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ABSTRACT

The TCR-pMHC interaction is the only antigen specific interaction during T lymphocyte activation. Recent work suggests that formation of catch bonds is characteristic of activating TCR-pMHC interactions. However, whether this binding behavior is an intrinsic feature of the molecular bond, or a consequence of more complex multimolecular or cellular responses, remains unclear. We used a laminar flow chamber to measure, firstly, 2D TCR-pMHC dissociation kinetics of peptides of various activating potency in a cell-free system in the force range (6-15pN) previously associated with catch-slip transitions and, secondly, 2D TCR-pMHC association kinetics, for which the method is well-suited. We did not observe catch bonds in dissociation, and the off-rate measured in the 6-15pN range correlated well with activation potency, suggesting that formation of catch bonds is not an intrinsic feature of the TCR-pMHC interaction. The association kinetics were better explained by a model with a minimal encounter duration rather than a standard on-rate constant, suggesting that membrane fluidity and dynamics may strongly influence bond formation.

SIGNIFICANCE STATEMENT

T lymphocytes use their T cell receptors (TCR) to discriminate between similar peptide-MHC (pMHC) antigens. The mechanisms employed to achieve this discrimination are debated. The TCR-pMHC interaction is subjected to forces and recent work in live T cells has suggested that force paradoxically increases TCR-pMHC bond lifetimes for activating antigenic peptides, forming so-called “catch bonds”, which facilitates discrimination from non-activating peptides. A question is whether this behavior is intrinsic to the TCR-pMHC bond or a cellular response. We measured TCR-pMHC lifetimes under force in a cell-free system: lifetimes correlated well with activation potency of the TCR-pMHC bonds, while no catch bonds were observed. We observed that a minimum encounter duration is necessary for bond formation, which could increase specificity.

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INTRODUCTION

T lymphocyte activation begins with the binding of the T Cell Receptor (TCR) on the lymphocyte surface to an antigenic peptide carried by a Major Histocompatibility Complex (pMHC) molecule on the antigen presenting cell (APC) surface, triggering a cascade of signaling events. The TCR is the only antigen-specific molecule of the T lymphocyte activation, making the TCR-pMHC interaction a decisive step. A long-standing problem was to understand the basis of the exquisite specificity of T lymphocytes. While discrimination between different pMHCs seems based on quantitative properties of the TCR-pMHC bond such as its lifetime[CITATION Matsui1994 \l 1036], bond rupture is a stochastic event, making a single lifetime measurement insufficient to discriminate between peptides forming bonds with only limited lifetime difference[CITATION Lin2019 \l 1036]. Several studies using surface

plasmon resonance mostly reported a correlation between a TCR-pMHC bond off-rate measured in solution (three-dimensional or 3D) and its lymphocyte activation potency [CITATION Matsui1994 \l 1036][CITATION Lyons1996 \l 1036][CITATION Kersh1998a \l 1036] [CITATION Aleksic2010 \l 1036][CITATION Dushek2011 \l 1036], leading to the kinetic proofreading model[CITATION McKeithan1995 \l 1036]. However, as the TCR-pMHC interaction takes place between two cell surfaces, it is subjected both to a disruptive force and to two-dimensional (2D) motions linked to membrane fluidity and dynamics, that may profoundly change the kinetics of molecular interactions[CITATION Bell1978 \l 1036][CITATION Marshall2003 \l 1036] [CITATION Huppa2010 \l 1036][CITATION Huang2010 \l 1036]. Furthermore, TCR triggering is sensitive to mechanical forces [CITATION Kim2009d \l 1036] [CITATION Li2010 \l 1036] [CITATION Hu2016 \l 1036] [CITATION Liu2016 \l 1036] [CITATION Feng2017 \l 1036]. Multiple bond lifetime measurements might be needed by the cell to overcome the stochasticity of bond rupture. However, due to the duration needed for such repeated assays and the time constraint of T lymphocyte activation, it has been proposed that force application might be a way to strongly reduce the lifetime of the TCR-pMHC bonds, to allow numerous, repeated measurements in a short time.(Bongrand He Front Immunol, 3:90 2012; Klotzsch Schutz BJ2013) Force might also unravel other discriminating parameters such as bond sensitivity to force, further improving discrimination between pMHC, while also reducing the variability of bond lifetimes (Klotzsch Schutz BJ2013). For these reasons, efforts have been made to measure TCR-pMHC 2D *dissociation* kinetics and the effect of mechanical force thereon. Independent studies using the biomembrane force probe[CITATION Liu2014 \l 1036][CITATION Hong2015 \l 1036] [CITATION Kolawole2018 \l 1036][CITATION Sibener2018 \l 1036] or optical tweezers [CITATION Das2015 \l 1036] on live cells, or using optical tweezers in cell-free experimental set-up[CITATION Das2015 \l 1036 \m Liu2015] reported that activating TCR-pMHC interactions exhibit a decrease in off-rate when exposed to mechanical force in the 10pN-15pN range, *i.e.* catch

bonds. It was therefore suggested that T cells might probe the TCR-pMHC bond by exerting a pull on the order of 10 pN, with activating peptides displaying a non-intuitive increase bond lifetime that made it 10 fold higher than the lifetime of non-activating peptides [CITATION Liu2014 \l 1036]. This raises two questions: i) bonds formed by T cells and APCs are not only bimolecular interactions but depend on complex cellular processes[CITATION Huppa2010 \l 1036][CITATION Huang2010 \l 1036]. Thus, a cell-free system is necessary to check that the catch bond effect is a cause, not a consequence, of high activation by efficient agonists. We previously measured dissociation kinetics of TCR and observed increases in off-rate with force, *i.e.* slip bonds. However, the forces applied were higher than 15 pN, precluding a direct comparison with aforementioned results[CITATION Robert2012 \l 1036]. ii) Since T cell discrimination may involve multiple TCR-pMHC interactions, measuring the kinetics of bond formation as well as dissociation is necessary to fully assess the properties of TCR-pMHC interaction.

While several groups have examined 2D dissociation kinetics, studies of the 2D *association* kinetics of the TCR and pMHC are scarce and were limited by the methods used. 2D association measurements need a quantification of both the binding events and the distribution of the durations of the molecular encounters that may lead to binding; these encounter durations need to be in a physiologically relevant range. Before pulling, both BFP and optical tweezers bring into contact TCR and pMHC-bearing surfaces for durations of several hundreds of milliseconds, much longer than membrane fluidity and dynamics would allow[CITATION Huang2010 \l 1036][CITATION Das2015 \l 1036]. The thermal fluctuation assay relies on spontaneous membrane fluctuations bringing into contact both surfaces[CITATION Liu2014 \l 1036]. While contact durations may be much shorter than in BFP or optical tweezers, neither duration of encounter nor applied force are controlled. In contrast, our laminar flow chamber enables control of a distribution of encounter durations in the millisecond

range[CITATION Robert2011a \l 1036][CITATION Limozin2016 \l 1036] and is thus well-suited for 2D association measurements after molecular encounters of short duration. The description of 2D bond formation is usually based on on-rates, corresponding to one free energy barrier leading to one free energy well. Probability of bond formation as a function of the duration t_e during which a receptor can interact with its ligand (referred later as "encounter duration") can be written as $P(t_e)=f_E\times\left(1-\exp\left(-k_{on}\times t_e\right)\right)$ where f_E is a proportionality factor and k_{on} is the on-rate[CITATION Limozin2016 \l 1036]. Using the laminar flow chamber, we observed that description of antibody-antigen association through an on-rate was not appropriate. We proposed a minimal encounter duration model [CITATION Robert2009 \l 1036][CITATION Robert2011a \l 1036] where bond formation results from crossing a rough initial part of the energy landscape, which occurs as a slow diffusion process, before a free energy well; probability of bond formation can be written as $P(t_e)=f_E\times erfc\sqrt{t_{on}/t_e}$ where $f_E$is a proportionality factor, *erfc* is the complementary error function, and t_{on} is a characteristic time of the bond. The similarities between TCR and antibodies suggest that this model might also describe TCR-pMHC association. We measured at the single molecular level in the laminar flow chamber the association and dissociation kinetics of five different agonist TCR-pMHC bonds under force from 6 to 45pN, in the range where catch bonds were observed by other groups. These TCR-pMHC interactions did not form catch bonds, but their dissociation kinetics correlated well with their activation potency. Minimal encounter durations (t_{on}) described bond formation better than on-rates (k_{on}.

RESULTS

Demonstration of single molecular TCR-pMHC association and rupture under 2D conditions- Single bond measurements were demonstrated using the usual method for laminar flow chamber experiments[CITATION Robert2012 \l 1036] with two necessary conditions that

are sufficient if both are realized: first, if single molecular bonds are observed, increasing the amount of ligand on the chamber surface must increase proportionally the BLD; second, survival curves must remain unchanged for the different amounts of ligand on the chamber surface as the same binding events are measured. Here, for each pMHC, flow chamber experiments were performed on substrates coated either without ligand as a negative control, or coated with eight different amounts of ligand, doubling from one condition to the next, thus varying the ligand amount 128-fold. For each pMHC, for three consecutive amounts of ligand (forming a fourfold range) plus a negative control, BLD varied proportionally to the amount of deposited ligand. This is shown by the high correlation coefficient of BLD versus amount of deposited ligand for these four conditions, with R^2= 0.97 for 3A, R^2=0.97 for H74, R^2=0.98 for 9V, R^2=0.80 for 3Y and R^2=0.92 for 9L (SI Appendix, Figure S1). Survival curves remained unchanged at least for the two highest consecutive amounts of ligand in this range (SI Appendix, Figure S2), arrests were therefore considered to be the consequence of formation of single molecular bonds.

Activating pMHC do not necessarily form catch bonds with TCR. We measured the dissociation kinetics of the five agonist TCR-pMHC pairs under force ranging from 6pN to 45pN. The slope of the survival curve (where the fraction of surviving bonds is plotted versus time) is equal to the off-rate in semi-log plots such as in SI Appendix Figures S2 and S3. To account simply for the change of slope with time suggesting a bond strengthening (SI Appendix, Figure S3), we chose to consider the average slope, or off-rate, on the 5 first seconds. This was justified by calculating for each force the correlation between the peptide activation potency and the average off-rate calculated at variable intervals (SI Appendix, Figure S4). An interval of 5s ensured a maximal correlation coefficient at each force. The corresponding average half-lives are calculated from the average off-rate between 0 and 5s ($k_{off(0\text{-}5s)}$) as equal to $\ln(2)/k_{off(0\text{-}5s)}$, Figure 1 shows the half-lives plotted versus force: 3A, 3Y

and 9L behave as slip bonds, with off-rate increasing with force. H74 and 9V are little influenced by force in this range.

Dissociation kinetics show good correlation with activation potency from 6 to 15pN. Activation potency was measured previously [CITATION Aleksic2010 \l 1036] as an EC_{50}, being the amount of pMHC on a surface necessary to trigger 50 percent of the maximum γ-interferon production by 1G4 T lymphocytes. Here, off-rates were significantly correlated with activation potency of each pMHC under 6pN, 10pN and 15pN force (Figure 2). It then reduced sharply at 30pN and 45pN. A poor correlation was also observed here between off-rate measured in solution (3D) earlier by surface plasmon resonance and activation potency. 2D and 3D series of off-rate values remain in the same order of magnitude.

Association kinetics supports a minimum encounter duration model. The BLD were measured for the five TCR-pMHC interactions with different values of shear stress. The distribution of encounter durations for these shear stresses was calculated using a numerical simulation of microspheres and binding sites motion (SI Appendix, Figure S5). The calculation of distributions of encounter durations allowed us to compare the ability of two different bond models to describe bond formation. One model uses a classical on-rate k_{on}, and a second model uses a minimal encounter duration t_{on} as described in our previous works, both use a single adjustable parameter. Our assumptions on the geometry of the bonds were checked by systematically varying in the simulation the maximal solid angle of diffusion φ_{max} and the maximal variability in radial length ΔR_{max}. Calculated association kinetics changed in a limited way except for very narrow angles (SI Appendix, Figure S6). The classical on-rate model (k_{on}) fitted the data poorly (Figure 3) except for the 3A pMHC, while the minimal encounter duration model (t_{on}) gave better fits. t_{on}values were 0.46ms for 3A, 0.72ms for H74, 0.65ms for 9V, 0.91ms for 3Y and 0.69ms for 9L.

DISCUSSION

What is the physiological relevance of single TCR-pMHC bond measurements under force? In physiology, both TCR and pMHC are linked to actively motile cell membranes, where they are surrounded by adhesive molecules roughly of the same short dimensions (such as CD4 or CD8, CD2, CD28 and their respective ligands) or moderately larger (such as LFA-1 and its ligand ICAM-1), but also by much larger sterically repulsive molecules such as CD45, CD43 and CD148[CITATION Burroughs2006 \l 1036][CITATION Springer1990 \l 1036]. The interaction takes place in a context of mechanical forces: the T lymphocyte crawling on the APC surface while TCRs probes their ligands creates a mechanical shear force in the order of 1000pN at the cell scale[CITATION Yang2015 \l 1036]. Motion in the axis perpendicular to membrane plane due to membrane fluctuations exist as in other cell types[CITATION AYu1990 \l 1036], but the T lymphocyte also probes the APC by extending and retracting microvilli which are enriched in TCRs[CITATION Jung2016 \l 1036]. Forces exerted by a T lymphocyte upon TCR engagement have been measured with biomembrane force probe, showing initial pushing and pulling forces around 25pN[CITATION Husson2011 \l 1036], by traction force microscopy, showing forces between 50pN and 200pN after initial spreading[CITATION Bashour2014 \l 1036], and by DNA sensors showing forces between 12 and 18 pN seconds after binding[CITATION Liu2016 \l 1036]. The 6-45pN range chosen is well-suited to mimic these observations.

In a laminar flow chamber, as well as in other methods, a first important limitation is due to the use of TCR or pMHC grafted on artificial surfaces. Adhesive or repulsive molecular environment is absent, as well as membrane reorganization that allow clustering of adhesive molecules as well as expelling of larger anti-adhesive molecules. A second limitation is due to the kinetics of force application, which is necessary to bond detection and may modify protein-protein binding. Many ligand-receptor pairs involving biomolecules display multiple

binding states, of which the most stable may not be reached instantaneously. Properties of a ligand-receptor bond depend thus on its history[CITATION Pincet2005 \l 1036]. Experimental evidence obtained on antigen-antibody bonds supports the general view that bond formation is a time- and force-dependent process involving a continual strengthening that may require milliseconds to seconds or more[CITATION Robert2009 \l 1036] [CITATION LoSchiavo2012 \l 1036] A third point is that, rather than unidimensional paths, energy landscapes should be represented as multidimensional surfaces allowing multiple reaction pathways (indeed proposed for catch-bonds[CITATION Pereverzev2005a \l 1036]). Consequently, it is important to recall that the flow chamber method displays the behaviour of bonds that are a few milliseconds old, whereas typical contact time used with atomic force microscopy or biomembrane force probe is on the order of 100ms. Thus, different experimental methods may explore different regions of the energy landscape.

Do agonist TCR and pMHC form intrinsic catch bonds? We did not observe catch-bonds in the dissociation of our five agonist TCR-pMHC ligand pairs in our cell-free experimental set-up, but three classical slip bonds found for either a very potent or poor activators (3A, 3Y and 9L), and two almost "ideal" bonds (*i.e.* showing no force dependence) being moderately potent activators (H74,9V). There is no doubt that catch bonds can be detected with the flow chamber[CITATION Thomas2002 \l 1036][CITATION Marshall2003 \l 1036], including by our group[CITATION Gonzalez2019 \l 1036]. Forming catch bonds at 10-15pN has been proposed to increase ligand discrimination by strongly increasing differences in bond lifetimes between catch bond forming agonists peptides and slip-bond forming irrelevant or antagonist peptides[CITATION Liu2014 \l 1036][CITATION Hong2015 \l 1036] [CITATION Kolawole2018 \l 1036][CITATION Sibener2018 \l 1036]. Using the laminar flow chamber, non-specific TCR-pMHC interactions could not be detected here, and so

presumably had lifetimes shorter than the detection threshold of 180ms. The specificity of agonist versus irrelevant peptide detection may therefore be very good even if agonist peptides form slip bonds with the TCR. Also, previous studies on catch-bond forming TCRs found that lifetime differences between strong or weaker agonist pMHC at 10-15pN were typically around two-fold[CITATION Liu2014 \l 1036][CITATION Das2015 \l 1036] [CITATION Kolawole2018 \l 1036][CITATION Sibener2018 \l 1036]. Here, differences in off-rates between strong and weaker agonists between 6pN to 15pN were also up to two-fold (Figure 2), illustrating that significant survival differences may also be produced by agonist pMHC without catch bonds. This different response of TCR-pMHC to force that we report here could be solely due to specificities in the TCR-pMHC interactions we studied. It is also possible that cellular [CITATION Hong2015 \l 1036][CITATION Liu2014 \l 1036] [CITATION Das2015 \l 1036] and cell-free[CITATION Das2015 \l 1036] experiments that observed catch bonds may have produced misleading results, for the following two reasons. First, whether an increase in average survival duration when force increases suffices to define an intrinsic bond strengthening is debatable. As the laminar flow chamber allows measurement of association kinetics, we were able to show that all the TCR-pMHC interactions studied show a very strong decrease in bond formation (2 to 3 log) when shear increases (Figure 3). This contrasts with L-selectin/ligand interactions, arguably the prototype of catch bonds, which show the opposite: an increase in observable bond formation when shear increases [CITATION Paschall2008a \l 1036], consistent with an increase in bond strength. In force-clamp experiments, a fraction of newly formed bonds may break before clamp force is reached. If this fraction changes when the chosen clamp force is modified, the statistics of bond lifetimes under clamp force will be measured on a different population of bonds. The observation of an increase of bond lifetime when clamp force increases might be possible for slip bonds if several bound states coexist in a population of bonds : increasing the

clamp force could select the stronger bound states as the sole able to be measured under clamp force, with longer observed lifetimes, while the number of bonds reaching clamp force would decrease. On opposite, if catch bonds are formed, when clamp force is increased the number of bonds reaching clamp force should increase as well as the bond lifetime under clamp force. Statistics of bonds observed under a given clamp force relatively to the number of adhesion assays would be needed to differentiate the two cases, as it is done with the laminar flow chamber. Second, to demonstrate single bond measurements, biomembrane force probe and AFM rely on ensuring that there is only a low proportion of binding events (classically less than 10%) relative to the total number of cell-surface or surface-surface contacts during the experiment : the proportion of double binding events is then the square of the proportion of single events, *i.e.* less than 1% [CITATION Johnson2018 \l 1036]. While this argument does indicate that the minimal observable binding event predominates under these conditions, it does not prove that this event corresponds to a single molecular bond: the minimal observable binding event could comprise multiple molecular interactions. The fact that a single TCR-pMHC interaction is measurable by the method is indeed an assumption in studies using low adhesion probabilities to demonstrate single molecular binding[CITATION Johnson2018 \l 1036]. Therefore, we believe that the laminar flow chamber uses currently the most stringent criteria to demonstrate measurement of single molecular bonds.

By contrast with cell-free experiments, the use of live T lymphocytes may complicate the interpretation of results in other ways. Biomembrane force probe or optical tweezers create cell contacts of micrometer scale lasting hundreds of milliseconds that may allow a cell reaction to modify the readout. Indeed, it has long been shown that the TCR is a mechanotransducer[CITATION Kim2009d \l 1036][CITATION Li2010 \l 1036] and that T cell can actively modulate the lifetime of TCR-pMHC association[CITATION Huppa2010 \l 1036]. A gathering of receptors due to a cellular response could for example quickly reinforce

the initial bond and make the critical force for cell sensibility appear as the peak lifetime force of a catch bond[CITATION Dushek2014 \l 1036]. Indeed, recent experiments show an increase in catch-bond forming TCR-pMHC survival time up to 15-fold for CD8-expressing T lymphocytes compared to non-CD8 expressing-T lymphocytes[CITATION Kolawole2018 \l 1036]. This increase could be a cellular response enhanced by CD8, as such a change seems unlikely to be caused mechanically by the very low affinity CD8-MHC interaction. Also, the change in the distribution of bond durations towards an increase in the proportion of catch bonds in[CITATION Liu2014 \l 1036] could be interpreted as a consequence of cell activation and not as its cause. A TCR-pMHC binding-triggered increase in the apparent affinity of other TCRs microns away has been recently interpreted as a facilitating cell reaction[CITATION Pielak2017 \l 1036]. Moreover, TCR binding to pMHC can exhibit slip or catch bonds depending on the molecular context of the interaction and active cellular processes[CITATION Hong2018 \l 1036], strongly suggesting that catch bond formation may not be an intrinsic feature of TCR-pMHC interactions.

10pN is a critical force for ligand discrimination by the T lymphocyte. We find a good correlation between TCR-pMHC bonds off-rate and their activation potency in the force range where previous studies found catch-slip transitions for activating peptides (*i.e.*, 10 to 15pN). Thus, bond lifetime around 10pN might be a critical parameter linked to T lymphocyte activation, irrespectively of the molecular mechanism. While it is difficult to determine the exact force exerted by the cell on individual TCR, these forces might be close to 10pN: experiments done at a scale smaller than the cell can show forces in the same order of magnitude (50pN)[CITATION Bashour2014 \l 1036], as were the initial pulling forces (25pN) of the T lymphocyte reported by Husson et all[CITATION Husson2011 \l 1036]. Most importantly, DNA sensors showed that a 12 to 18pN force is indeed applied on TCR by

the T lymphocyte seconds after binding and before calcium signalling, and seems to be necessary for ligand discrimination[CITATION Liu2016 \l 1036].

A minimal encounter duration might be an important prerequisite for lymphocyte activation. A form of control of the distribution of encounter duration is needed to test association kinetics models. The laminar flow chamber is uniquely well-suited to this, as the displacement of binding sites imposes a distribution of short encounters in the millisecond range, that may be estimated through relatively simple numerical simulations. The poor fit of our 2D TCR-pMHC association data by a k_{on} model is similar to our observations for several antigen-antibody bonds[CITATION Robert2009 \l 1036][CITATION Robert2011a \l 1036] [CITATION Limozin2016 \l 1036]. We propose the use of another association model in which binding occurs after a minimal encounter duration varying accordingly to a characteristic duration t_{on}. To compare models with an equal number of free parameters, we set a common value for $f_{E.}$, an assumption comforted by trials with two free parameters showing little change for f_E. The t_{on}association model appears to describe our results better than the k_{on}association model.

We interpret the rough part of the energy landscape, responsible for the minimal encounter duration, as the diffusive rearrangements necessary for peptidic chains to form the bond. Among membrane mechanics that may control the duration of molecular encounters between TCR and pMHC, localized fluctuations of microvilli tips, with a typical amplitude of several tens of nanometers[CITATION AYu1990 \l 1036][CITATION Brodovitch2015 \l 1036] and a frequency comprised between 0.2 and 30 Hz [CITATION AYu1990 \l 1036], could impose encounter durations in the order of magnitude to select bond formation by the fastest of the TCR-pMHC. A major question is whether this feature is relevant to TCR signaling. A striking point is that any encounter between TCR and pMHC of duration below the minimal duration

would fail to produce a force-resistant interaction and prevent signal transduction: the minimal encounter duration could act as a specificity threshold.

Overall, our data suggests that a complete description of the kinetics of the TCR-pMHC interaction must take into account the time of bond formation,and illustrates the importance of simultaneously measuring association and dissociation.

MATERIALS AND METHODS

Molecules- As described in [CITATION Aleksic2010 \l 1036], Human Leukocyte antigen (HLA) A2 molecules were expressed in *E.coli* as inclusion bodies from amino acid 1 to amino acid 278; a biotinylation sequence for BirA enzyme was added at the C-terminal end. Five different peptide and MHC molecules were used, differing by a single amino-acid in either the peptide (3A, 9V, 3Y, 9L) or the HLA A2 molecules (H74). The 1G4 TCR α and β subunits were expressed in *Escherichia coli* as inclusion bodies, refolded in vitro, and purified using size exclusion chromatography.

Microspheres- Dynabeads M450 Tosylactivated microspheres (diameter: 4.5µm, Invitrogen, France) were coated with a monoclonal mouse anti-His tag antibody (MCA485G, Serotec, France) according to the manufacturer's protocol[CITATION Limozin2016 \l 1036], then incubated with 1G4 TCR bearing a 6-histidine tag.

Surface preparation-The functionalized surfaces used in the flow chamber were prepared as described before[CITATION Limozin2016 \l 1036]. Briefly, 75×25 mm^2 glass slides (VWR, France) were cleaned in a "piranha" solution, a heated mix of 70% H_2SO_4 solution (95-98% in water, Fisher Bioblock, France) and 30% H_2O_2 solution (50% in water, Sigma-Aldrich, France), then coated with a poly-L-lysine solution (150000-300000Da, Sigma-Aldrich, France), rinsed then incubated with a glutaraldehyde solution (2.5% in 0.1M borate buffer, pH 9.5, Sigma-Aldrich, France) , rinsed then incubated with a solution of biotinylated bovine serum albumin (BSA) (100 µg/ml, Sigma-Aldrich, France) in PBS, rinsed then

incubated in a blocking solution of glycine (0.2M) and BSA (1mg/ml) in PBS, rinsed then incubated in a streptavidin solution (10 µg/ml in PBS, Sigma-Aldrich, France), then rinsed with PBS.

Flow chamber experiments-We used a unique automatized laminar flow chamber set-up [CITATION Limozin2016 \l 1036] on an inverted microscope (Diavert, Leica, Germany) with a CCD camera (IDS, Germany) and a ×10 lens. Movies were recorded at 50 images per second and compressed by the IDS U-Eye software using a M-JPEG codec. Experiments were performed at 37°C on substrates coated with various densities of pMHC on average 6 times per density under 5 shear rates each from 5 s^{-1} to 45 s^{-1}. Force on bond was calculated as $F=\sqrt{a/2R}\left(T+\Gamma/a\right)$ with $T=1.7005\times 6\pi\mu a^2 G$ and $\Gamma=0.9440\times 4\pi\mu a^3 G$ (with T the traction on the microsphere, Γ the torque on the microsphere, a the microsphere radius (2.25µm), R the total bond length (32nm), μ the medium viscosity (7x10^{-4} Pa.s at 37°C), and G the shear rate) [CITATION PierresBenolielZhuEtAl2001 \l 1036].

Trajectory analysis and arrest statistics- Statistics of bond formation were determined by counting the number of microspheres arrests and the total distance travelled by microspheres after sedimentation, as previously described [CITATION Robert2012 \l 1036][CITATION Limozin2016 \l 1036]. Statistics of bond rupture were determined by measuring the durations of microspheres arrests defined if their position did not change by more than $0.5\,\mu m$ during 0.2s, and if its velocity before the arrest was within the velocity range of moving sedimented microspheres. The binding linear density (BLD) under a given condition (i.e., a given shear rate and a given ligand surface density) was defined as the number of arrests divided by the total distance travelled by sedimented microspheres. The BLD of specific association was calculated by subtracting from the BLD measured with assay surface the BLD obtained with control surface. Bond rupture under a given condition was described by survival curves of the bonds, obtained by counting the fraction *s*of arrests exceeding the

duration t versus t, and corrected by subtracting estimated non-specific arrests[CITATION LoSchiavo2012 \l 1036].

Numerical simulations- The distribution of TCR and pMHC encounter durations as a function of shear rate was calculated by combining dynamics of a microsphere in laminar flow with an estimate of the diffusion volumes of TCR and pMHC reactive sites (SI Appendix, Erreur : source de la référence non trouvée). A molecular encounter was defined to begin and last while the diffusion volume of a TCR binding site intersects the diffusion volume of a pMHC, calculated as follows. We assume that in both molecules polypeptidic linkers outside of immunoglobulin domains give some length variability and degrees of rotational freedom. TCR binding site was at the extremity of the TCR (of length L3=8nm), itself bound at the extremity of the anti-His tag antibody Fab fragment (of length L2=8nm) that is hinged to the Fc fragment (of length L1=8nm); distance from its anchoring point is equal to L1+L2+L3+ΔR_{TCR}, where ΔR_{TCR} is the length variation with $0<\Delta R_{TCR}<\Delta R_{TCRmax}$ and solid angle φ_{TCR} its rotational freedom with $0< \varphi_{TCR}< \varphi_{TCRmax}$.. pMHC binding site is on the distal parts of domains α_1 and α_2 of the HLA A2 molecule (of length L4=8nm); the C-terminal end of the HLA α_3 domain is linked to the biotin; binding site is separated from its anchoring point by L4+ΔR_{pMHC}, where ΔR_{pMHC} is the length variation and solid angle φ_{pMHC} its rotational freedom with $0< \varphi_{pMHC} < \varphi_{pMHCmax}$. Both binding diffuse rapidly in shell-shaped volumes described by their thicknesses ΔR_{TCRmax} and $\Delta R_{pMHCmax}$ and by their solid angles φ_{TCRmax} and $\varphi_{pMHCmax}$ respectively (we define $\Delta R_{max}= \Delta R_{TCRmax} + \Delta R_{pMHCmax}$ and $\varphi_{max} = \varphi_{TCRmax} + \varphi_{pMHCmax}$.) Validity of this simulation was systematically tested in a previous work[CITATION Limozin2016 \l 1036].

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FIGURES LEGENDS

*Figure 1: **A-E**: Effect of varying forces (6 to 45pN) on half-life of agonist TCR-pMHC bonds. Half-lives of bonds calculated from off-rate measured between 0 to 5 s (left) are plotted versus time (bottom). Error bars are experimental SEM. **F**: schematics illustrating examples of typical slip bonds (green) and catch bonds (blue).*

*Figure 2: Off-rates of TCR-pMHC bonds between 0 and 5seconds for force increasing from 6 to 45 pN for each pMHC (**A-E**), plotted versus corresponding activation potencies measured as the EC_{50} for γ-interferon production. Errors bars are experimental SEM. In **F**, off-rates were measured in surface plasmon resonance and plotted versus corresponding activation potencies. $R^2$is the square Pearson correlation coefficient, p is the result of a Student test on Pearson coefficient.*

Figure 3: Binding linear density (BLD) of each experiment plotted versus most probable encounter duration (red dots). Most probable (MP) encounter duration D_e was calculated as $D_e=L/V_{avg}$ where L=35 nm is the molecular length of the bond, including intermediate antibodies, and V_{avg} is the peak of the microspheres velocity distribution. Data fits for two bond models are shown: dashed lines show the fit with the classical k_{on} model, while full lines show the fit with the minimal encounter duration (t_{on}) model.

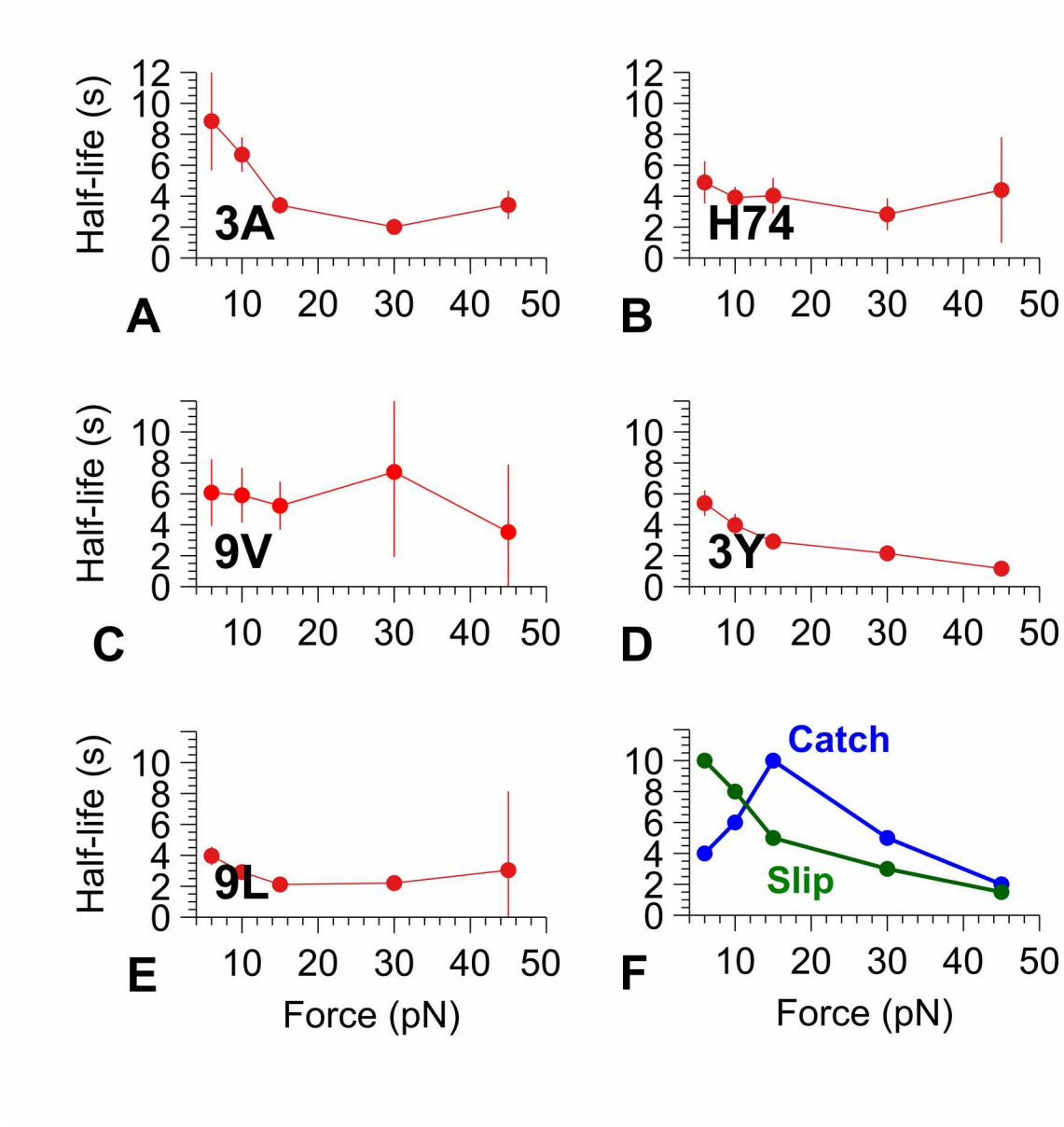

FIGURE 1

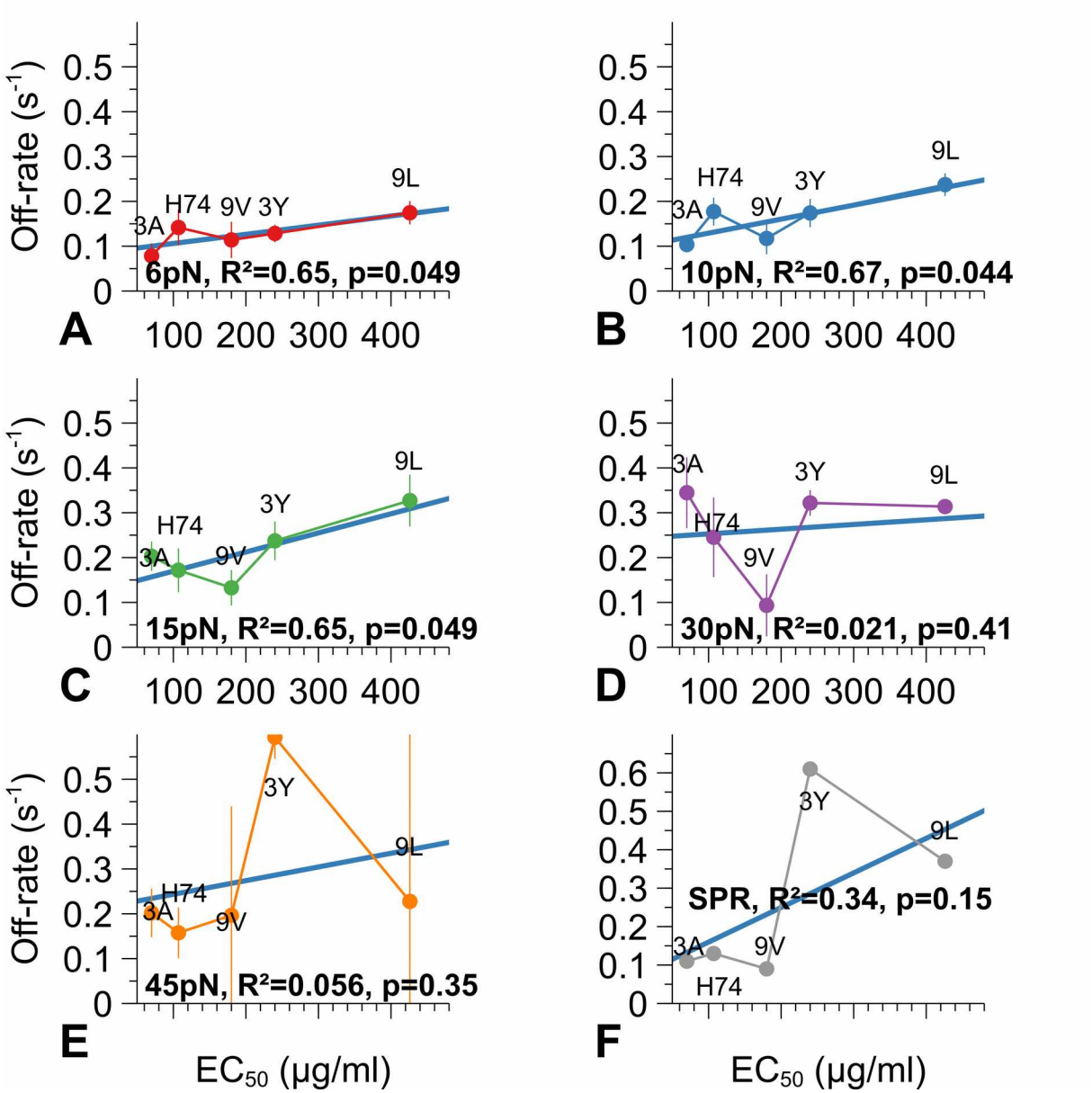
Off-rate (s-1)
0.5
0.4
0.3
0.2
0.1
0
3A
H74
9V
3Y
9L
6pN, R²=0.65, p=0.049
A
100 200 300 400
10pN, R²=0.67, p=0.044
B
15pN, R²=0.65, p=0.049
C
30pN, R²=0.021, p=0.41
D
45pN, R²=0.056, p=0.35
E
EC50 (µg/ml)
0.6
SPR, R²=0.34, p=0.15
F

FIGURE 2

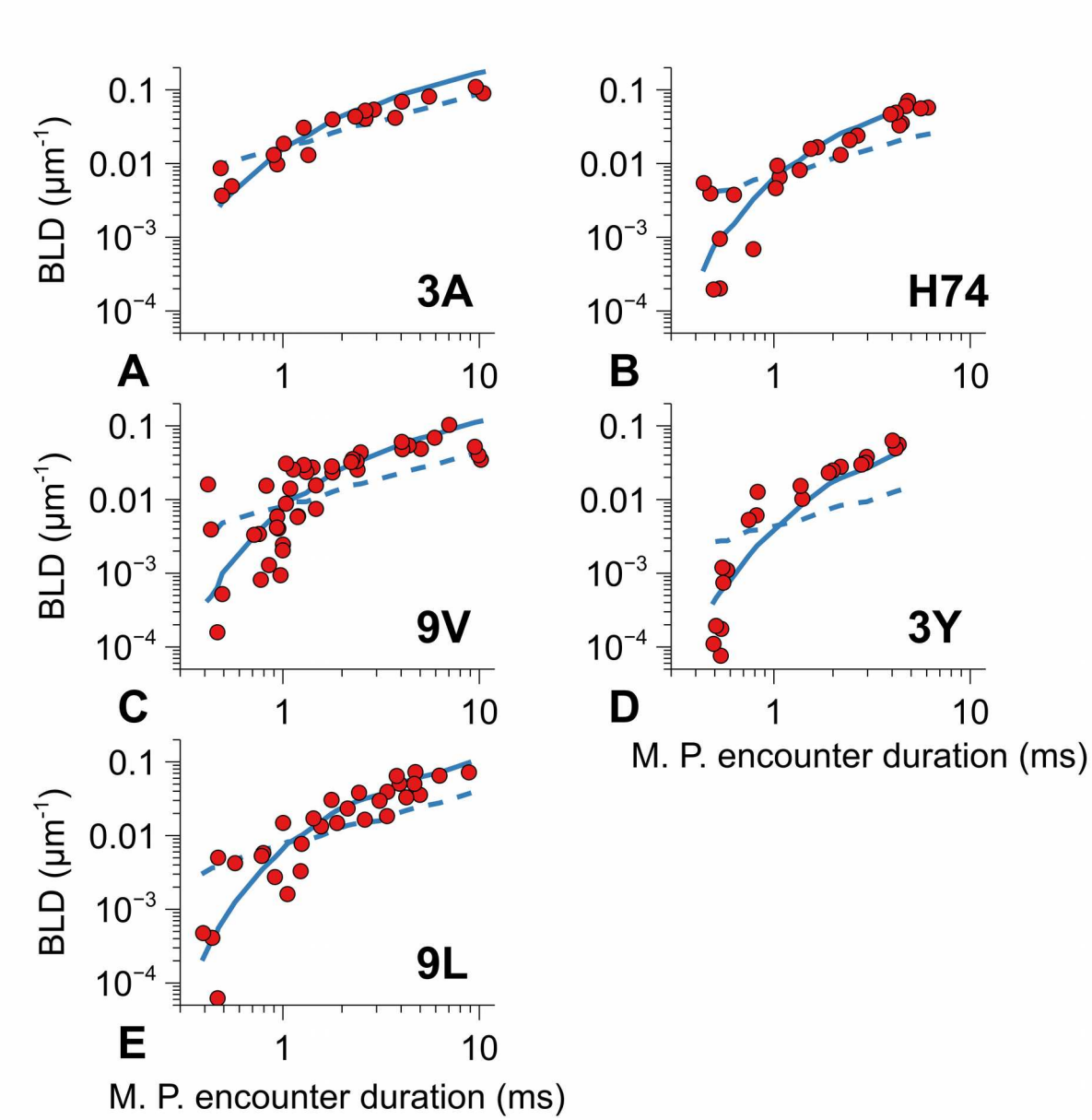
BLD (μm-1)
0.1
0.01
10−3
10−4
3A
A
1
10
H74
B
9V
C
3Y
D
9L
E
M. P. encounter duration (ms)
M. P. encounter duration (ms)

FIGURE 3