that transferrin and its receptor, a marker of reticulocytes maturation, were recycled back to the plasma membrane after endocytosis and indicated the unique mechanism of selective externalization [52]. Later, Johnstone and his team confirmed that that liberated intraluminal vesicles (ILVs) generated in multivesicular bodies (MVBs) could be released to the extracellular environment through fusion with the plasma membrane (Johnstone 1987). These small-sized vesicles released into the extracellular milieu are the original detailed description of what we know as exosomes today [49].

After a decade, several ground-breaking researchers discovered the unique role of exosomes in immunity and focused attention on this. For example, it has been shown that B lymphocytes release similar vesicles bearing antigen-presenting molecular to alter T cell response [53]. The following study indicated a novel means of genetic information transferring through exosome exchanging the mRNA and microRNA between parent and recipient cells [54, 55]. Function and application of exosome have increased from that time. Therefore, exosomes, initially considered as a means of cellular waste disposal from reticulocytes, were largely ignored; now they draw enormous attention since they are thought as a cell-to-cell communication, signal mediation, the disease diagnoses, and as a potential for drug development.

Biogenesis and sorting mechanism of exosome

Exosomes originate in endosomal compartments called MVBs. MVBs form during the maturation of endosomes and they can be visualized by electron microscopy (EM) with size 100–600 nm, and size around 50 nm ILVs are present in MVBs. Some MVBs end up fusing with lysosomes and lead to degradation and recycle of ILV content by lysosomal hydrolases [56-58]; while others, with surface markers of late endosomes like tetraspanin CD63, lysosomal-associated membrane proteins (LAMP1 and LAMP2), and MHC class II in antigen-presenting cells [59], can fuse with the plasma membrane and release ILVs like exosomes. By forming an inward budding vesicle and then releasing in the endosomal lumen of the membrane vesicle with a small portion of cytosol, the ILVs are able to segregate cargo at the endosomal membrane. This formation of ILVs consists of highly dynamic membrane compartments involved

in at least two known mechanisms, one depends on the endosomal sorting complex required for endosomal sorting complex required for transport (ESCRT) and/or ubiquitin machinery, whereas the other is ESCRT-independent.

Exosomes contain many ubiquitinated proteins and they are incorporated with MVBs sorting pathway and ESCRTs [60]. ESCRT is highly a conserved protein from yeast to mammals [61] and it is composed of four different complexes (ESCRT-0, -I, -II and -III) with several associated proteins (VPS4, VTA1 and ALIX) [62, 63]. ESCRT-0, ESCRT-I and ESCRT-II mostly assemble within cytoplasm while ESCRT-III and Vps4-Vta1 are mainly present on membranes for late processing [64] [65]. The ESCRT-0 complexes are the first subunits to recognize and segregate the ubiquitinated transmembrane proteins. A hepatocyte growth factor–regulated tyrosine kinase substrate (HRS) is the main component of ESCRT-0 that takes charge of the recognition in ubiquitinated cargo proteins. HRS also helps an alliance with the other ESCRT-0 components, signal transducing adaptor molecule (STAM), and other non-ESCRT proteins, such as Eps15 and clathrin. A deficiency of HRS results in failure of dendritic cells to release exosome and restrain the antigen-presenting activity through exosomal regulation [66]. Moreover, HRS helps to recruit TSG101, a GPI-anchored protein, of the ESCRT-I complex and ESCRT-I will participate in the following recruitment of ESCRT-III via ESCRT-II or ALIX. The ESCRT-I and -II complexes are able to help membrane deformation into buds with sorted cargo in endosomal membrane, and ESCRT-III components subsequently drive vesicle scission [61]. Therefore, with the help of ESCRTs-I, -II, and –III, the cargoes start to accumulate on the endosomal membrane. There are other membrane proteins with chaperones such

as Hsc70 [67] that are proposed to help the inclusion of protein cargo into ILVs. At the end of the sorting, an AAA-type ATPase, VPS4, will disrupt the ESCRT complexes and the inward membrane with its accumulated cargo forms the MVBs. Most of the cargoes in MVBs are destined for degradation. For example, the majority of the ubiquitinated protein like epithelial growth factor (EGF) receptors, c-Met, and gp130 will be degraded by lysosomal proteases. However, some ILVs in MVBs will release as exosomes by direct fusion with the plasma membrane. Here, the Rab GTPases members, Rab5, Rab11, Rab27a and Rab27b, play an essential role in formation and secretion of exosomes [68].

However, ILVs are still able to form in the absence of ESCRTs. When applying siRNA oligonucleotides against HRS, Tsg101, Vps22 and Vps24 to abolish the key subunits of ESCRTs family, ILVs are still produced. As a result, other means of generating ILVs must exist, while the ESCRTs are still required for the biogenesis of growth factor-induced MVEs [65].

The mechanisms for their generation are less clear. Normally, the proteins sorting into exosomes are through ESCRT machinery, whereas generation of the ESCRT-independent cargoes, like tetraspanin CD63, is dependent on ceramide instead of HRS. In Jurkat T cells, CD 63 is a protein abundant on ILVs but with unclear function, and it can lead to secretion and sorting of ILVs and therefore exosome through the ceramide-dependent pathway [69]. Another example of the ESERT-independent pathway is MAL, a tetraspanin membrane protein which is a critical element in constitutive exosome secretion in Jurkat T cells. In the absence of MAL, the exosome releasing is greatly impaired and exosomal markers expression were diminished upon

HIV-1 Nef protein induction, which is known to induce exosomal markers accumulation in exosome [70]. Furthermore, phospholipase D2 (PLD2), activity in rat basophilic leukemia 2H3 cells is correlated with the amount of exosome released upon stimulation [71]. Other research indicates that the ESCRT-independent pathway may be facilitated by lipids such as ceramide, and lipid metabolism enzymes like neutral sphingomyelinase (nSMase) since the blockade of nSMase 2 will inhibit the exosome production [69].

Moreover, the certain selective ways to sort the various exosomal cargoes can reflect its original cell properties while further details still need to be confirmed. For example, Jurkat T cells activated by anti-CD3 will release exosomes contained T cell receptor subunit and adhesion molecular which are commonly found in normal T cells [72]. Also, exosomes derived from APC have similar properties as APC which could directly influence the naïve T cells through the MHC/peptide complex and the costimulatory molecules on the surface [59]. Not only certain molecules are thought as specifically found in cell-type of exosomes, the detailed sorting mechanism in various cells have also been proposed. For example, the co-stimulatory molecule CD86 and several tetraspan proteins, such as CD53, CD63, CD81, and CD82 are selectively enriched in exosomes secreted by B-cell. [73] In CD81 deficient mice, the secreted exosomal content had diminished tetraspanin CD-81 associated proteins like CD20, ICAM and B-cell receptor. Mittelbrunn and his team have shown that miRNAs are selectively delivered from active T cell to APC via exosome on immune synapsis by ceramide-dependent secretion [55]. Further research has indicated that the exchange of genetic materials can modulate gene expression in recipient cells and it happens during

immunological synapse. Furthermore, recent findings are focusing on the novel sorting mechanism and packing system of the exosome and it will help to illuminate the importance of exosome.

Characterization of Exosome

Electron microscopy is mainly used for characterizing the exosomes and exosome marker proteins are another important means to identify exosomes [74]. Due to their endosomal origin, most exosomes are enriched in proteins associated with the specialized membranes transport and fusion such as Rab-family GTPases, Annexins and flotillin-1. Tetraspanin molecules (CD63, CD9, CD81 and CD82) are commonly used as protein markers of exosomes. They can regulate the internalization and sorting of proteins towards exosomes [75] For example, Alix and TSG101 are endosomal sorting proteins required for transport (ESCRT)-associated proteins and involve in multivesicular body biogenesis. Other common exosomal cargos include major histocompatibility complex (MHC I and MHC II), heat shock proteins (Hsc70, Hsp 90), adhesion molecules, as well as lipid-related proteins and phospholipases. The major proteins in exosomal content are cytoskeletal, ribosome and mRNA granule proteins which help the biogenesis of exosomes. Also, lipids such as cholesterol, sphingomyelin, ceramide and ganglioside GM3 are routinely found in exosome content due to their origins. Some of them will concentrate especially in the membrane to form membrane rafts [76]. Moreover, activated CTLs expressed typical exosomal markers such as ALIX, TSG101, and Flotillin-1[50].

Although scientists heavily rely on these exosomal marker proteins to confirm the presence of exosomes, the complexity of cell type-specific proteins makes us cannot always find the markers in exosomes and unexpected cargoes may be present in the exosomes [77]. Moreover, proteomic analysis has shown that majority of the exosome are the same while few are selectively enriched under the diverse circumstance. For example, activated CTLs expressed massive proteins which their functions were relative to related to nucleic acid binding and enzyme modulators [50]. The selective proteins only specific to certain cell type under particular environment will determine the biological functions of exosomes. Overall information about protein and RNA being identified in exosomes has been reported to the database and open to access [77, 78]

Current findings and applications of exosome

The increasing focus on exosome has exploded due to their aid in communication among cells. First of all, several studies have found unexpected cargo in exosomes. For example, mRNA was found in secreted exosomes by Glioblastoma tumor cells and it delivers the genetic information to the recipient cell [79]. In embryonic stem cells, mRNA and several transcription factors are selectively enriched in secreted extracellular vesicles and they could be transferred into recipient hematopoietic progenitor cells and translated to corresponding proteins [80, 81]. Small non-coding RNAs, like microRNA, are also found in exosomes from mast cells [82]. Hence, exosomes are linked with cell-to-cell signaling, including antigen presentation, molecular and genetic information transfer. Secondly, exosomes can protect their

cargoes inside the bilayer lipid membrane while circulating in body fluid. This makes exosomal cargoes impervious to circulating nucleases [83] and proteases and avoid being attacked by the immune system by digestion of phagocytes because exosomes are usually recognized as 'self'. The small-sized structure of exosomes also enables them to pass through a certain barrier, such as the blood-brain barrier, in our body effortlessly. More importantly, CTLs stimulated by IL-12 have been shown to secret bio-functional exosome to facilitate the communication between activated CTLs and naïve CTLs [50]. It was the first report that CTLs stimulated by three signal model could deliver messages to bystander-CTLs and enhanced the T cell activation through secretion of exosomes [50].

Taken altogether, exosomes can transport and deliver cargoes enclosing sophisticated functional molecules to neighboring cells or even distinct receptor cells. The exosomal cargos can be taken up by direct fusion with cells and they subsequently affect recipient cells. The discovery of their function in the exchange between cells has brought increasing attention to exosomes because they are considered as an effective regulator and intercellular communication.

Exosomes not only become a way for intercellular communication but also become a useful tool for the diagnostic purposes because their cargoes suggest the nature of the original cells [84]. Since we already know that the exosome secretion will be altered during the infection, the components inside the exosome will provide us a hint about the status of the cell cycle and the important information regarding an infection process. Besides, exosomes are extensively present in almost all biological fluid, so they provide the opportunity to use noninvasive or minimally invasive

approaches for detecting and diagnosing the disease with greater speed and lower cost of analysis. Take glioblastoma-derived exosomes for an example; they carry a diagnostic set of mRNAs (EGFRvIII) in the serum of glioblastoma patients and this is a potential for brain tumor diagnosis [54] In addition, recent studies have shown that cancer patients exhibit different patterns of RNA and miRNA. Total 8 microRNAs (miR-21, miR-141, miR- 200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214) were compared in exosomes isolated from various stages of ovarian cancer and played significantly distinct profiles from that observed in the benign disease. Therefore, miRNA from circulating tumor exosomes are proposed as promising diagnostic markers for detection of ovarian cancer, extending its utility to screening asymptomatic populations [85]. Taken all, exosomes are the new promising approach to detect and diagnose many diseases, including imperceptible cancer.

Lastly, exosomes are a potential means of drug discovery [86]. With the ability to package and deliver active drugs, exosomes derived from mesenchymal stromal cells can be exploited to selectively deliver therapeutic genes or drugs to tumors [87]. By using immature DCs derived from the bone marrow of mouse as a source of exosomes, scientists tested the possibility of loading exosome with the MAGE tumor antigens(MAGE-A3, -A4, -A10, and MAGE-3DPO4 peptides) in patients with non-small cell lung cancer (NSCLC) as a safe, feasible and efficient drug delivery system [88]. Exosomes also have the ability to concentrate and sequester different antitumor drugs to overcome drug-resistance, which is a key determinant of cancer chemotherapy failure. For example, cancer cell resistance to drugs and antibodies are linked with an ABCA3-dependent pathway of exosome secretion. B-cell lymphoma cells released

exosomes regulated by ABCA3 can carry CD20 to shield target cells from antibody attack, and both pharmacological blockade and the silencing of ABCA3 enhanced susceptibility of target cells to antibody-mediated lysis [89, 90]. Moreover, since many tumor antigens, such as carcinoembryonic antigen (CEA) and mesothelin, are found in tumor-derived exosomes, many studies have proposed the promising development of tumor exosome-based cancer vaccines. For example, Hsp70-enriched exosomes from heat-treated tumor cells can increase immune stimulating activities and Th1 typed anti-tumor effects in both autologous and allogeneic mouse models. This could be a novel MHC cancer vaccine providing tumor antigenic [91].

To conclude, exosomes have drawn significant attention since they help the transporting of functional molecules for communication, diagnosis and disease treatment. Also, like the previous statement, many types of research have indicated that exosomes are present in almost all biological fluid, such as plasma, urine, breast milk. The extensive presence of exosomes can contribute because they are secreted by many somatic cells, including epithelial cells, immune cells and tumor cells. Among them, immune cells release considerable exosomes into the mammalian fluid, and therefore they are thought as a critical role in immunity.

The important role of the exosome in immunity and CTLs

Exosomes have significant roles in immune responses and several immune cells secrete functional exosomes, under both normal and stimulated environment. B cells, dendritic cells and mast cells of the immune system appear to not only release exosomes constitutively but may also be stimulated to secrete massive exosomes for

the intercellular interactions. For example, murine dendritic cells, which can activate T lymphocytes, secrete higher levels of exosomes upon engagement with antigen-specific CD4+ T lymphocytes and can promote anti-tumor immune responses in mice. Tumor peptide pulsed DCs-derived exosomes, which expressed MHC class I, suppressed tumor growth in the tumor bearing mice and further mediated specific cytotoxic T lymphocytes activities. This suggests that DC-derived exosomes play a potential role in cancer immunotherapy as a novel therapeutic cancer vaccine and indicate that exosomes can communicate to T lymphocytes [92]. Exosome-like vesicles released by melanoma cells bears FasL and help the tumor to escape and hinder the anti-tumor activity of lymphocytes[93]. Human B cells and T cells release more exosomes upon interaction with antigen-specific CD4+ T cells and antigen receptors, respectively [94]. Lastly, activated T cells released great amounts of exosomes during activation[95, 96].

In addition, exosomes from APC have been suggested to promote antigen recognition; CD4+ T cells have been demonstrated to deliver activation signals [97]. Other research showed it cannot find the exosome in the supernatant stimulate with PMA with ionomycin, which is known to induce NF-AT activation and IL-2 production. This indicated that the production of exosome may be specific to TCR activation. Therefore, exosomes bearing TCR/CD3 complexes at surface had the potential to specifically trigger the target human T cell activation through MHC I/TCR complex [72] Furthermore, exosomes directly loaded with OVA323-339 peptide induced stronger T-cell proliferation both in vivo and in vitro, and stimulate OVA-specific Th1 response in mice [98]. Exosomes derived from B cells can also assist diverse subset populations to communicate and modulate the T lymphocytes immune

function [99]. Many findings have revealed that exosomes are potent immune regulators and are relevant for the development of a therapeutic treatment to modulate immune responses, especially in T cells.

However, little is known about how exosomes mediate T cell function through cell-to-cell communication. The previous studies mainly focus on exosomes secreted by APC that can induce T cell response. Now, more and more research has discovered the T cell autologous activation through exosomes. Exosomes originating from activated CD3+ T cells together with IL-2 were able to generate proliferation in autologous resting CD3+ T cells and have the higher proportion of CTLs and alter the cytokine profile. This indicated the intercellular communication between activated and resting T cell through exosome to prevent the tolerance state and enhance the T cell activation [100]. Activated human T cells upon PHA stimulation can release exosomes containing bioactive Fas ligands (FasL) and APO2 ligands which subsequently induce self-apoptosis of T cells to prevent the autoimmune disease [96]. Further research showed that this secreting exosome will improve tumor expansion and damage the CTLs antitumor activity in a FasL-dependent way [101]. In addition, exosomes derived from antigen-specific T cells suppressed CTL cytotoxic responses and antitumor immunity[102]. Lastly, as mentioned before, activated CTLs triggered the bystander activation in the resting CTLs without the presence of antigen [50] and the exosomes enhanced and helped to maintain the CTL immune response. This confirmed the critical role of exosomes in enhancing the communication between activated CTLs and weak stimulated-CTLs to generate immense immune response against infection. Together,

exosomes derived from T cells can serve as tumor treatment and improve T cell function in the future.

As a result, we hypothesized that exosomes derived from CTLs stimulated by 3 signals model (3SI) using highest-affinity peptide will enhance the activation of low-affinity CTLs. We would like to investigate the function of Exo in activating CTLs with low-affinity. Also, since CTLs stimulated by 3 signals model have shown to secret bio-functional Exo in the late stage of activation [50], we would also identify whether CTLs can also generate functional Exo in the early stage of activation.

Chapter 2: Materials and Methods

Experiment Scheme

See Fig 1.

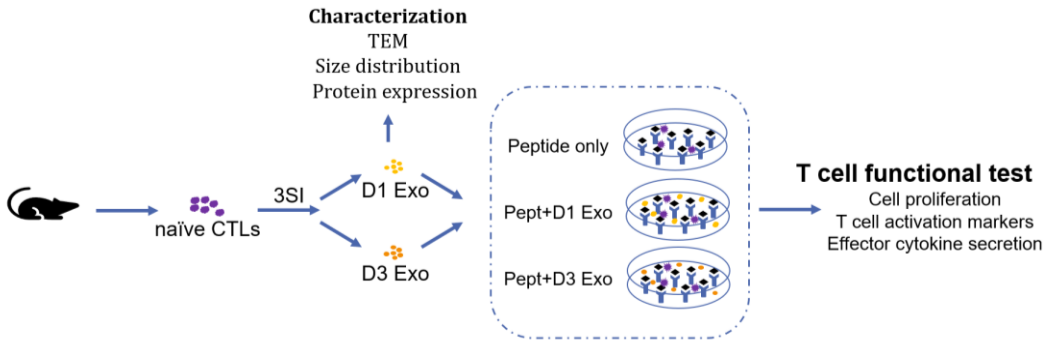


Figure 1. Experimental scheme. Naïve CD8+ T cells were purified from OT-1 transgenic mice through negative selection. After one-day and three-day stimulation of 3SI, exosomes (Exo) were purified from the supernatant (exosomes purified from day one, D1 Exo; exosomes purified from day three, D3). The purified Exo was characterized by their morphology and protein expression. In addition, naïve CTLs were stimulated by peptides with/without Exo. All activated CTLs were stained with antibody and analyzed by FACS to determine T cell function.

Purification of naïve T cell

The mice were euthanized, and peripheral lymph nodes were collected. The harvested lymph nodes were homogenized in 15 ml glass grinders in Allos medium. After washing with Allos several times and filtering with 0.75uM nylon filter, the cells are incubated together with FITC-labeled antibodies that are specific for B220, CD4, CD44, CD11c and I-Ab for negative selection. The suspension was subsequently incubated with FITC binding magnetic MicroBeads (Miltenyi Biotech, Auburn CA), and pass through separation columns attached to a MACS magnet. Cells that do not bind to the column were collected and use FACS to confirm the purity (>95% CD8+ and <0.5% CD44hi cells).

Activation of naïve CTLs

Purified naïve T cells are stimulated *in vitro* with Allos in 48 wells flat-bottom microtiter wells coating with antigen (peptide A) (DimerX H-2Kb: Ig fusion protein loaded with OVA257-264 peptide; BD Pharmingen), recombinant B7-1/Fc chimeric protein (R&D Systems) and 2U/ml of murine rIL-12 (R&D Systems) as previously described [50, 103]. 3×10^5 cells in 1.5 ml Allos media are placed in each well and 2.5U/ml IL-2 added to all wells. Treatment without IL-12 will serve as control. In affinity test, purified naïve T cells are treated with different affinity peptides A, B, C, D, E, F with or without exosomes stimulation. The peptides are different in affinity from high (peptide A) to low (peptide F).

Purification of exosomes

Exosome free medium was generated by centrifugation at 100,000 g for overnight and supplemented with 10% FBS. Naïve T cells were seeded and incubated for day1 and day 3, we harvested the cell supernatants for exosome purification by several steps of centrifugation and filtration [50]. Briefly, cells were centrifuged at 300g for 5min followed by 10,000g for another 10 min to remove the debris. The supernatant was collected to filter through 0.22 μM membranes. After, exosomes were precipitated by PEG for overnight and are pelleted by ultracentrifugation at 100,000 g for 90 min at 4°C (Beckmen XL-90, Beckman Coulter). Purified exosomes were determined for protein concentration by Bradford microassay method (BioRad Laboratories) and safe in -80°C for following use. Size distribution of in vitro T cell-derived exosomes from various time points were estimated by Malvern Zetasizer Nano ZS90 (Malvern, UK).

Nanoparticle tracking analysis and transmission electron microscopy

The presence and concentration of exosomes from activated T cells were determined by using a nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). NTA was carried out by using the NanoSight LM10 on purified exosomes which were diluted 20-fold in 400ul PBS. After loading into NanoSight chamber, data of each sample will be collected from different views and analyzed by NTA2.1 software. Exosomes containing 0.03–0.3 pg protein will suspend in 2% glutaraldehyde and apply to a Formvar-coated grid and negatively stained with uranyl acetate. Electron micrographs was collected using a Zeiss EM10 transmission electron

microscope at an accelerating voltage of 80keV26. Exosomes validated by protein markers and electron microscopy will be further used for the following experiments.

Western Bolt

Protein (10µg) extracted from purified exosomes were separated by electrophoresis on 10% SDS-polyacrylamide gel and analyzed by western blotting. First, the protein was transferred to a Polyvinylidene Difluoride (PVDF) membrane (BioRad, Hercules, CA) and blocked with a 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween-20 blocking solution (TBST) containing 1% bovine serum albumin (BSA). Incubate the membrane with the first antibody at room temperature (RT) for 1h and wash for 3 times using TBST to remove the exceed antibody. Following incubation of the membrane use horseradish peroxidase (HRP)-conjugated anti-bovine IgG secondary antibody for 1h at RT. Signals were detected by the SuperSignal West Pico Chemiluminescent Substrate and a Gel Doc imaging system.

Cell proliferation

The purified naïve T cells were washed in HBSS, resuspended in HBSS (1 x 107 cells/ml) containing carboxy-fluorescein diacetate succinimidyl diester (CFSE) at a final concentration of 0.5 µM. Incubated for 5 minutes at 37°C and transferred to cold Allos medium. Subsequently cells were washed twice times with Allos medium and resuspended at a concentration of 2 x 10^5 cells /ml. The cell proliferation was monitor by FACS.

Cell staining and T cell activation analysis

Naive CD8+ T cells are stimulated by exosome isolated from day1 and day 3, respectively. The quantity and quality of CD8+ T cells activation will be determined by flow cytometry (BD Biosciences) and analyzed using the FlowJo software. T cell activation markers and production of functional molecules, such as CD25 and CD69 expression IFN-γ, TNF-α, and granzyme B will be examined to determine the activation process. CD25 and CD69 will use the normal surface according to protocol and intracellular staining will be used for IFN-γ, TNF-α, and granzyme B secretion analysis. Particularly, IFNγ expression will be induced by incubating in RP-10 with 0.2 μM OVA257–264 peptide and 1 μL Brefeldin A (BioLegend) for 3.5 hrs at 37°C. After peptide and BFA stimulation, cells are stained based on protocol. Briefly, we add 4% Fixing buffer to the cells at one to one ration for 15 minutes at 4°C, after the cells are permeabilized in Saponin-containing Perm/Wash buffer (Biolegend) for another 15 minutes at 4°C, then stained with color-conjugated antibody for 30 minutes at 4°C. Finally, the cells are washed once with Perm/Wash buffer and Resuspend in 2% FBS Staining buffer. The analysis will be done using a FACS Diva flow cytometer and FlowJo software. Finally, to further test the optimal activation of T cell, the exosomes from a certain time point will be used to stimulate the effector function.

Chapter 3: Results

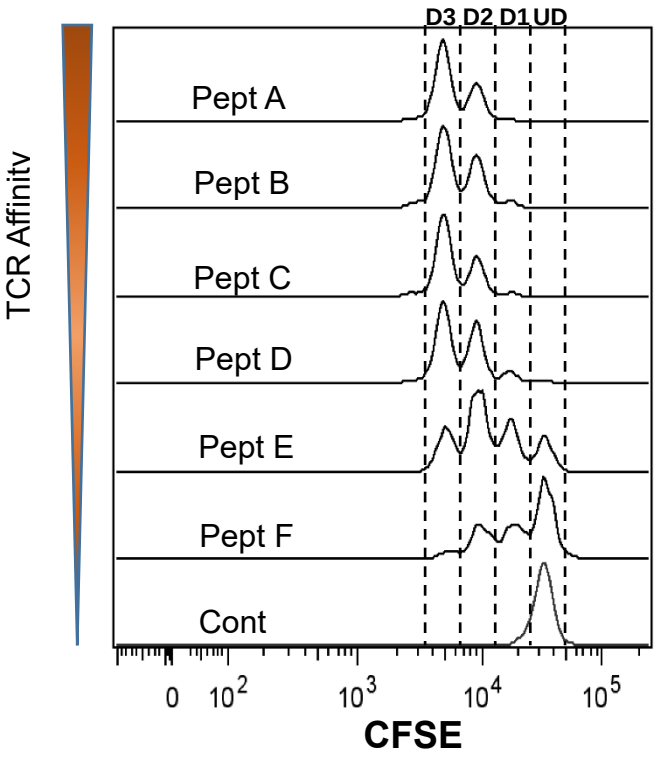
Exosomes enhanced cell proliferation in low affinity peptide treatment

Robust cell proliferation takes place after CTL activation in response to antigen stimulation. Generally, T cells with high affinity had increasing cell proliferation [104]. Although T cells with low affinity may initiate the same proliferative rate as T cells with high affinity, it showed limited expansion during the *in vivo* response to microbial infection [30]. However, their study was done in the different system where naive CTLs derived from OT-1 mice were grafted into B6 wild-type mice and various peptides were presented by recombinant *Listeria monocytogenes.* Therefore, more factors are introduced in the systems while we were only focusing on the difference of peptide affinity. Because peptide/TCR affinity is the main factor contributed to immerse CTLs response to antigen stimulation [26], we first tested the effects of various affinity peptide treatment on cell proliferation. CFSE is often used for tracking of multiple generations during proliferation through dye dilution analyzed by FACS [105, 106]. After purification, CTLs were labeled with CFSE to track the cell generation through dye dilution analyzed by FACS [105]. CFSE-labeled CD8+ T cells were stimulated by peptides with the diverse affinity (peptide A, B, C, D, E, F, respectively) only and co-stimulators. With diluted CFSE signals indicating cell division, CD8+ T cells treated with higher and intermediate-affinity peptides (peptides A, B, and C) underwent similar rounds of cell cycles after 48-hour stimulation. Low-affinity peptide stimulation (peptides D, E, and F) had fewer cells entering the cell cycle with most of the cells staying undivided in peptide E (Fig. 2A). These results indicated that the extension of

the proliferation of CTLs was dependent on the peptide/TCR affinity. Since in Zehn's study, the low-affinity peptides stimulation could lead to the similar rate of proliferation compared with high-affinity peptides stimulation at the initial stage of infection [30], we speculated that endogenous T cells may respond to peptides and help the activation of low-affinity CTLs from TCR transgenic mice.

Next, we purified the exosomes from the vesicle-depleted supernatant of fully activated CTLs treated with the highest-affinity peptide (peptide A), co-stimulators (IL-2, Dimer X and B7) and IL-12 after three days of stimulation (D3 Exo) [50]. The purified D3 Exo together with the peptides of various affinities were added *in vitro* to stimulate the CFSE labeled CD8+ T cells. We examined the percentages of divided CTLs in the same round of the cell divisions based on their diluted CFSE intensity (UD, D1, D2, D3) and compared the exosome treatment with the control. In the groups of high to intermediate-affinity peptides (peptide A, B, and C), the treatments with the peptide and the D3 Exo showed most of the cells had divided three times, similar to cells in the peptide only control. However, in the group of low-affinity peptides, D3 Exo pushed more cells into cell cycle progression, especially in peptides E and F. And, for those dividing cells, D3 Exo enhanced more cells to enter division 3 in peptide E, and division 2 in peptide F (Fig. 2B). Therefore, the D3 Exo enhances the proliferation of CTLs stimulated with low-affinity peptides and has no effects on high-intermediate peptide-stimulated cells.

A


D3 D2 D1 UD
TCR Affinity
Pept A
Pept B
Pept C
Pept D
Pept E
Pept F
Cont
0
10^2
10^3
10^4
10^5
CFSE

B

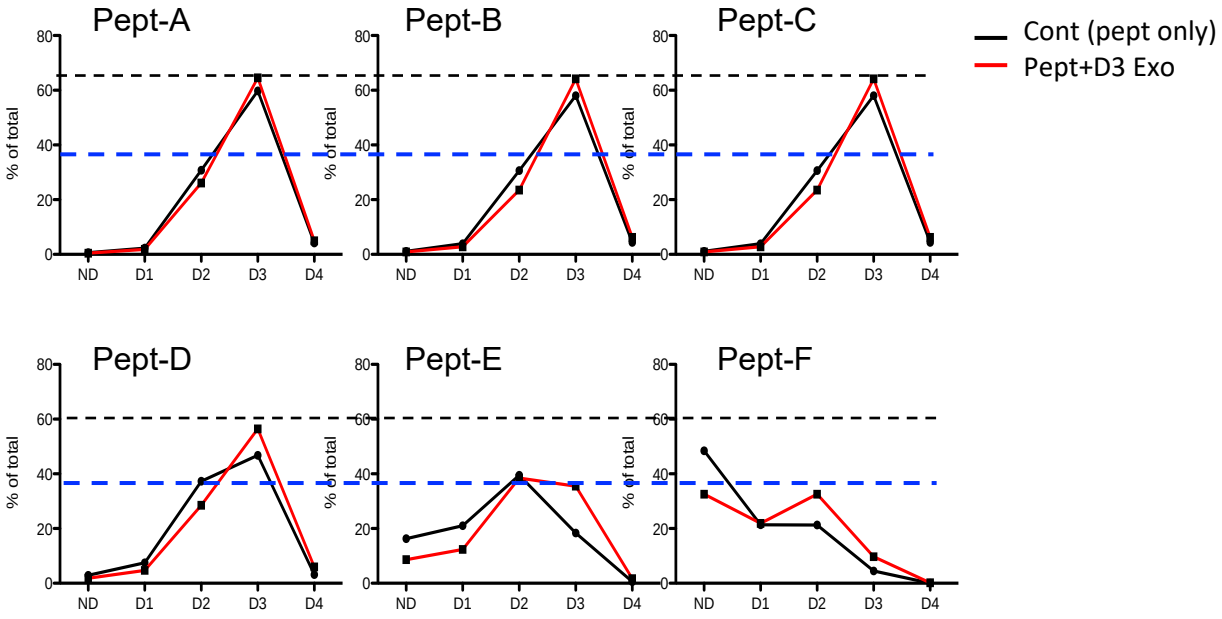

Pept-A
Pept-B
Pept-C
Pept-D
Pept-E
Pept-F
% of total
0
20
40
60
80
ND
D1
D2
D3
D4
Cont (pept only)
Pept+D3 Exo

Figure 2. Exosomes increased the cell proliferation in low-affinity peptide treatment. Purified naïve CD8+ T cells were first labeled with CFSE and stimulated with peptides in the presence or absence of D3 Exo to monitor cell division by diluted fluorescent signals. The peptides were ranging from high affinity (peptide A) to low affinity (peptide F). (A.) Cell division of in vitro activated CTLs, which were stimulated with peptides with diverse affinities were examined by FACS two days after stimulation (B.) Total cell number of each generation of divided CTLs was analyzed in the control (peptide only, black lines) and the treatment with peptides and D3 Exo (Red lines). (ND: undivided cells; D1: cells entering first division cycle; D2: second division; D3: third division.)

Exosomes increased the survival of CTLs treated with low-affinity peptides

To sustain the CTL population during an immune response, the number of CTLs can be affected by either rate of proliferation or ability to survive, or both [107]. We had demonstrated that exosomes stimulated the proliferative rate in the treatments with lower-affinity peptides during the two-day expansion (Fig. 2B). We wonder whether these D3 Exo affected the total number of cells through the involvement of difference in survival.

When we compared the D3 Exo treatment with the control, which was treated with peptide only, D3 Exo enhanced cell number in every peptide treatment, by 1-2 fold between A to D. However, the increase was raised to 4-8 folds in low-affinity peptides E and F (Fig.3A). There was no difference observed in CFSE dilution in peptides A-C in the presence of D3 Exo, suggesting that D3 Exo positively influence the survival in CTLs stimulated in high to intermediate peptides. Despite the fact that the D3 Exo pushed more cells into cell cycle, and furthered cell cycle progression (Fig.2B), the increase of cell number by D3 Exo in peptides E and F, was much bigger than the effects explained by the difference in proliferation rate driven by D3 Exo, indicating D3 Exo may enhance the cell survival in CTLs treated with low-affinity peptides, possibly to an extent bigger than high to intermediate peptides (Fig.2B and Fig.3A). IL-2 plays a major role in providing survival signal to T cells, through triggering IL-2 signaling upon ligating with a IL-2 receptor. CD25 is the alpha subunit of IL-2 receptor, which is upregulated once T cells are activated, which enhances the IL-2 signaling to facilitate T cell survival. To test if D3 Exo enhances peptide-stimulated CD8 T cell through upregulation of CD25, we analyzed the CD25

expression in each dividing cycle in each peptide treatment. Indeed, the presence of D3 Exo enhanced CD25 expression in cells in every cell cycle, from undivided, to cells in division 3 (Fig.3B), and these effects of D3 Exo were observed similarly in all peptide stimulation (data not shown). Therefore, D3 Exo can enhance the proliferation and survival of peptide-stimulated CTLs, preferentially with low affinity-peptides. And, the survival effects could be contributed by enhanced IL-2 signaling by D3 Exo, which will be further examined using IL-2 blocking assay.

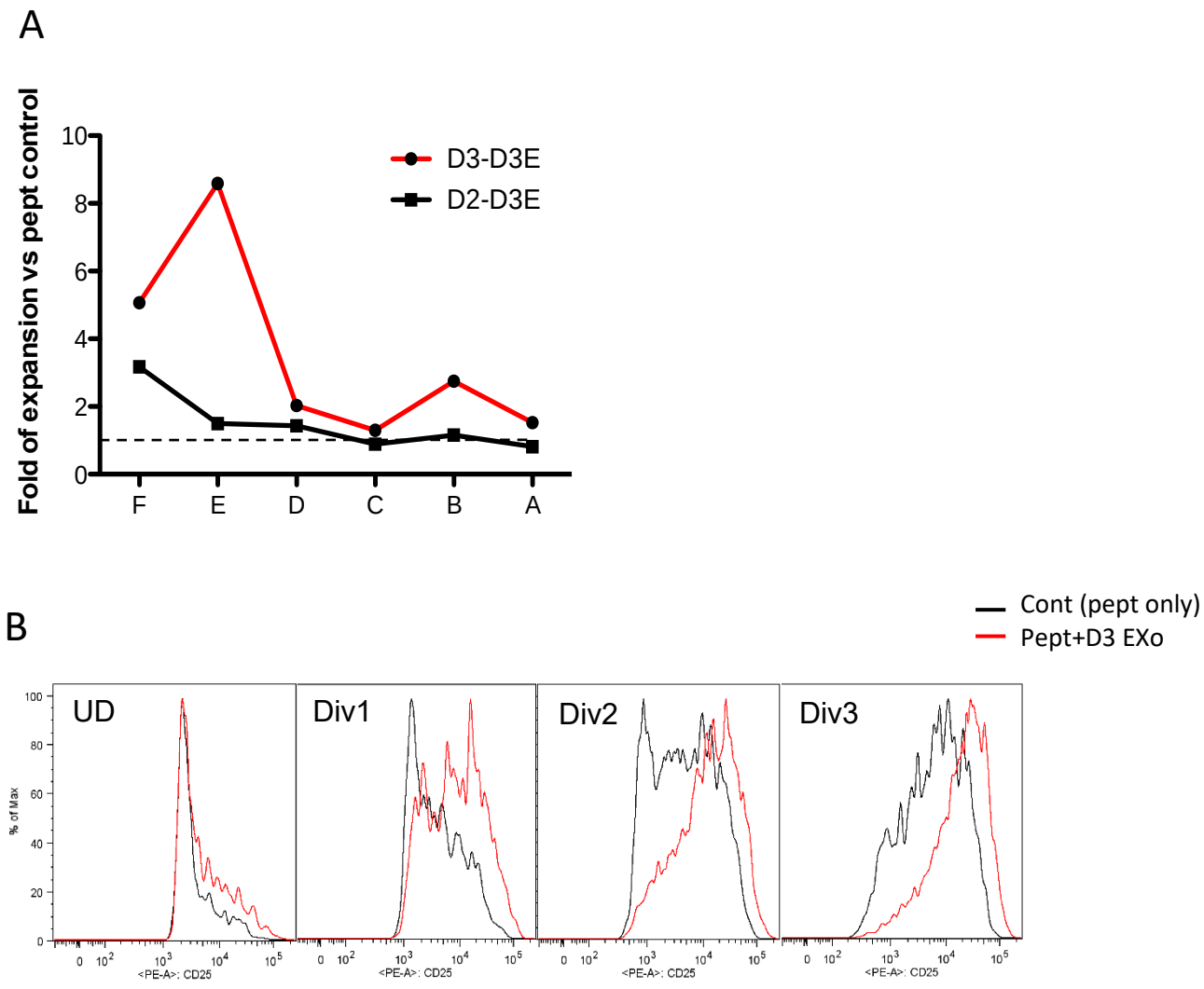


Figure 3. D3 Exo enhanced the survival of CTLs treated with low-affinity peptides by upregulating CD25. Naïve CD8+ T cells were stimulated with peptides in the presence or absence of D3 Exo. (A.) Cell expansion affected by D3 Exo. Data were expressed as the ratio of cells number of (D3 Exo+ peptide) divided by peptide only control, following two-day and three-day stimulation (D2 and D3). (B.) The expression of CD25 in each division (indicated by CFSE dilution) of CTLs treated with exosome was compared with CTLs treated without exosome (UD: undivided; Div1: cells undergo first division; Div2: second division; Div3: third division).

Exosomes stimulated the generation of IFN-γ in low-affinity CTLs

After antigen stimulation, CTLs initiate the cell proliferation and the development of CTL effector function [108]. CTLs must receive comprehensive activation signals including cytokine to develop the effector function [16, 109]. To understand the effects of exosomes on the development of CTL effector function, we test the production of effector cytokine, IFN-γ [110].

Our results showed that although encountering with high-affinity peptide, the stimulation of peptide only could only induce the cell division (Fig. 2A) while failed to produce IFN-γ (Fig. 4A). Surprisingly, the treatment of the D3 Exo overcame the disadvantages of low-affinity peptide stimulation and induce the production of IFN-γ. Like the results of CTL expansion, the D3 Exo enhanced IFN-γ production mostly in CTLs stimulated with low-affinity peptides instead of high-affinity peptides (Fig. 4A). Moreover, we analyzed the relationship between IFN-γ production and cell generations. We found that the effects of the D3 Exo stimulation to induce IFN-γ production were significant in the later rounds of cell division (Fig. 4B).

In summary, CTLs stimulated by high-affinity peptide failed to generate the IFN-γ in all cell generations. However, the exosome promoted the IFN-γ production in the dividing CTLs treated with low-affinity.

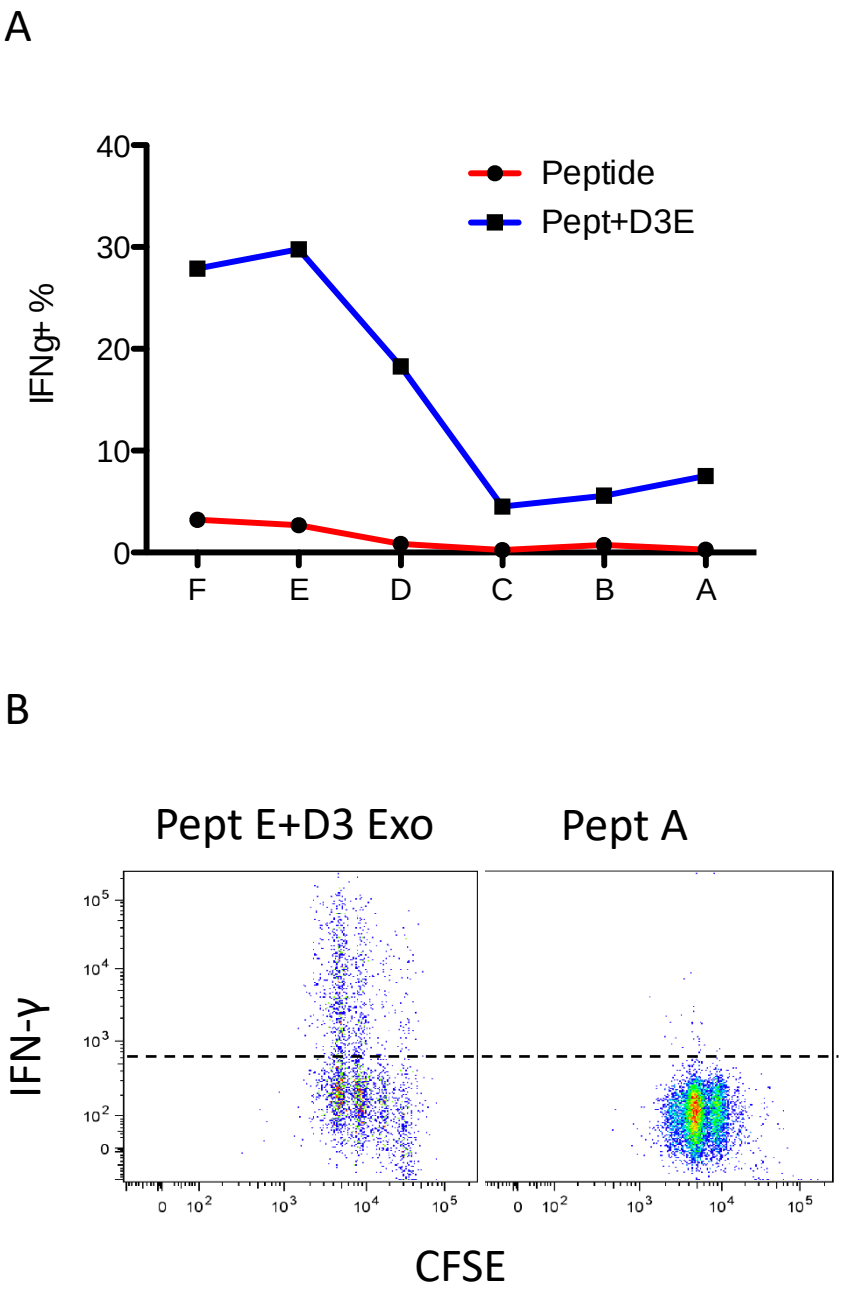


Figure 4. D3 Exo treatment induced IFN-γ production. Naïve CTLs were labeled with CFSE and stimulated peptide, and with/ without the D3 Exo for three days (A) or two days (B). Cells were harvested and stimulated with the corresponding peptide for 3.5 hrs. The cells were then stained for intracellular IFN-γ. (A.) IFN-γ expression on activated CTLs (B.) IFN-γ production and cell generation of activated CTLs was compared between the treatment of the peptide E (E) and the D3 Exo and the treatment of the highest-affinity peptide, peptide A (A).

Low-affinity CTLs stimulated with exosomes exhibit higher GZB, corresponding to CD25

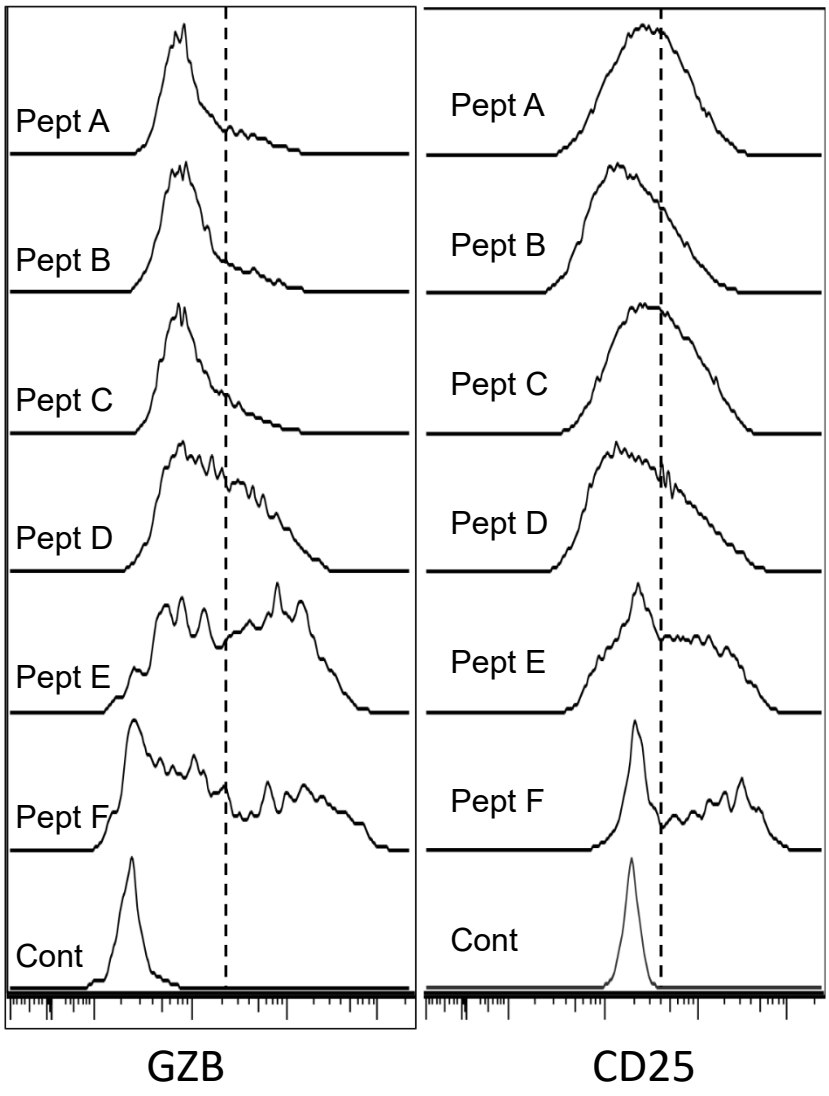
Production of GZB which responsible for the cytotoxic activity of CTLs is another major effector molecule [1, 110]. Since CTLs must receive comprehensive signals to develop the CTL effector function [111], different peptide affinity did not significantly contribute to the induction of IFN-γ (Fig. 4A). The previous study showed that *in vivo* stimulation of CTLs by microbial did not result in the significant difference between high-affinity peptide treatment and low-affinity peptide treatment[30]. However, we demonstrated that stimulation of low-affinity peptides, not high-affinity peptides, led to induction of GZB (Fig.5A). To validate the effects of low-affinity peptides on the effector function of CTL, we tested the T cell activation marker, CD25. The expression of CD25 was upregulated when treated with low-affinity peptides (Fig. 5A).

Based on our results, the stimulation of high-affinity peptide can trigger the cell proliferation (Fig. 2A) while fail to induce the effector function (Fig. 4A and 5A). In contrast, the stimulation of low-affinity peptide showed limited cell proliferation (Fig. 2A) while promoted the development of effector CTLs, with enhanced survival rate (Fig. 3A), IFN-γ production (Fig. 4A) and higher expression of GZB and CD25 (Fig. 5A).

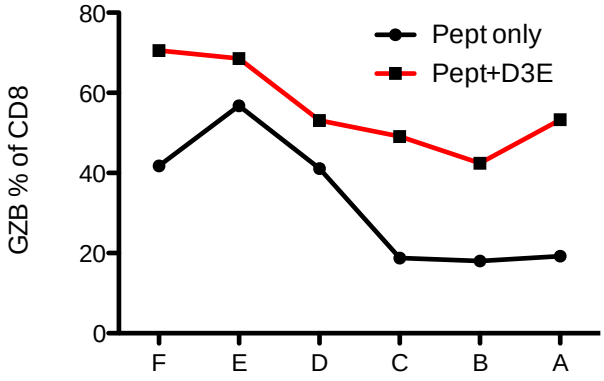
After observing the strong correlation between GZB production and CD25 expression (Fig. 5A), we tested the role of exosomes in activating low-affinity CTLs. When treated with the D3 Exo and the peptide, CTLs produced more GZB than the peptide treatment only (Fig. 5B). Similarly, CTLs stimulated with D3 Exo and peptides

expressed higher CD25 (Fig. 5C). Thus, exosomes enhanced GZB production in all peptide affinities, which is associated with upregulation of CD25.

A


Pept A
Pept B
Pept C
Pept D
Pept E
Pept F
Cont
Pept A
Pept B
Pept C
Pept D
Pept E
Pept F
Cont
GZB
CD25

B



C

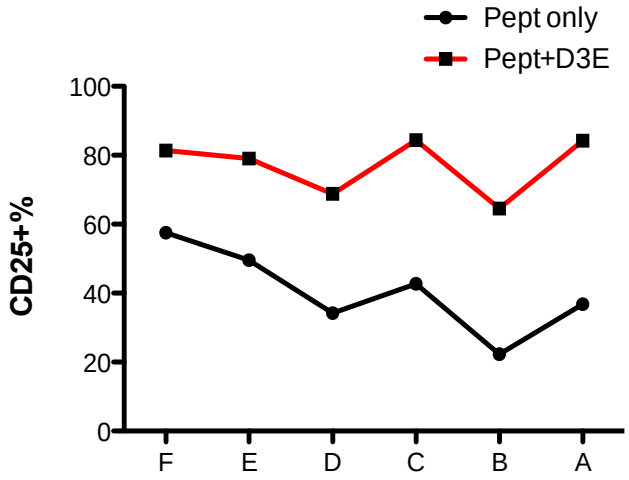


Figure 5. Exosomes increased the production of GZB, which was corresponding to CD25 expression, in the treatments of low-affinity peptides. Naïve CTLs were stimulated with different peptides for three days and stained for expression level. The expression of intracellular GZB and surface CD25 was obtained by FASC based on fluorescent intensity. (A.) The GZB and CD25 expression in response to the treatment of peptides with diverse affinities. (B.) The production of GZB and (C.) the CD25 expression was measured in the treatment with/without the D3 stimulation.

Activated CTLs secreted exosomes since the early stage of activation

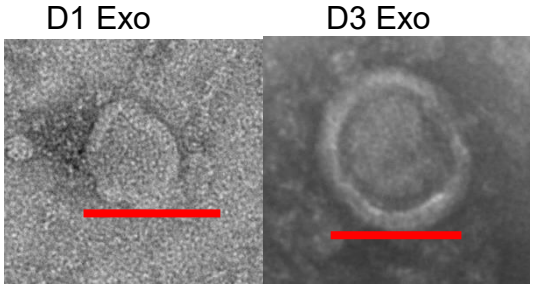
To induce the maximum immune responses, the effects of the exosome i.e. bystander activation of naïve CTLs and stimulation of low-affinity CTLs should take place as early as possible. In addition, cells, especially immune cells, are reported to continuously generate the exosomes during infection [112]. Therefore, we investigated the weather early activated CTLs could generate exosomes as a short-term response to infection.

We isolated the two populations of exosomes from an early stage and a late stage of activation. First of all, purified naïve CD8+ T cells from OT-I mice were fully activated with antigen and co-stimulators plus IL-12 in vesicle-depleted media [50]. The supernatant was collected from one day or three days following stimulation to purified extracellular vesicles. The purified extracellular vesicles displayed round shape structure with a double layer using TEM (Fig.6A). The protein yield was obtained by total protein concentration divided by total collected supernatant. D3 Exo yielded protein as 2.23 μg/ml. Surprisingly, the D1 Exo yielded abundant protein (0.9 μg/ml) considering that CTLs only started few cell proliferation (only 1/10 cell numbers compared with D3 CTL) at D1(Fig. 6A). The vesicles derived from CTLs stimulated for one day (D1 Exo) were smaller than three days stimulation of CTLs derived vesicles (D3 Exo), with mean sizes of 30.25 nm and 45.62 nm, respectively, as detected by a Nano ZS90 (Fig. 6A). The vesicles demonstrated size ranges and morphology consistent with exosomes [50, 57]. As a result, we confirmed that the extracellular vesicles from activated CTLs with one day or three days stimulation was exosome according to the morphology.

Next, we tested whether vesicles derived from CTLs of one-day stimulation expressed typical exosome markers [75]. Equal amounts of protein from purified vesicles and activated CTL lysates were examined by western blot. Although we there is no exclusive house housekeeping genes for EVs (exosomes and microvesicles) currently, we chose the most widely used one as the reference gene, β-actin. Both the D1 Exo and the D3 Exo contained comparable amounts of β-actin (Fig. 6B). Even though D3 Exo displayed the selective enrichment of exosomal proteins like Flotillin, Tsg101, and Alix, D1 Exo expressed extreme low (Flotillin and Alix), or even not detectable (Tsg101) exosomal markers (Fig. 6B).

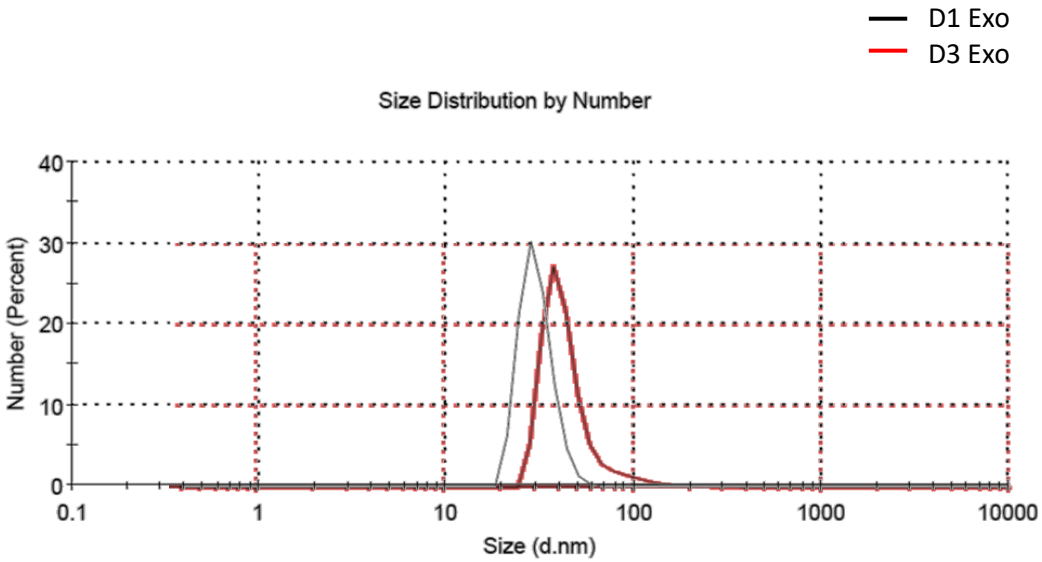
Overall, the D3 Exo was larger and more enrich with exosomal markers, CTLs activation signal (Zap70), and effector molecular (GZB) than the D1 Exo. The results indicated that D1 Exo was smaller and only expressed limited protein expression. Also, we exhibited that can produce abundant exosomes since early activated CTLs.

A



Exosomal protein yield (μg/ml)

D1 Exo	D3 Exo
0.90	2.23



B

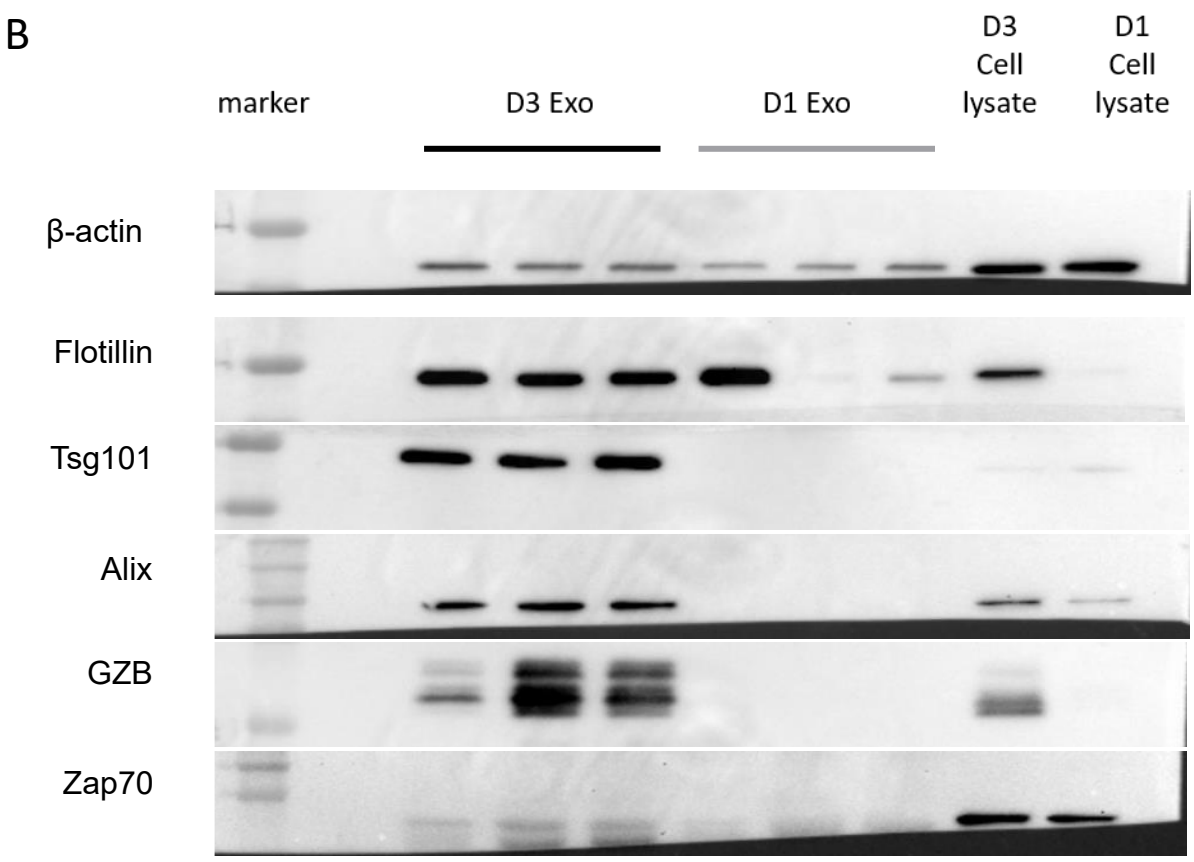

marker
D3 Exo
D1 Exo
D3 Cell lysate
D1 Cell lysate
β-actin
Flotillin
Tsg101
Alix
GZB
Zap70

Figure 6. Activated CTL-derived vesicles in the early stage of activation expressed typical exosome morphology and protein markers. Naïve CD8+ T cells purified from OT-I mice were in vitro activated by peptide A, B7, IL-2 and IL-12 for one day and three days. The extracellular vesicles were purified from each supernatant and characterized. Each experiment was replicated at least three times with similar results. (A.) Purified vesicles were observed under Zeiss EM10 transmission electron microscope. The graphs vary on amplification magnitude, and the bar in each graph indicates 50 nm. Size distribution of D1 Exo and D3 Exo measured by Malvern Zetasizer Nano ZS90. Protein yield was measured by total protein concentration/ supernatant (μg/ml). (B.) Representative blots showed exosome markers and CTL-associated protein. Purified exosomes from one day (D1 Exo) and three days (D3 Exo) activated CTLs were loaded at 10 μg protein in each lane and processed together for each protein marker.

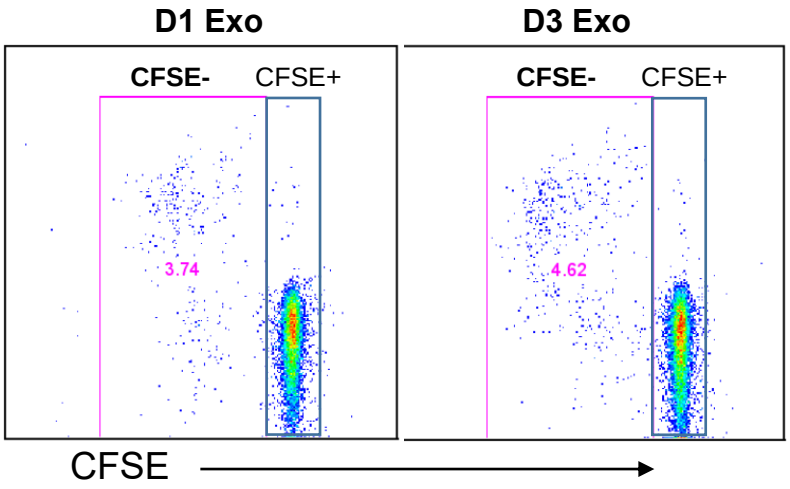
Early secreted exosomes triggered bystander activation in naive CTLs

In the previous report, we had discovered that D3 Exo derived fully activated CTLs can induce the bystander activation in naïve CTLs [50]. We would like to know whether the early secreted exosomes have the same function of inducing bystander activation like late secreted exosomes.

Purified D1 Exo and D3 Exo were added to the culture media supplied with IL-2 to stimulate naïve CTLs. The CTLs were harvested after three-day culture and stained with the corresponding antibody. Based on the dilution of CFSE dye, The CTLs were divided into CFSE- as cells entering the cell cycle or CFSE+ group as undivided cells. The percentage of CTLs entering the cell cycle was 3.74% in the group of the D1 Exo while it was 4.68% in the group of the D3 Exo (7A). Exo drove bystander-CTLs proliferation and dividing while D3 Exo was stronger than D1 Exo.

We next investigated the common used CTLs activation markers, CD25 and CD69, using FASC since activated CTLs expressed increasing CD25 phenotype [113] . We found out that the expression of CD25 was increased in both D1 Exo (67.3%) and D3 Exo (72%) and CD69 was also enhanced in D1 Exo (69.3%) and D3 Exo (69.2%) (Fig. 7B). We also observed the strong enhancement of GZB (13.3% in D1 Exo and 23.5% in D3 Exo) and IFN-γ production GZB (46.3% in D1 Exo and 62.9% in D3 Exo) (Fig. 7B). In short, D1 Exo had very similar but somewhat weaker effects to induce the expression of activation markers and effector molecules. CFSE+ expressed little enhancement in the expression of activation markers and effector molecules (Fig. 7B). Together, exosomes derived from an early stage of CTLs activation was sufficient to deliver activation signals to resting CTLs and help to induce the immune response.

A



D1 Exo—**CFSE-**

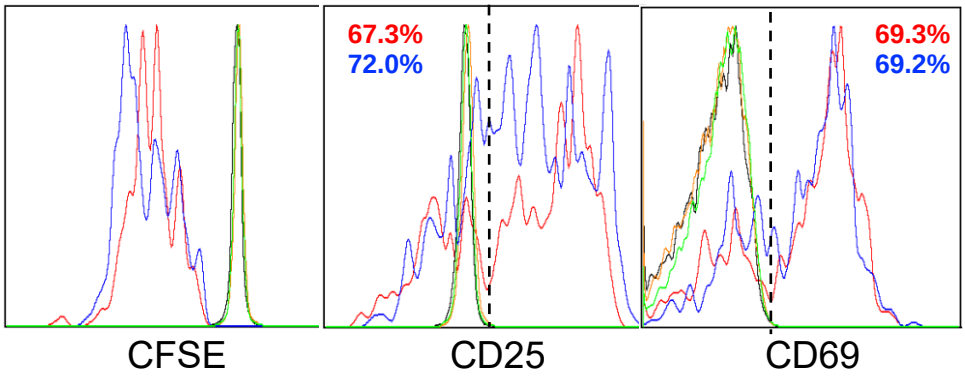
D3 Exo—**CFSE-**

D3 Exo—CFSE+

D1 Exo—CFSE+

No Exo control —CFSE+

B



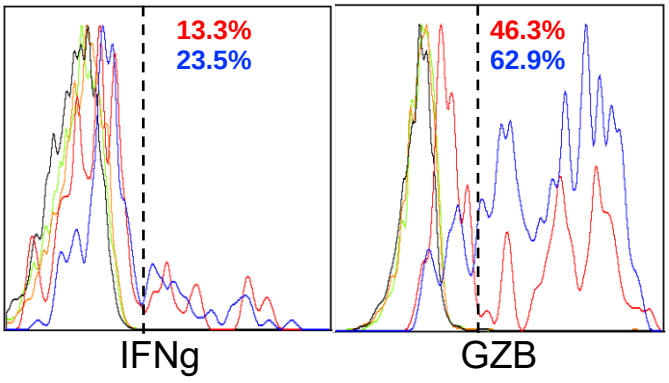


Figure 7. D1 Exo and D3 Exo induced the bystander activation in naïve CTLs. Exosomes were purified from the supernatant of one-day and three-day stimulated CTLs (D1 Exo and D3 Exo). Exo was added to the culture media supplied with IL-2 to stimulate naïve CTLs. The expression of the cytolytic and activation related molecules were assessed using FACS three days after exosomal stimulation. (A.) Cell proliferation of CTLs labeled by CFSE. CTLs were termed as CFSE positive (CFSE +) or negative (CFSE -) based on the CFSE signal. (B) Expression of CFSE, CD25, CD69; and effector molecules like GZB and IFN-γ. CTLs were stimulated with the presence or absence (No Exo control) of Exo (D1 Exo or D3 Exo).

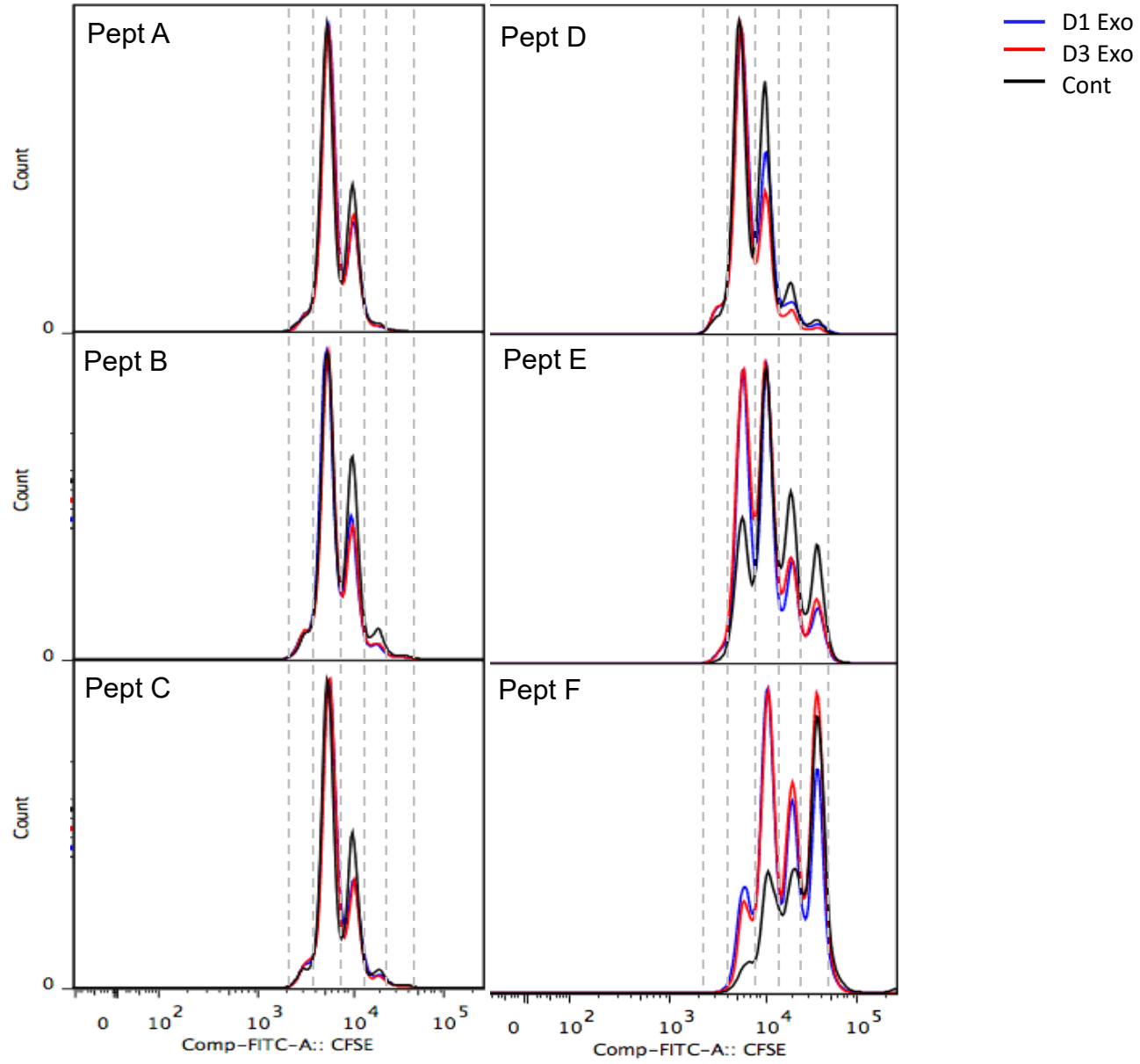
Exosomes derived from the early activated CTLs promoted low-affinity CTL activation

We had shown that the D3 Exo can stimulate the CTL enter cell division, especially in low-affinity peptide treatment (Fig 2B). We wonder whether D1 Exo had the same effects on CTLs. Purified D1 Exo and D3 Exo were incubated with CTLs treated with different peptides. As D3 Exo was more powerful than D1 indicating by previous results (Fig. 7B), exosomes helped the cell division while the D3 Exo was stronger in stimulating the cell dividing compared with the D1 Exo. The help was drastically in the low-affinity peptide treatment (Fig. 8A).

We next investigated the expression of several CTL activation markers to analyze the D1 Exo function. In comparison to the peptide stimulation only, CTLs treated with the exosome and the peptide generally exhibited a higher level of CD25, IFN-γ and GZB (Fig. 8B). Also, the production of effector molecular (GZB and IFN-γ, especially GZB) in low-affinity CTLs was significantly altered by the exosome stimulation which D3 Exo was stronger the D1 Exo (Fig. 8B). This was consistent with the previous observation that exosome had more profound and significant effects in low-affinity CTL than high-affinity CTL to promote the activation.

To conclude, activated CTLs use exosome as a novel means to communicate to the resting CTLs since the early stage of activation. Moreover, the early-derived exosome can help the CTLs with low affinity to proliferate and increase the expression of effector molecules.

A


Pept A
Pept D
Pept B
Pept E
Pept C
Pept F
Count
0
10^2
10^3
10^4
10^5
Comp-FITC-A:: CFSE
D1 Exo
D3 Exo
Cont

B

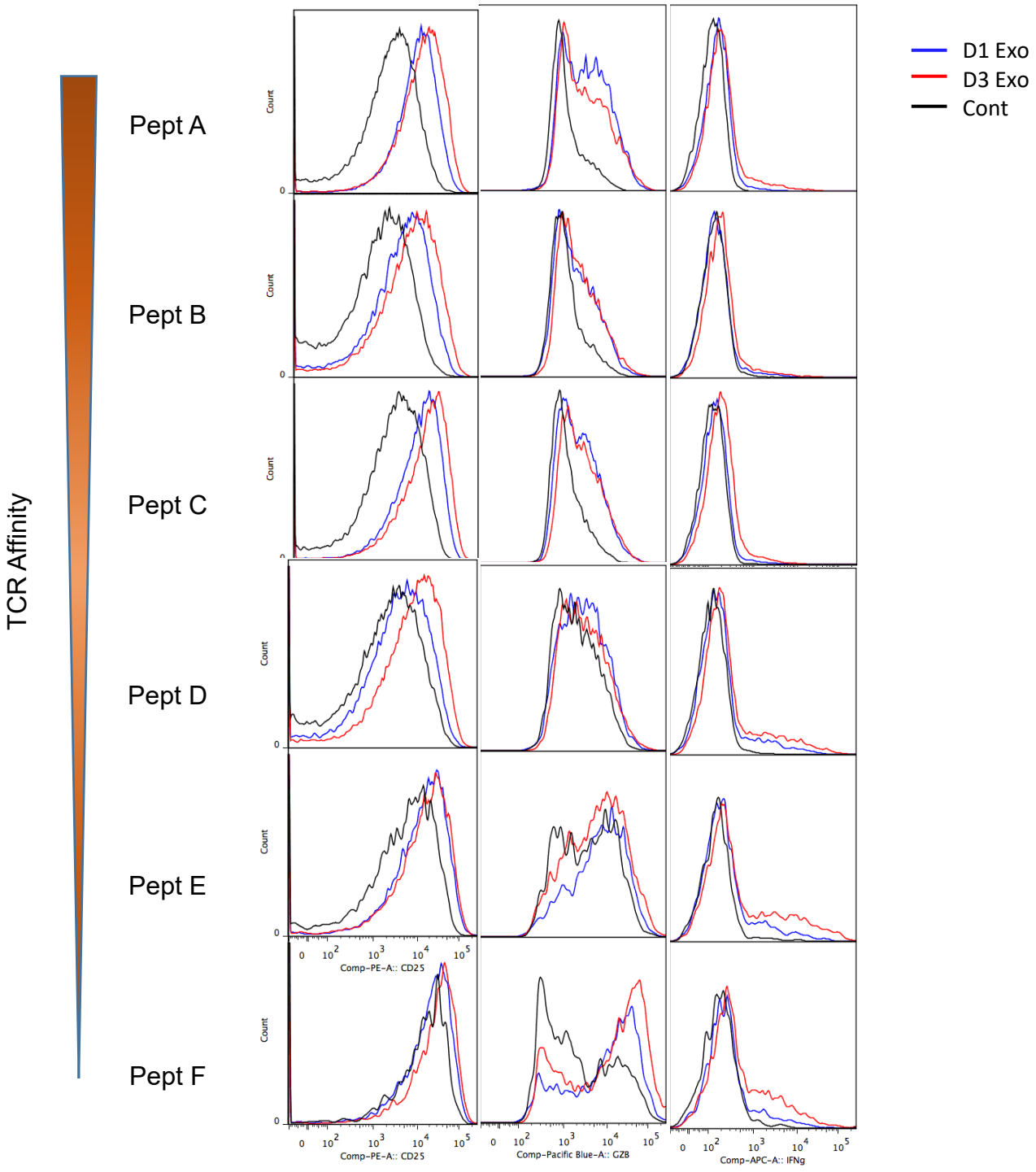

TCR Affinity
Pept A
Pept B
Pept C
Pept D
Pept E
Pept F
D1 Exo
D3 Exo
Cont
Count
Comp-PE-A:: CD25
Comp-Pacific Blue-A:: GZB
Comp-APC-A:: IFNg

Figure 8. D1 and D3 Exo enhanced the CTLs activation in low-affinity peptide treatments. Naïve CTLs were treated with diverse affinities of peptides (peptide A, B, C, D, E, F) with/without the stimulation of the Exo. After three-day incubation, the stimulated CTLs was harvested, stained with antibody cocktails and analyzed by FACS. (D1 Exo: day-one Exo plus peptide; D3 Exo: day-three Exo plus peptide; Cont: peptide only.) (A.) Cell dividing evaluated by dilution of CFSE labeling. (B.) Expression of CTL activation markers, CD25, GZB and IFN-γ.

Exosomes increased T-bet expression

Finally, exosome had been reported to mediate the transcription signals during an immune response [114]. We investigated one of the important transcriptional regulators of CTL differentiation and activation, T-bet [43]. In all the treatments with Exo, the expression of T-bet increased while the most significant one showed in the treatment with low-affinity peptides (Fig. 9). In conclusion, Exo can induce the expression of the transcriptional regulator, T-bet, in CTLs treated with high to low-affinity peptides.

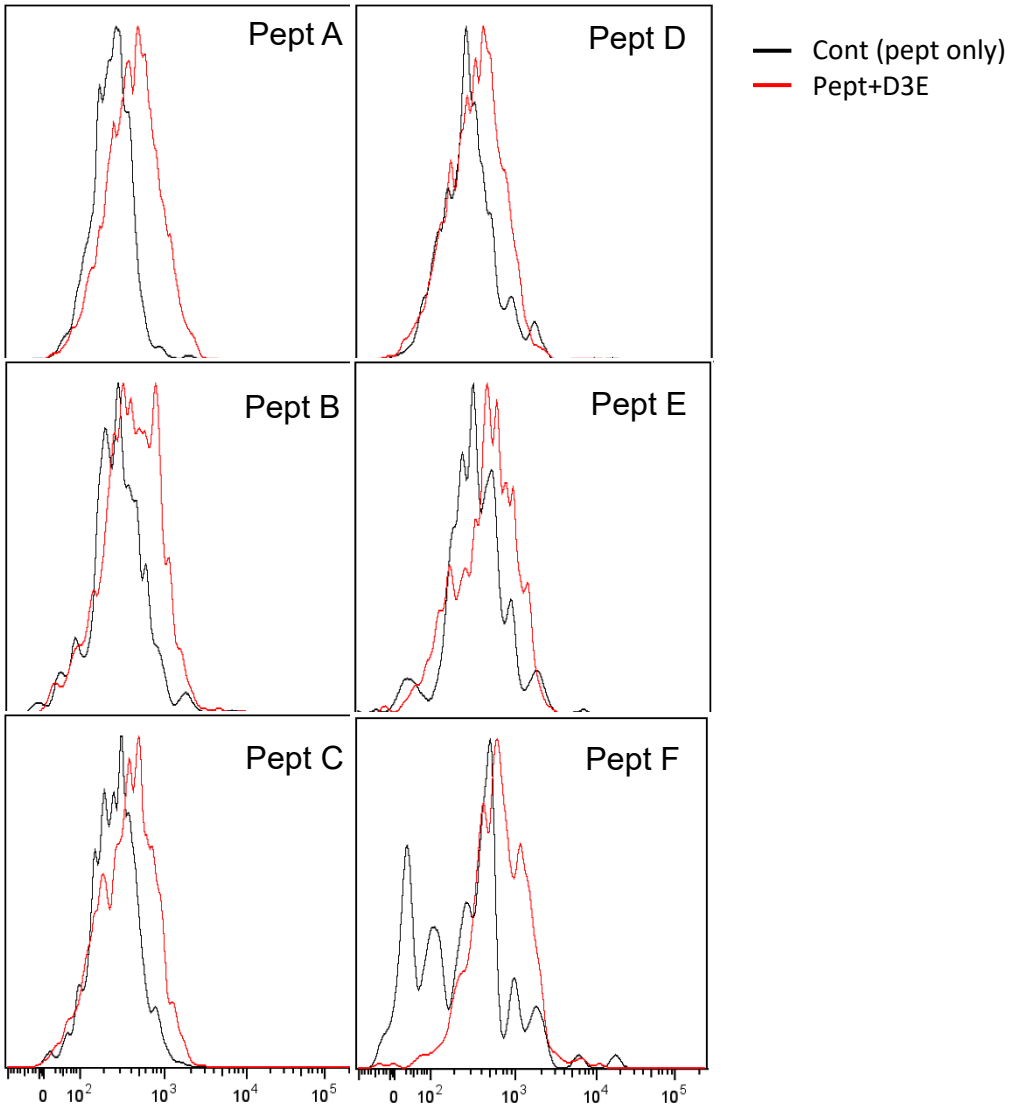


Figure 9. D3 Exo up-regulated the T-bet expression in low-affinity peptide treatments. Naïve CTLs were stimulated with D3 Exo plus peptide and peptide only for three days. Peptides were different in affinity. Next, CTLs were harvest and stained for detecting intracellular expression of T-bet by FACS.

Chapter 4: Discussion

Since there was an indication about the strong co-relationship between the pMHC/TCR affinity and the magnitude of CTL expansion and function [115], many studies focused on identifying the highest affinity to induce CTL the response. Identifying the highest affinity of TCR has long been at the center of the research in activating CTL. For example, the goal of cancer therapy is to develop chimeric antigen receptor (CAR) T cells with the highest affinity to tumor cells [116, 117]. Also, CTLs must receive comprehensive signals to induce cell expansion and differentiate into effector T cells [111]. However, in the real world, CTLs cannot always contact high-affinity peptides and receive the sufficient activation signals [103]. Actually, low-affinity CTLs, that will only receive weak activation signals, can still sustain a substantial population during a CTL response and help to maintain diverse antigen repertoire. Without further assistance, low-affinity CTLs exhibit impaired cell expansion and limited function [22, 30]. More importantly, mounting evidence indicated that low-affinity CTLs are important to induce effector response and maintain a diverse memory repertoire against viral infection and cancer [39]. Therefore, to optimize the CTL activation and summon maximum effector response, it's urgent to identify the mechanism of how signals can be delivered from activated T cells to resting T cells and help low-affinity CTLs to overcome the disadvantage of deficient activation signals. Here, we discovered that exosome stimulation improved the optimal CTL activation under weak stimulation (i.e. low-affinity peptide stimulation).

To induce the appropriate immune response against intracellular infection, T cells must be activated and start the robust expansion. The previous study has shown

that the weak stimulation of the antigen was sufficient to drive CD8 T cells through multiple rounds of division [2, 3]. This was termed as autopilot response which CTLs was activated due to a short-term exposure to the antigen [118]. However, the weak stimulation cannot induce a proper CTL cytotoxic activity against intracellular infection [16]. In our study, we discovered the novel mechanism to stimulate the activation of CTLs which was exposed to low-affinity peptides by exosomes derived from activated CTLs.

The affinity between the peptide and TCR is a critical key to induce robust proliferation of effector CTLs against the antigen. Weak pMHC /TCR interaction can induce a typical exhaustion phenotype and lead to dysfunction and susceptibility of CTLs [119]. We showed low-affinity CTLs could overcome the disadvantages of weak proliferation signals by treating with exosomes and more cells entered the cell cycle (Fig. 2B).

However, the cell proliferation is important while the maintenance of cell population is also critical to sustaining the vigorous immune response of CTLs. Antigen stimulation alone could lead to multiple rounds of cell division, as indicated by CFSE dye dilution, but cell numbers did not increase indicating impaired cell expansion [111]. As a result, the cell proliferation is not enough to determine the expansion of CTL, survival is also an important factor.

Although high-affinity CTLs generally showed superior proliferation with more cell entering the cell cycle (Fig. 2B), low-affinity CTLs treated with exosomes exhibited significant cell expansion and maintained the cell population during stimulation indicated by an increasing cell number (Fig. 3A). It was because the

exosome treated CTLs have better survival rate, so the total number of cell increased along with time. Interestingly, the treatment of D3 Exo stimulated the greatest expansions in peptide E, not the lowest affinity, peptide F. (Fig. 3A). This supports the idea that T cell expansion is not fully reliant on the peptide/TCR affinity and it is associated with exosomal signals. Thus, a major contribution of the exosome stimulation is to promote survival of the dividing cells.

There are many factors that will affect the magnitude of CTL activation. For example, the cytokine is one of the key signals that regulate the immune response. We found that the upregulation of CD25 expression was possibly the mechanism of exosome to stimulate the cell division and survival in low-affinity CTLs by increasing the IL-2 survival signal.

CD25 is the α chain of the IL-2 receptor [120]. IL-2 has long been considered as a T cell growth factor since it stimulates the proliferation and drives more T cells to enter the cell division cycle [121] [122]. IL-2 not only modulates the expansion of T lymphocytes but also can be produced by activated T lymphocytes [123]. Moreover, the presence of IL-2 during primary antigenic exposure is required for the development of effector T cells [124, 125]. Removal of IL-2 will result in extensive cell death [126]. Therefore, supplying IL-2 is necessary for studying CTL activation in order to provide the survival signals and to mimic the inflammatory environment during an infection.

The expression of the IL-2 receptor will be up-regulated upon CTL activation via TCR signaling [127]. The resting T cells express intermediate-affinity receptors to IL-2, IL-2Rβ/γ chains. After initiating the signal transduction trigger by pMHC/TCR interaction, T cells express high-affinity IL-2 receptor, which is IL-2 α /β/γ [128]. To

assemble a high-affinity receptor (IL-2 α /β/γ), the α chain of the IL-2 receptor is essential. Similar to previous results that IL-12 is necessary to promote the cell division by sustaining high-affinity IL-2 signaling [129] our data showed the exosome stimulates the cell expansion in low-affinity CTLs by upregulation of CD25 expression in response to IL-2.

We also observed that exosomes boosted the development of effector T cells by regulation of the CD25 expression. The upregulation of CD25 has a positive influence on the production of GZB in low-affinity TCRs (Fig 5A). The connection between CD25 and GZB was further demonstrated since the treatment of exosome stimulated the expression of both CD25 and GZB (Fig 5B, C).

IFN-γ is produced by activated effector CTLs and helps to stimulate the immune response [17]. CTLs must be stimulated with antigen, co-stimulators and IL-12 to obtain optimal activation [13, 15, 16], weak stimulation cannot induce the IFN-γ production. Therefore, the production of IFN-γ is used to recognize the CTL effector cells [130]. The production of IFN-γ was increased in the later rounds of cell division (Fig. 4B). This indicated that robust CTL expansion did not result in the development of effector function since peptide A treatment underwent several rounds of cell division while it produced low IFN-γ. Likewise, the augmentation of CD25 expression was more obvious in the late cell division (Fig. 3B). Upregulation of CD25 may enhance the IL-2 signal and lead to higher production of IFN-γ while detail mechanisms need further investigation.

In short, our study indicated that the stimulation of a high-affinity peptide can trigger the several rounds of cell division (Fig. 2A) while failing to induce the strong

effector function (Fig. 4A and 5A). *in vivo* stimulation of low-affinity peptides exhibited limited cell expansion of CTLs [30] while the stimulation of low-affinity peptides together with exosomes showed improvement of cell survival (Fig. 2A). This supported the theory that Exo enhanced the cell survival of CTLs. Exosome treatment also increased the IFN-γ production (Fig. 4A) and GZB expression (Fig. 5A). Besides, all the results support that the expression of CD25 might play an important role in activating low-affinity though IL-2 regulation.

The prompt immune response against infection is needed to prevent the disease. The exosomes derived from the late stage of CTL activation has substantial effects on provoking the bystander activation of naïve CTLs [50] while we also showed early-secreted exosomes also have the function.

The contents of exosomes reflect the status of original cells. Cells may adopt various ways to sort and generate exosomes in responding to a diverse environment. Through examining the morphology of exosomes, a suggestion will be revealed about the way that the cells respond to stimulation and explain the intracellular changes. The differences in size and morphology of secreted exosomes suggests that the stimulation time may direct exosome formation and content. Although both one-day stimulation and three-day stimulation can induce the production of abundant exosomes in CTLs, the size and the specific type of proteins differ noticeably between D1 Exo and E3 Exo (Fig. 6B). D1 Exo had the similar but weaker capability to induce the bystander activation of naive CTLs. When encountering the infection, T cells must respond timely to antigens and undergo massive proliferation. Therefore, early-secreted Exo was an

efficient communication between activated and resting T cells as a timely response to pathogens.

Secondly, considering that one-day stimulation only resulted in slow cell proliferation and three-day stimulation resulted in significant total cell number increase, the one-day simulation yielded abundant exosomes based on the protein concentration (Fig. 6A). Also, there were no available exosomal markers currently due to the specificity of cell type and distinct environment of stimulation. The markers we used were previously reported in the D3 Exo while other markers should also be tested. The production of exosomes can generally be divided into two pathways, ESCRT-independent and ESCRT-dependent [65]. Tsg101 and Alix are the major components of ESCRT pathway [64]. Lacking the expression of these proteins (Fig. 6B) suggested D1 Exo might take other pathways to generate exosomes, as a prompt response to the antigen, whereas D3 Exo formation in CTLs is ESCRT-dependent. However, further experiments are needed to understand the mechanism of secreting Exo by activated CTLs.

Flotillin is the common marker extracellular vesicle that abounds on the membrane [131]. The various expression in D1 Exo could be due to a bench difference and purification condition so more replica and more accurate assay should be adopted. The stable expression of activation-relative markers, Zap70, in D1 and D3 Exo indicated that the exosomes carried the downstream TCR signal molecule. Moreover, exosomes have been reported to carry the effector molecules to induce the apoptosis in a cancer cell [96]. Similarly, the abundance of GZB suggested (Fig. 6B) that exosome may help the cytotoxic activity of CTLs against infected cells while it needs the further

trials to confirm. Also, considering the low expression level in D1 Exo, we are currently analyzing the proteomic data which has a higher sensitivity and provides more comprehensive protein information and help us to understand the unique signals transferred by Exo from activated CTLs to naïve CTLs in the future. Furthermore, there was a large difference in protein expression, such as flotillin, between Exo and CTL lysate (Fig. 6B), supporting that CTL can selectively sort and pack the distinct components of exosomes. Understanding the sorting mechanism of the exosome in activated CTLs will benefit the future study of improving immune response. It would also be interesting to determine what contents of these early secreted exosomes might trigger the activation signal cascade. As a result, more candidates of protein markers should be tested on the early-secreted Exo to understand the biogenesis and signaling pathway in activating CTLs.

Similar to D3 Exo, D1 Exo triggered bystander activation of CTLs without the engagement of the peptide/TCR. D1 Exo stimulated naïve CTLs proliferation while D3 Exo stimulated more cell division. Both D1 and D3 Exo induced the production of IFN-γ and GZB although D3 Exo was more powerful than D1 Exo. The increased expression of CD25 and CD69 also supported the role of D1 Exo in inducing CTL response (Fig. 7B). Further understanding of the function of CTLs to directly destruct infected cells and evaluation of exosomal effects on a physiological range should be confirmed *in vivo* in the future.

When treating naïve CTLs with peptide and Exo, although the function of early-secreted exosomes was weaker than late-secreted exosomes, we still detected that D1 was sufficient to initiate and then sustain the great expansion in the low-affinity peptide

(Fig. 8A). As the previous observation, the tendency of CD25, GZB and IFN-γ in D1 Exo was identical to D3 Exo due to increased expression, especially in the treatment with low-affinity peptides (Fig. 8B). The early-secreted exosomes also showed increasing CD25 expression which will contribute to immense immune response. Moreover, both early and late secreted exosomes changed the transcriptional profile of CTL. The increment of T-bet expression (Fig. 9) suggested that exosome altered the transcriptional profile and more research is needed in the future to identify the downstream signal pathway of exosomal stimulation.

While there are undoubtedly many factors involved in the maintenance of robust immune response, our data indicated that the exosomes secreted by CTLs activated by strong-affinity peptides could result in communication between CTLs and help the activation of low-affinity CTLs. Although the regulation of exosome formation and secretion in CTLs is still unknown, we demonstrated that CTLs generated exosomes within D1 of stimulation as an immediate immune response to infection. Along with time, the secreted-exosomes became more powerful in improving the cell expansion and developing the effector function of CTL. To conclude, the research provides the first evidence that exosomes derived from 3SI stimulation contribute to mount a robust immune response of CTLs treated with the low-affinity peptide. (Fig. 10).

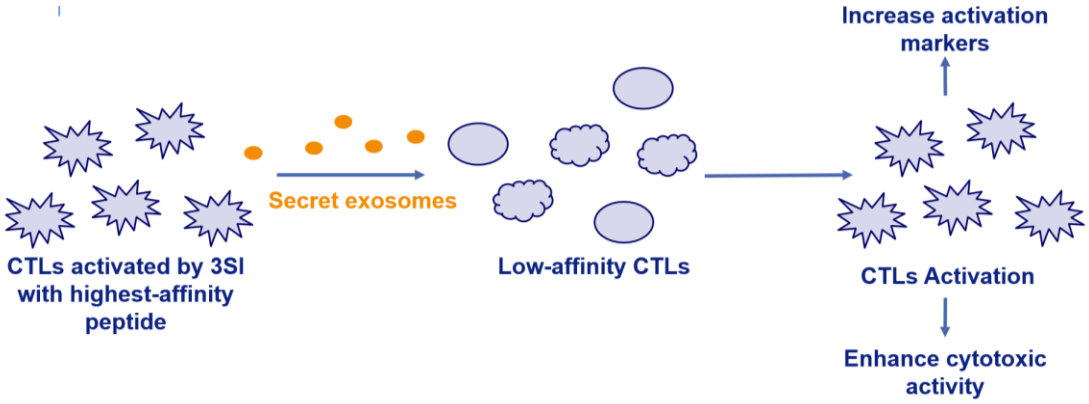


Figure 10. CTLs stimulated by 3SI with highest-affinity peptide can secret bio-functional exosomes to communicate and subsequently stimulate the naïve CTLs, especially those who received weak activation signals from low-affinity peptide stimulation.

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