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In Vivo Imaging of T Cell Priming

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The rules by which naïve T cells decide whether and how to respond to antigenic stimuli are incompletely understood. Using multiphoton intravital microscopy (MP-IVM) in lymph nodes (LNs), we have shown that CD8$^+$ T cells are primed by antigen-presenting dendritic cells (DCs) in three consecutive phases. During phase one, T cells undergo brief serial contacts with many DCs for several hours after homing into the LNs. Subsequently, during phase two, T cells engage in prolonged stable interactions with DCs. Finally, in the third phase, T cells return to transient interactions with DCs as they begin to proliferate and eventually leave the LNs. We have examined the influence of antigen dose on the duration of phase one by systematically varying both the number of cognate peptide–major histocompatability (pMHC) complexes per DC and the density of cognate pMHC complex–presenting DCs per LN. The duration of phase one and the kinetics of CD8$^+$ T cell activation were inversely correlated with both parameters. Very few pMHC complexes were needed for full T cell activation and effector differentiation. Furthermore, there was a sharp threshold of antigen dose below which T cells did not transition to phase two but continued to migrate until they exited the LN, unactivated. The stability of peptide binding to MHC was a critical determinant of this threshold antigen dose in vivo. Our results suggest an integrative mechanism that allows T cells to reach an informed decision about whether to respond, based on the overall antigen dose encountered.

Presentation Notes

Slide 1: Title page

This talk focuses on how T cells find dendritic cells (DCs) in vivo, how the interactions between these two types of cells change over time, and the rules that govern their interactions.

Slide 2: T cell homing

Naïve T cells recirculate constitutively from the blood to lymph nodes (LNs) and other secondary lymphoid organs (*1*). Homing to LNs (the apple-shaped tissue seen in the center of the slide) occurs via high endothelial venules. Lymph fluid is channeled from the peripheral tissues to LNs, where DCs collect antigenic material. In inflamed tissues, DCs receive maturation signals and then carry antigen to lymph nodes, where they stimulate antigen-specific T cells. Upon stimulation, T cells clonally expand and differentiate into effector cells, which express receptors that allow them to migrate to peripheral sites of inflammation. Although most effector cells are short-lived, a few cells will become long-lived (memory cells). These memory cells are made up of two populations, based on their migratory ability: The effector memory cells migrate to peripheral tissues, whereas central memory cells migrate preferentially to lymphoid organs based on their expression of homing molecules. Though we are studying many aspects of this model, the generation of effector cells, both short-lived and long-lived, is not a focus of this talk. Instead, the focus is on how T cells find DCs in vivo, how those interactions change over time, and the rules that govern those interactions.

Slide 3: The popliteal LN model for multiphoton intravital microscopy

After years of in vitro studies, it has been illuminating to observe how T cells and DCs interact in their physiological three-dimensional (3D) environment, the intact LN, which has been made possible by recent advances in intravital microscopy technology. Our lab has developed a microsurgical preparation of the popliteal lymph node (popLN) in anesthetized mice that allows prolonged intravital multiphoton microscopy (MPM-IVM) (*2*). The popLN has many advantages for these studies. Its small size allows imaging of the majority of the LN, its anatomical location facilitates stabilization of the tissue, and it drains lymph fluid from the lower leg, allowing easy control of antigen delivery. Our experimental protocol begins at −18 hours, with the injection into the right footpad of red fluorescently labeled DCs that either were or were not pulsed with peptide antigen. At 0 hours, we inject green fluorescently labeled, antigen-specific T cells intravenously (i.v.). Two hours after T cell injection, we inject antibody against L-selectin i.v., which prevents further T cell homing to LNs, thus synchronizing the T cells in the LN and allowing us to define a clear timeline of T cell activation.

Slide 4: Multiphoton time-lapse video microscopy

When using MPM-IVM to image the popLN, a *z* stack of *xy* sections is imaged at a set rate and those stacks can then be assembled into a volume for each time point. For example, if we take a *z* stack every 15 s (four frames per minute), we can then sequence the volumes from each time point and assemble them into a time-lapse movie. Our movies are typically played at 15 frames/s, so they play 225 times faster than real time.

Slide 5: Distribution of transferred cells in LNs

When we transfer red fluorescently labeled DCs and green fluorescently labeled T cells into a recipient and perform MPM-IVM in the popLN (at more than 150 μm below the outer surface of the LN, the capsule, which encloses the LN), we find a high density of T cells and DCs colocalized in the deep paracortex (*3*). Collagen in the LN capsule is seen in blue, made visible by second harmonic generation. On the left is a view of the whole LN, and on the right is

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a movie of the zoomed-in view of the deep T cell zone.

Slide 6: Homed T cells migrate in the extravascular space of the LN

This movie shows active migration of T cells in the extravascular space of a LN in an amoeboid fashion, consistent with random walk behavior.

Slide 7: Early T cell motility in LNs (phase one)

At early time points (here 2 hours after T cell transfer), there are short-duration interactions between green fluorescent, antigen-specific T cells and red fluorescent DCs without antigen (movie on the left), which are indistinguishable from the short-duration interactions between green fluorescent, antigen-specific T cells and red fluorescent DCs pulsed with cognate antigen (movie on the right). This behavior is characterized as phase one–type DC–T cell interaction and is prevalent for the first 8 hours after T cell transfer.

Slide 8: Stable T cell–DC Interactions (phase two)

Eight hours after T cell transfer (when the T cells have been in contact with antigen-pulsed DCs for at least 6 hours, based on the use of antibody to L-selectin at 2 hours after T cell transfer), the green fluorescent, antigen-specific T cells form stable interactions with red fluorescent peptide-pulsed DCs (see movie). This behavior is characterized as phase two–type DC–T cell interactions and remains prevalent until ~20 hours after T cell transfer.

Slide 9: Late return to transient T cell–DC Interactions

Beginning ~20 hours after T cell transfer, and seen in the movie at 44 hours after transfer, green fluorescent, antigen-specific T cells have returned to short-term interactions with red fluorescent peptide-pulsed DCs. These interactions are characterized as phase three–type interactions.

Slide 10: T cell priming by DCs occurs in three phases

When the MPM-IVM data were combined with flow cytometry studies of activation markers and cytokine production and proliferation, it became clear that there are three phases of $CD8^+$ T cell priming by DCs (*2*, *3*). For the first 8 hours after entry into a LN with antigen-presenting mature DCs, there are brief interactions between T cells and DCs. By the end of the first phase, these brief interactions result in up-regulation of early T cell activation markers (CD69). The second phase lasts approximately 12 hours and is characterized by stable DC–T cell contacts, the pattern of activation marker up-regulation characteristic of activated T cells, and cytokine production [interleukin-2 (IL-2) and interferon-γ (IFN-γ)]. On the second day after T cell transfer (phase three), there is a return to transient interactions between the T cells and DCs, with full cytokine production and proliferation of the newly generated effector T cells, which are eventually able to exit the LN and home to peripheral tissues to exert their effector functions.

The first phase was of particular interest, because many studies have previously shown that the initial contact between a T cell and a DC often yields an immediate transition to a stable interaction. Thus, we pose the question: What controls the transition from phase one to phase two, and how does antigen dose play a role in this transition?

Slide 11: Altered peptide ligands for P14 T cells

In order to study the role of antigen dose in controlling the timeline of T cell activation, it is necessary to carefully control the antigen dose. Our initial study was done with the transgenic mice that express the P14 T cell receptor (TCR), which recognizes the lymphocytic choriomeningitis virus (LCMV) peptide gp33-41 (KAVYNFATC, or "C-peptide") presented by the class I MHC $H\text{-}2D^b$. However, when this peptide is synthesized and used to pulse DCs, it is prone to oxidation of the cysteine residue and dimerization via cysteine-cysteine disulfide bond formation, which make antigen dose difficult to control. Therefore, many groups have switched to using an altered peptide ligand for this system, KAVYNFATM, or "M-peptide" (*4*). This altered ninth residue is an MHC contact residue, and it has been shown that the M-peptide has a higher affinity for $H\text{-}2D^b$ and a similar TCR affinity, when compared to C-peptide (*5*, *6*).

Slide 12: Multiphoton imaging of M- versus C-peptide

We began by comparing the dynamics of green fluorescent P14 T cell interactions with red fluorescent DCs pulsed with 10 μM C-peptide (see left movie) versus 10 μM M-peptide (see right movie) at early time points. Although the antigen-specific T cells maintain phase one–like interactions with the 10 μM C-peptide–pulsed DCs, there is a clear transition to phase two–like interactions for the antigen-specific T cells (green), but not the control T cells (blue), with the 10 μM M-peptide–pulsed DCs at this early time point. The identical behavior of control cells in both experiments shows that the surgical preparation is intact and that the preparation itself is not the cause of the slowed T cell migration.

Slide 13: P14 T cell interactions with peptide-pulsed DCs

In order to quantify the differences between these two peptides, we measured the duration of the interactions between P14 T cells and 10 μM M-peptide– versus 10 μM C-peptide–pulsed DCs (*7*). The 10 μM M-peptide–pulsed DCs led to a transition to phase two by 2 to 4 hours, whereas 10 μM C-peptide–pulsed DCs did not transition to stable interactions until 6 to 8 hours.

Slide 14: Measuring M- and C-peptide half-life in $H\text{-}2D^b$

Although pulsing DCs with M- or C-peptide at high concentrations may lead to initially similar replacement of peptide in endogenous pMHC complexes, a difference in the affinity of MHC for the two peptides could lead to very different numbers of retained pMHC complexes at later time points. However, the 18-hour delay between DC transfer and T cell transfer, necessary for DC homing, could greatly affect the number of peptide-pulsed (nonendogenous) pMHC complexes remaining when T cells first interact with DCs.

In order to determine whether the differences in the T cell priming dynamics are due to different pMHC affinities, we worked with Hing Wong and Bai Liu at Altor BioScience to develop a P14 TCR multimer, which allowed us to measure pMHC complex levels on peptide-pulsed cells (*7*). In order to determine the pMHC complex half-life of C-peptide and M-peptide in $H\text{-}2D^b$, we pulsed a tumor cell line with 10 μM of either M- or C-peptide for 3 hours and then washed the cells and maintained the cells at 37°C in a peptide-free solution until they were assayed for pMHC complex levels, using the TCR multimers, at various time points. We found empirical half-lives of 1.2 hours for C-peptide and 2.6 hours for M-peptide. Arup Chakraborty's lab at the Massachusetts Institute of Technology developed a computational model to correct for aspects of the experimental protocol that may artificially lower the TCR multimer binding (*7*) (including a 15-min step at room temperature to allow Annexin V staining), and we have calculated corrected half-life values of 2.36 hours for C-peptide and 6.01 hours for M-peptide with $H\text{-}2D^b$.

Slide 15: Estimating M peptide–MHC copy number

In order to estimate the number of pMHC complexes left on DCs when they first encounter T cells in our experiments, we first measured the number of H-2D^b's on immature DCs and found that there are approximately 25,000 H-2D^b's per immature DC. If we make the assumption that our peptide-loading conditions (10 μM M- or C-peptide) have sufficiently high concentrations to load all available H-2D^b's on a given DC, we can assume that DCs loaded with C- or M-peptide begin with 25,000 cognate pMHC complexes. This is probably an overestimate, but it allows us to define an upper limit. Using our calculated half-lives for C- and M-peptide, we can calculate that there are ~3000 M-peptide pMHC complexes and ~130 C-peptide pMHC complexes per DC at 18 hours, when we transfer T cells (*7*). This is a remarkably small number!

Slide 16: Relationship of peptide loading concentration and effective antigen dose

Although we have considered how many cognate pMHC complexes there are per DC, it is also important to consider how many pMHC complexes might be accessible to a given T cell when it initially meets a DC. This can be estimated by assuming that the contact zone is approximately 50 μm^2. Although our subsequent calculations may underestimate the eventual size of the T cell–DC contact zone (if endogenous pMHC complexes are drawn into the contact zone with the cognate pMHC complexes), we can estimate that there are no more than 13 (C-peptide) or 313 (M-peptide) cognate pMHC complexes per initial contact zone at the time of T cell transfer when DCs are pulsed with high concentrations of each peptide. Later experiments with even lower doses of peptide yielded full T cell activation with less than 50 pMHC complexes per DC, based on estimates from our model (*7*).

Slide 17: In vivo proliferation of M- versus C-peptide

To begin to estimate the in vivo effect of different doses of antigen, an assay based on dilution of the vital dye carboxyfluoroscein succinimidyl ester (CFSE) was performed to measure T cell proliferation. CD45.1 congenic mouse recipients received peptide-pulsed DCs, followed 18 hours later by CFSE-labeled CD45.2$^+$ antigen-specific T cells. Two hours later, antibody to L selectin was injected into the recipients to block further T cell homing to LNs. At 48 hours, the popLNs were removed and CD45.2$^+$ (transferred) cells were analyzed for CFSE dilution. To get maximal proliferation of P14 T cells, 6 μM C-peptide was required; whereas 30,000 times less peptide (200 pM) M-peptide yielded the same maximal proliferation.

Slide 18: CTL cytotoxicity is independent of peptide dose

Effector function was also found at this threshold concentration of 200 pM M-peptide, much like that observed for 10 μM M-peptide. In order to assay effector function, we performed an in vivo cytotoxicity assay by transferring peptide-pulsed DCs into congenic CD45.1 recipients, followed 18 hours later by antigen-specific T cells. At 48 hours, two populations of B cells were injected at a 1:1 ratio, one population pulsed with peptide and labeled with high levels of CFSE and one labeled only with low levels of CFSE. Six hours later, the popLNs were removed and the ratio of peptide-pulsed (CFSE-high) to unpulsed (CFSE-low) target cells was analyzed to assess cytotoxicity. DCs pulsed with 10 μM M-peptide or 200 pM M-peptide both led to the production of cytotoxic T cells, whereas DCs pulsed with 10 μM SIINFEKL (control peptide) did not.

Slide 19: Varying M-peptide concentration

In order to understand the effect of low antigen dose on the dynamics of T cell priming, we compared the interactions between T cells and DCs pulsed with 200 pM M-peptide and those pulsed with 10 μM M-peptide at early time points. There are transient interactions between antigen-specific T cells (in green) and 200 pM M-peptide–pulsed DCs (in red) at early time points (see left movie), which are characteristic of phase one. However, when antigen-specific T cells (in green) interact with 10 μM M-peptide–pulsed DCs (in red) at this early time point, T cell–DC interactions are prolonged, characteristic of phase two, whereas control T cells (in blue) maintain transient interactions (see right movie). Clearly, antigen dose can alter the duration of phase one. This suggests that phase one exists as a period for signal integration by T cells and may be prolonged in low-antigen environments to allow accumulation of the relevant signal.

Slide 20: P14 T cells undergo prolonged phase one–type interactions with DCs pulsed with low-dose M-peptide (200 pM)

Although green fluorescent, antigen-specific T cells engage in phase one–like interactions with 200 pM M-peptide–pulsed red fluorescent DCs at early time points (see left movie), they transition to phase two–like interactions by later time points (see right movie). Therefore, at this threshold dose, there is a lengthened phase one, which still yields full T cell activation, proliferation, and effector function.

Slide 21: Naïve T cell–DC interactions

In order to quantify the effect of antigen dose on the timeline of T cell activation, we used MPM-IVM to study the interaction times of antigen-specific T cells with DCs pulsed with various concentrations of M-peptide. DCs pulsed with concentrations of 500 pM or greater yielded a transition to phase two (as defined by interaction times of 30 min or more in a 60-min movie, marked by the red dotted line) by 2 to 4 hours. DCs pulsed with the threshold concentration of 200 pM yielded phase two by 6 to 8 hours. DCs pulsed with 100 pM did not yield phase two–type interactions even after 10 to 12 hours (and these cells also did not stimulate maximal T cell proliferation). So, clearly, the duration of phase one is inversely correlated with antigen density on the DCs. What is the role of the density of DCs?

Slide 22: How to modulate DC density?

Because the number of DCs arriving in the LN after subcutaneous footpad injection may not be linearly dependent on the number of DCs injected, we injected a constant number of DCs in each experiment but modulated the fraction of cognate antigen–bearing DCs.

Slide 23: Effect of DC dilution

To determine the effect of antigen-specific DC density on T cell activation, a constant number of DCs was transferred to congenic CD45.1 recipients, followed 18 hours later by CFSE-labeled, antigen-specific T cells. The DCs were 100, 10, 1, 0.1, or 0% cognate antigen–bearing—the remaining DCs in each condition were pulsed with SIINFEKL, a control peptide. The cognate antigen–pulsed DCs were pulsed with either a high dose (10 μM) or a threshold dose (200 pM) of M-peptide. At 18 and 48 hours after T cell transfer, popLNs were removed and assayed for CFSE dilution and IFN-γ secretion. When the DCs were pulsed with 10 μM M-peptide, 100 and 10% cognate antigen–pulsed DCs stimulated full proliferation of and IFN-γ secretion by the T cells. When only 1% of the DCs were cognate antigen–bearing, IFN-γ secretion by the T cells was reduced, and when only 0.1% of the DCs were cognate antigen–bearing, then T cell

proliferation and IFN-γ secretion were essentially eliminated. However, the response to DCs exposed to the threshold dose of antigen (200 pM) was much more sensitive to the density of DCs: Whereas 100% of cognate antigen–pulsed DCs yielded essentially full T cell proliferation and IFN-γ secretion, 10% of cognate antigen–pulsed DCs produced greatly reduced T cell IFN-γ secretion, and 1% of cognate antigen–pulsed DCs did not stimulate T cell proliferation or IFN-γ secretion. So the lower dose of antigen yielded a 10-fold higher sensitivity to DC density.

Slide 24: Do T cells or DCs control the transition from phase one to phase two?

It is also important to examine whether T cells or DCs control the transition from phase one to phase two. DCs were pulsed with both 10 μM SIINFEKL and 200 pM M-peptide, and then MPM-IVM was performed to study the interactions of P14 T cells (specific for M-peptide) and OT-I T cells (specific for SIINFEKL). The P14 T cells (in green) maintain transient, phase one–like interactions with the DCs (in red), whereas the OT-I T cells (in blue) maintain stable phase two–like interactions with the same DCs (see left movie). This argues against DCs deciding for T cells whether to engage in phase one or phase two interactions, because a single DC can support both phase one and phase two interactions with two different populations of T cells.

Trivial explanations for these results include competition between peptides for loading in H-2K^b's or between T cells for access to DCs. An argument against this is that SIINFEKL loads only in H-2K^b, whereas M-peptide can load in H-2K^b and H-2D^b (although the P14 TCR recognizes M- or C-peptide only in H-2D^b), and the levels of M-peptide are unlikely to compete with the high levels of SIINFEKL. Nevertheless, to rigorously rule out the possibility that the results were caused by competition between peptides or T cells, we performed an MPM-IVM experiment wherein P14 and OT-I T cells interacted with DCs pulsed with 10 μM SIINFEKL and 10 nM M-peptide (a concentration of M-peptide that is high enough to yield a fast transition to phase two, but not so high as to interfere with SIINFEKL loading in H-2K^b). This strategy (see right movie) yielded a fast transition to phase two for both P14 (in green) and OT-I (in blue) with the dually peptide-pulsed DCs (in red). Therefore, it is unlikely that the ability of a single DC to maintain both phase one and phase two interactions with different T cell populations was due to a difficulty in loading pMHC complexes or accessing the DCs.

Slide 25: Analysis of T cell interactions with dual-peptide–pulsed DCs

To quantify the difference between P14 T cells (blue squares) and OT-I T cells (green circles) in the left movie from the previous slide, the T cell interactions with DCs pulsed with both high-dose SIINFEKL (10 μM) and threshold-dose M-peptide (200 pM) were measured. There is a significant difference between the stable interactions at early time points between OT-I T cells and DCs and the transient interactions between P14 T cells and DCs. This argues against a primary role for DCs in determining the time for T cells to transition from phase one to phase two.

Slide 26: Signal integration by T cells

The results suggest that phase one is a period during which T cells integrate antigenic signal from interactions with DCs presenting low amounts of antigen. As a T cell serially interacts with three DCs (DC 1, 2, and 3), it gets a small stimulus from each DC, which can be thought of as a small increase in the T cell activation cumulative signal. When it reaches the fourth DC, in this cartoon, it has received sufficient cumulative signal to trigger a transition to phase two. However, the distribution of antigen may be less homogenous than we might expect—there can be heterogeneity in antigen dose per DC or antigen density on the surface of a given DC. If signal integration is a physiological phenomenon, then the following prediction can be tested: If T cells encounter two populations of DCs with different levels of antigen (one with a high level and one with a low level), then the time to transition to phase two interactions would be shorter than when the T cells encounter a single population of DCs with the low level of antigen.

We showed this experimentally by allowing P14 T cells to interact with DCs pulsed with either 200 pM M-peptide or 10 μM M-peptide in a single LN or separately. T cells interacted with transient interactions with 200 pM M-peptide–pulsed DCs from 0 to 2 hours after T cell transfer, whether or not there were 10 μM M-peptide–pulsed DCs present. However, bewteen 2 to 4 hours, T cells that interact with DCs pulsed with 200 pM M-peptide in the context of 10 μM M-peptide–pulsed DCs had transitioned to phase two. T cells transitioned to phase two in 6 to 8 hours when exposed only to DCs pulsed with 200 pM M-peptide. We conclude that it is the history of prior antigenic experience, not just the antigenic density on a single DC, that affects the kinetics of T cell activation. This is an argument for the importance of signal integration in T cell activation by DCs.

Slide 27: T cell decision-making, part one

Although our experiments were performed with a large number of adoptively transferred TCR transgenic T cells with a TCR with high affinity for the model peptide, physiological responses involve a T cell population that is polyclonal, containing cells with TCRs with diverse affinities for any given antigen. How do our observations apply to the physiological setting?

Slide 28: T cell decision-making, part two

In a physiological setting, the polyclonal T cell population contains many TCRs with diverse affinities for a given antigen presented in a LN by a mature DC. Each T cell must make a binary decision to participate in the immune response or not, based on the analog signal of antigen dose. A prolonged phase one allows these cells to measure the antigen dose in the LN carefully and decide between joining the immune response or leaving the LN, unactivated.

Slide 29: People who did the work

This work involved members of the von Andrian lab and collaborators in Arup Chakraborty's lab at MIT and from Altor BioScience.

Editor's Note: This contribution, in which primary data are shared with other readers, is not intended to be equivalent to an original research paper. Note, in particular, that the text and associated slides have not been peer reviewed.

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