Synthesis of chemical tools to study the immune system

Graaff, M.J. van de

Chapter 1

General introduction[1]

Immune response during host infection and its orchestration

The defense of the host against infection is critically dependent on the *innate* immune system. Mucus, tears, the skin, endothelial cells, and the enzymes of the digestive tract are just a few of the mechanisms with which the host attempts to avoid infection and combat intruders.[2] This multi-layered system can be seen as the defense of a fortress; with moats, palisades, and wall all contributing to keeping out the invading hordes. Despite these multiple layers, breaches of this defense can still occur. This is where the *adaptive* immune system plays its vital role. The host constantly samples the interior with cells bearing Pattern Recognition Receptors (PRRs); best described as guards patrolling behind the stone buttresses of the fortress.

PRRs recognize Pathogen-Associated Molecular Patterns (PAMPs) that are evolutionary highly conserved.[3] They have evolved to recognize molecules that are critical for pathogen survival. A mutation in the PAMPs will almost always result in decreased pathogen viability, which prevents the pathogen to escape detection by mutagenesis. It has become apparent that a wide variety of PAMPs exist, enabling the host to detect a large group of pathogens quickly – without having encountered the pathogen before - via this innate immunity (Figure **1**).[4] PRRs can be divided into two groups: the cytoplasmic receptors and the membrane bound receptors.

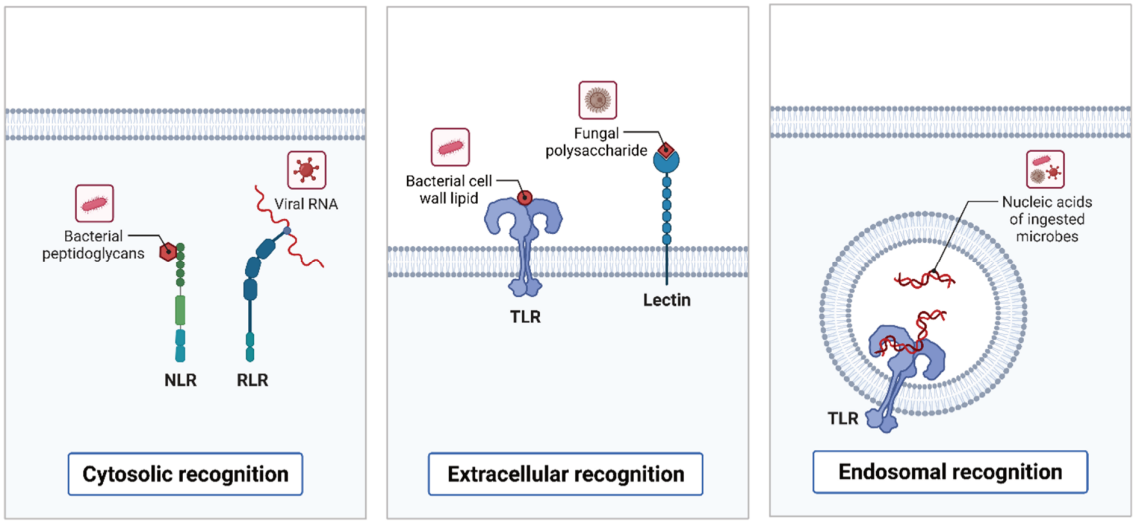


Figure 1. Overview of various PRRs and their respective ligands and cellular localization. Cytosolic recognition is initiated by NLRs and RLRs, whereas the membrane-bound receptors are responsible for either endosomal (TLRs) or extracellular (TLRs and C-type lectins) recognition.

The first group includes the nucleotide-binding oligomerization domain-like I (NLRs)[5] and retinoic acid-inducible gene-I-like receptors (RLRs)[6], and the second group includes receptors such as the C-type Lectin Receptors (CLRs)[7] and the Toll-Like Receptors (TLRs).[8]

Activation of these receptors lead to the start of the immune response, which includes the innate response (e.g., neutrophil recruitment), but also the adaptive immune response, in which a more tailored response to the pathogen is mounted.

For this latter response, the pathogen is proteolytically processed and one or more epitopes are generated from this process and presented on the surface of an antigen presenting cell, which can be sampled by T-cells. The activation of the PRR on the APC then translates to two additional signals: the co-stimulatory signal (upregulation of CD80 and CD86)[9], and the secretion of cytokines that induce responses appropriate for the pathogen family that induced the initial PRR trigger. Once activated by these three signals, the T-cells can orchestrate the anti-pathogen response, through direct killing of infected cells or the activation of the appropriate other families of immune cells through cytokine signaling. They can also form long-lived memory cells, which allow a faster recognition and immune response during a second infection with the same pathogen.[10] This combination of PRR ligands and a source of epitopes provides the framework within which some modern synthetic vaccines are conceptualized[11]: one or more ligands for these PRRs are co-delivered with a source of pathogen polypeptide (from whole cell to minimal peptides) that allows the activation of T-cells in a manner appropriate for the pathogen against which the vaccine is aimed; albeit bacterial, viral or tumor.[12] The proper combination of PRR-ligands remains a hurdle and often lies at heart of vaccine failure.[13]

One of the reasons for this is that certain aspects of PRR-biology remain poorly understood. The effect of the location of binding, kinetics, and multivalency of the ligation (when multiple receptors are activated at the same time) on signaling outcomes are not yet known. One PRR can bind multiple ligands at different sites within the receptor leading to different responses, whereas one ligand is able to bind to multiple different PRRs[14] thereby modulating the overall response.[15] PRRs engage in crosstalk and cellular localization of the PRR at the moment of activation can lead to vastly different signaling outputs.[16] Taken together, intracellular – but also intercellular – signaling events within the context of the immune system is quite complex. The toolbox from which the experimental biologist has to pick from to investigate these signaling events is not fitted to deal with such complexity without having to compromise conclusions with significant assumptions. The use of fusion proteins renders many PRRs inactive, and the modification of fluorophores to the ligands – in many cases chemically non-trivial – may also significantly alter the properties of the ligands. The use of inhibitors of specific pathways is also fraught with dangers relating to unexpected side-effects and/or off-targets.[17]

The recent emergence of a multidisciplinary approach to answering biological questions aided by design and synthesis of molecules can procure tools that are neatly fitted to the experiment. This thesis describes the process of designing and synthesizing chemical tools that adhere to specific experimental conditions, culminated through continuous feedback from close-collaborating immunobiological experimentalists. This process is contextualized within Toll-Like Receptors, combined with their respective ligands using photo- and/or chemo-protecting groups. The following paragraphs will explore these topics, with a focus on the Toll-Like Receptors, more deeply to aid in appreciating the experiments performed and described throughout this thesis.

Toll-Like Receptors

There are 13 TLR subsets within the mammalian family, of which TLR1 to TLR9 are conserved between humans and mice.[18] Humans have lost the ability to produce TLR11 to TLR13 whereas mice have lost functional TLR10.[19] The structural feats of the TLRs are similar, consisting of an N-terminal ectodomain, one transmembrane domain and a C-terminal cytosolic Toll-interleukin-1 receptor (TIR) domain.[20] The ectodomain is characterized by leucine-rich repeats which adopt a structural motif resemblant of horseshoe, and are involved with ligand recognition. The TIR domain is responsible for the recruitment of signaling proteins in the cytosol upon TLR activation.[21] Based on the predominant cellular localization of the TLRs prior to activation, they can be grouped into two classes: TLR1, TLR2 and TLR4 to TLR6 mainly reside on the plasma membrane, whereas TLR3 and TLR7 to TLR9 are localized intracellularly (Figure 2).[16]

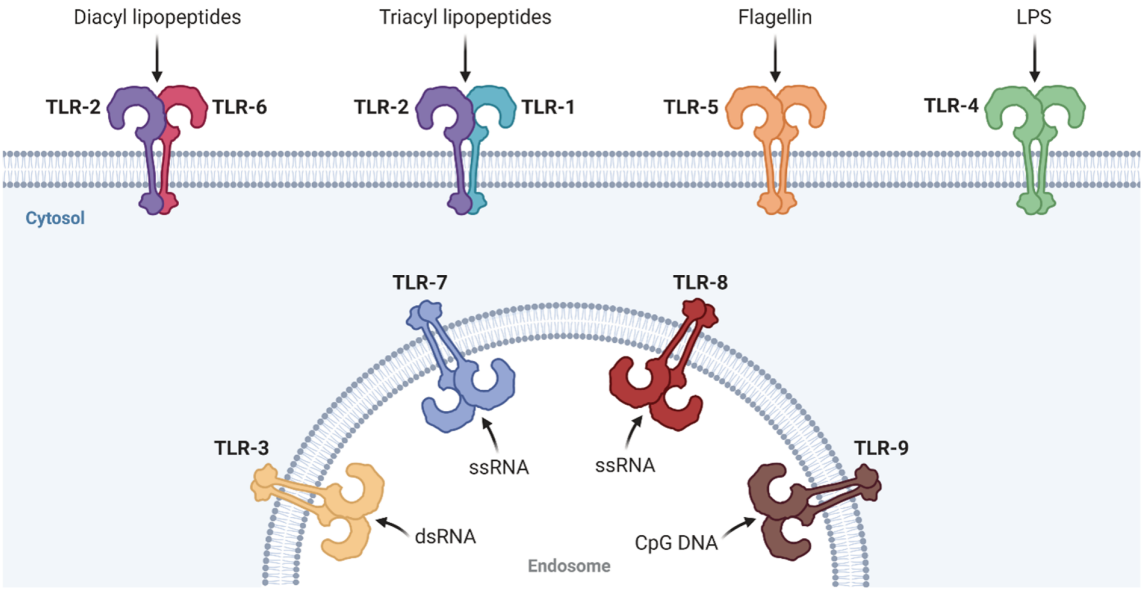


Figure 2. Localizations of TLRs when activated by their respective canonical ligands.

TLRs dimerize upon ligand binding.[20] Most TLRs homodimerize, one exception being TLR2 and its interaction partners: it can heterodimerize either with TLR1 or TLR6.[22,23] PAMP-binding-induced dimerization initiates signal transduction pathways leading to the transcription of genes crucial to host defense. These genes code for pro-inflammatory cytokines and type I interferons (IFN-I).[24] Pro-inflammatory cytokine production is induced by the transcription factor nuclear factor κB (NF-κB) which translocates to the cell nucleus upon TLR activation.[25] Pro-inflammatory cytokines mature cells of the innate immune system and appropriately initiate and organize the adaptive immune response. Production of IFN-I is in turn induced by activation of the members of the interferon regulatory transcription factor (IRF) family.[26] IFN-I is involved in a more direct way of inhibiting microbial cell growth, through the transcription of genes with direct antiviral activities.

Plasma membrane-residing TLRs are perpetually exposed to the extracellular environment in which encountering certain PAMPs is more likely. TLR2/1 and TLR2/6 are able to detect the presence of Gram-positive cell wall components such as peptidoglycans and glycolipids as well as viral structural proteins, whereas TLR4/TLR4 can detect Gram-negative cell wall components such as lipopolysaccharide (LPS).[27–29] TLR5/TLR5 senses the presence of flagellin, another highly evolutionary conserved molecular motif critical for microbial locomotion.[30]

The TLRs that are exposed to the intracellular environment are sensitive towards nucleic acids derived from microbial origin. The main entry for pathogens is through endocytosis or phagocytosis, and thus these TLRs are found predominantly along these pathways. Presence of these receptors in other subcellular sites may lead to self-sensing of host-derived nucleic acids and may explain why these receptors are restricted to endosomes and phagosomes.[31] TLR3 senses viral double-stranded RNA[32], TLR7 and TLR8 recognize viral single-stranded RNA[33,34] and TLR9 senses DNA with a high recurrence of unmethylated 5'-Cytosine-Guanine-3' (CpG) motifs.[35]

Although TLRs are still grouped today as residing in either the plasma membrane or endosomes, it recently became evident that some TLRs have the ability to signal both from the plasma membrane and intracellular vesicles.[16] More interestingly, the cytokine production profile is different when the TLR is activated from these distinct localizations.[36] This effect was first discovered for TLR4, which was shown to be able to induce both NF-kB and IRF activation depending on its localization upon ligand engagement (Figure 3).

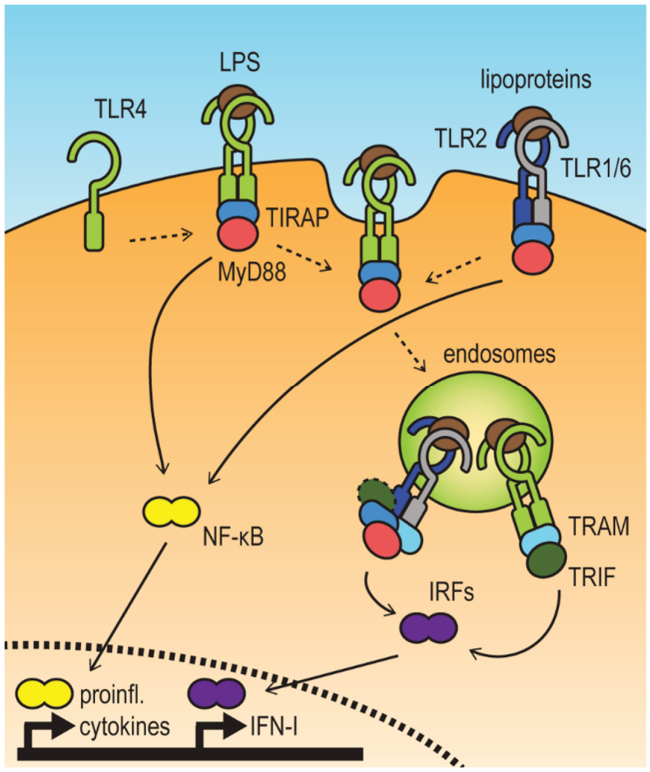


Figure 3. Proposed mechanism for distinct signal transduction pathways for TLR4. Dashed lines represent trafficking pathways; solid lines represent signaling pathways.

Several strategies have since emerged to explore this spatial-dependent activation more deeply. Endocytosis inhibitors restrict trafficking of the TLRs and thus offer insight in sequestered populations of TLRs.[37,38] Anti-TLR antibodies not only enable the visualization of TLRs during activation-induced translocation of the receptors, but can also prevent ligands to interact with certain receptor populations. Although valuable, these tools come with their disadvantages: specific antibodies against TLRs are difficult to obtain and endocytosis inhibitors do not only affect TLR trafficking events. TLRs have been recombinantly modified with tags against which an antibody *is* available, or fused to fluorescent proteins to address the issue of obtaining anti-TLR antibodies. However, fusion of TLRs with a tag or fluorescent moiety may interfere with receptor signaling and/or ligand binding. Thus, these techniques are not able to observe TLR signaling processes without substantial perturbation of the natural signaling mode-of-action. To study isolated TLR signaling events with minimal alteration to the TLR-signaling platform an alternative strategy is explored in this thesis, namely the use of conditionally controlled ligands: molecules that are only able to activate TLRs when exposed to a non-natural, spatial restricted trigger.

Conditionally controlled ligands

Conditionally controlled TLR ligands are a class of reagents new to field of TLR that show promise of unraveling the above-mentioned spatial – but also temporal – complexity exhibited by the TLR signaling processes. Spatial context can never be imposed when using canonical or synthetic TLR ligands, as they can only activate the TLR-expressing cells in bulk and thus, spatial restrictions have to be implemented in ways that can perturb the system such as co-administering endocytosis inhibitors as described above. A more elegant approach has recently surfaced, in which spatial restrictions have been incorporated as an inherent property of the TLR ligands. This is done by shielding a position within the ligand that is imperative for activation to occur, effectively inactivating the ligand. This protecting group can be conditionally removed; for example, by irradiating a specific volume of the sample with light when using a photolabile protecting group ('photocage') or, as proposed in this thesis, by treating the sample with uncaging reagents that are directed to endosomes or phagosomes when using a chemically labile protecting group ('chemocage').

Photocages

Photocages have been applied (Figure 4) in the design of caged Resiquimod (TLR7/8)[39] **1**, Pam_2CSK_4 (TLR2/6)[40] **2**, pyrimido[5,4-b]-indole **3** (TLR4)[41] and CpG-containing oligodeoxynucleotides (TLR9)[42] **4**. In the case of compound **2** it was found that the ligand was still able to bind TLR2 but inhibited dimerization with TLR6. This property allows for an even distribution of the ligand prior to activation and enables TLR dimerization and cytokine monitoring within minutes after 'pulling the trigger' (Chapter 2). These chemical tools enabled constraining a volume within the sample to be activated, usually by virtue of a strategically placed mask over the sample. However, the experiment is limited by the scattering of UV photons, as BMDCs rapidly move outside of the irradiation area, and phototoxicity induced by the high-energy UV-irradiation[39] necessary for these specific photocages.

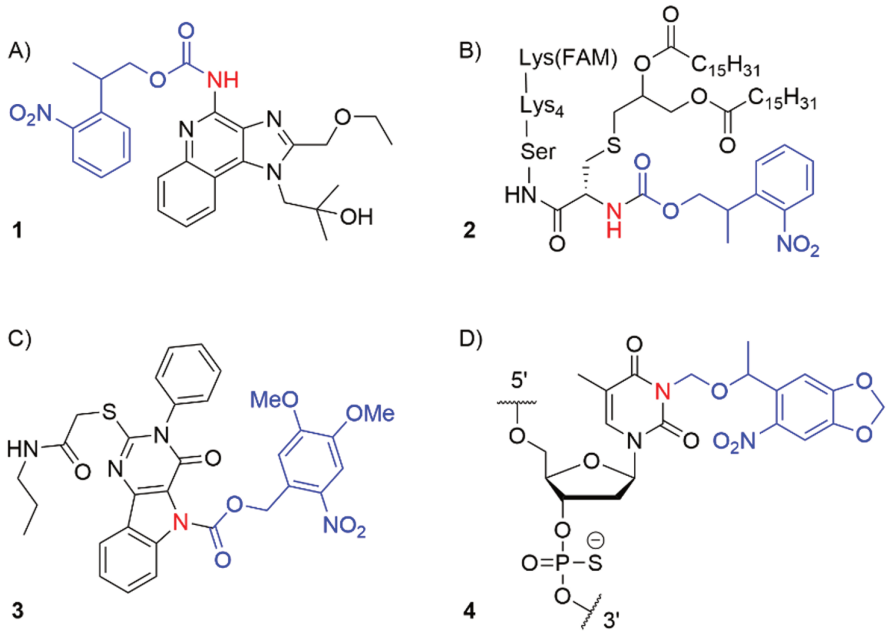


Figure 4. Photocaged TLR ligands. Photocages are indicated in blue whereas the amine critical for activity is indicated in red. A) Resiquimod protected with 2-(2-nitrophenyl)propoxycarbonyl (NPPOC). B) Fluorescein (FAM)-conjugated Pam_2CSK_4K(FAM) protected with NPPOC. C) A pyrimido[5,4-b]-indole protected with nitroveratryloxycarbonyl (NVOC). D) A phosphorothioate-linked thymidine residue as located in a CpG-bearing oligodeoxynucleotide protected with 6-nitropiperonyloxymethyl (NPOM).

Two-photon labile cages pose an obvious answer to some of these limitations, although they would have to be compatible with the synthetic route as the photoprotection step is not usually the final reaction in the synthetic plan. These cages contain highly conjugated systems often with strong electron donor/acceptor couples.[43] These properties increase both the number of photons that can be absorbed within a timeframe, and the time within which two photons can arrive in the system to allow electron transition. In such a system, two photons of 700 nm can be absorbed to allow an energetic transition of 350 nm, thus limiting UV-induced phototoxicity. Two photon absorption is quadratically dependent on the light intensity, whereas single photon absorption is linearly dependent on light intensity. This nonlinear scaling means that it will dominate over one photon absorption only at higher intensities. This necessitates the use of ultrashort pulse lasers, as normal LEDs or lasers are unable to reach the photon intensities required to observe significant two-photon absorption. Moreover, the light emitted has to be coherent for 2-photon absorption to occur, a property expressed by lasers but not LEDs. Pulse lasers build up energy over a microsecond timescale and release a pulse of coherent light over an interval in the femtosecond timescale, having a repetition rate of 50-500 MHz. Although these setups are costly, they are capable of irradiating volumes in the femtoliter range.[44]

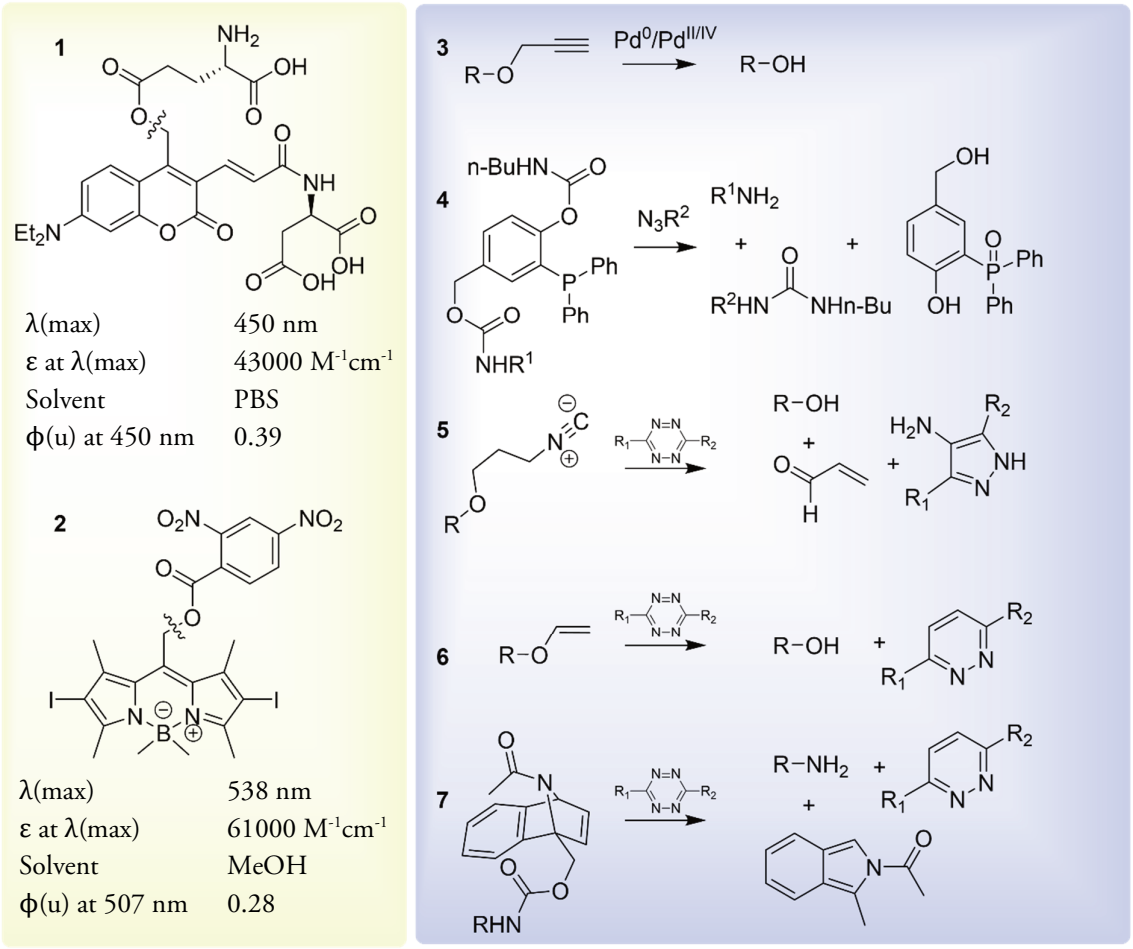
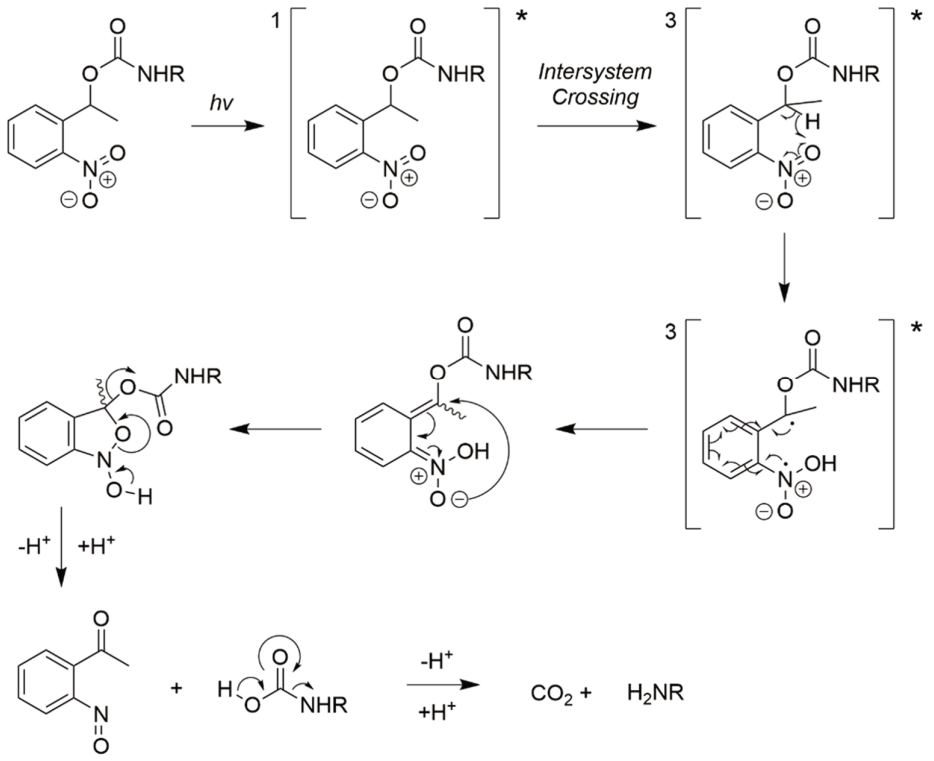


Figure 5. Examples of photo- and chemocages. Yellow box: compound **1** is a coumarin-4-ylmethyl based photocage[45] and compound **2** is a halogenated BODIPY-derived photocage[46]. Blue box: Several examples of bio-orthogonal chemo-uncaging, including removal of propargyl[47] by transition metals (**3**), modified Staudinger reactions(**4**)[48,49] or inverse electron-demand Diels-Alder (IEDDA) reactions employing isocyanides(**5**)[50], vinyl ethers(**6**)[51,52], benzonorbornadienes(**7**)[53] with tetrazine derivatives.

Nitrobenzyl cages

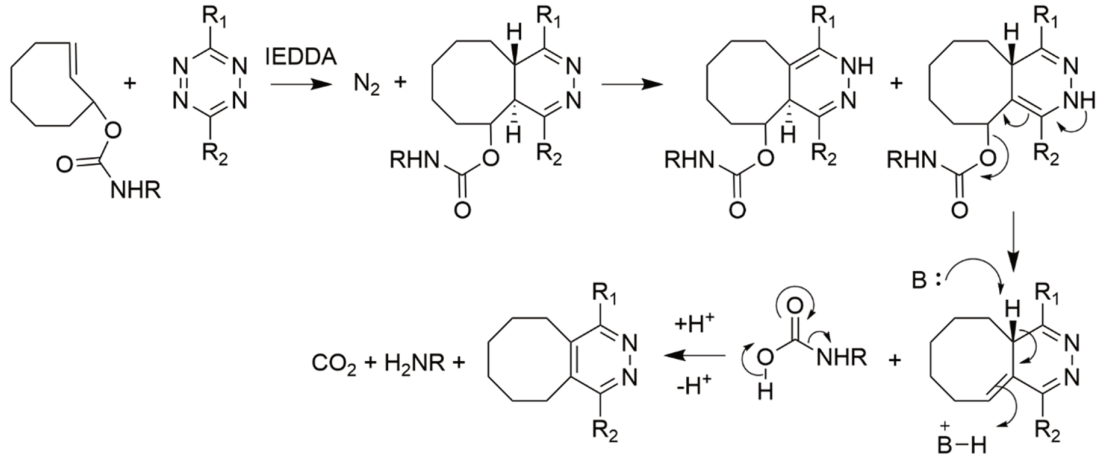
Many conditionally controlled ligands used in biological experiments still make use of 2-nitrobenzyl derived photocages. In the past decade a lot of attention has been focused on photocages with improved photochemical properties over the 2-nitrobenzyl derivatives. These include coumarin-4-ylmethyl[45]-, BODIPY[46]-, arylcarbonylmethyl[54]- and metal[55]-based photoremovable protecting groups (Figure **5**). Alternatively, the 2-nitrobenzyl core can be extended with a conjugated ring system to enhance its molar attenuation coefficient ε (its efficiency in absorbing photons). This can enable two-photon absorption as it dramatically reduces the light intensities required before significant uncaging occurs.[56,57] The chemical robustness of these next-generation 2-nitrobenzyl cages make them appealing to use in synthetic routes in which the product has yet to be exposed to multiple reagents. The simplified mechanism with which the 2-nitrobenzyl cages are cleaved can be seen below (Scheme **1**) and has been reviewed extensively.[58–60]



Scheme 1. Deprotection mechanism of benzylic-substituted 2-nitrobenzyls.[58] Irradiation leads to an excited singlet state, which quickly crosses to an excited triplet state. From here, a hydrogen atom is abstracted from the benzylic carbon to the nitro-group. The radicals rearrange to form the aci-nitro intermediate. This intermediate forms a 5-membered ketal ring which collapses to form the nitroso byproduct and liberates the carbamic acid.

Chemocages

An alternative to photocaging is the use of chemocages. They can be viewed similarly as protecting groups used in organic synthesis, with the important criterion that the uncaging conditions have to be bio-orthogonal. Early examples include removal of alloc[61] or propargyl[47] by transition metals, modified Staudinger reactions[48,49] or inverse electron-demand Diels-Alder (IEDDA) reactions employing isocyanides[50], benzonorbornadienes[53], vinyl ethers[51,52] or cyclooctynes[62] with tetrazine derivatives. Extra criteria must be added to warrant the use of chemical protecting groups in the context of conditionally controlled TLR ligands. A suitable cage would have to 1) be stable, as TLRs are highly sensitive for small amounts of free, uncaged ligand due to a positive feedback loop exerted by the signaling cascade, 2) uncage quickly, as transcription of the first cytokines start within 15 minutes of activation[63,64], and 3) be spatial controllable in its uncaging, in other words its uncaging reagents have to be directable specifically to endosomes. One such reaction was discovered in 2008 by Fox and co-workers: *trans*-cyclooctenes (TCOs) ligate with tetrazines with a rate of 2000 $M^{-1}s^{-1}$, enabling fast and quantitative reactions at low concentrations.[65] In 2013, Robillard and co-workers modified the TCO to include a hydroxyl functionality at the 2-position[66], onto which a payload can be loaded that is released upon tetrazine ligation. The mechanism with which a TCO releases its payload is depicted in Scheme **2**.[67–70]



Scheme 2. The ligation and consequent elimination of 2-O-substituted trans-cyclooctenes with tetrazines.

Outline of this thesis

The lack of chemical tools to study spatiotemporal effects in TLR signaling is addressed in Chapter 2. The TLR2/6 ligand Pam_2CSK_4 has been caged with a *trans*-cyclooctene on its N-terminal amine. This resulted in a ligand that binds to TLR2, but only induces activation through heterodimerization with TLR6 when tetrazine is present. This is verified through assessment of functional outcome (cytokine production) as well as visualization of nuclear translocation of fluorescently labeled NF-κB in mouse cells. Interestingly, when the experiment was repeated in human cells, significant TLR2 activity was displayed also in the absence of tetrazine. A variety of modifications on the ligand has been tested and resulted in a ligand that is desensitized to activate TLR2/6 in the absence of tetrazine, even in primary human cells.

Chapter 3 describes the synthesis of several TLR ligands that have a TCO on a position that is critical for TLR activation. The strategy described in Chapter 2 is extended and applied to ligands for TLR7, TLR8 and TLR9. A TCO-caged deoxycytidine has been made amenable for solid-phase oligonucleotide synthesis to produce caged CpG-containing DNA fragments. Lastly, the concept of caged TLR ligands is extended towards an *in vivo* application in which an anti-tumor antibody has been conjugated to a TLR7/8 ligand, Resiquimod, through a bifunctional TCO. Some tetrazine derivatives have been shown to be non-toxic in concentrations necessary to effect dissociation of TCO and its payload[71] and thus potentially can be exploited to initiate TLR downstream signaling within the tumor environment.

In Chapter 4, various known photocages are modified to include a second functionality that enables conjugation to a nucleophile. This effectively led to a light-labile linker, or 'photo-linker'. The second functionality broadens the scope of its utility to be included in-line as a building block in solid-phase peptide and DNA/RNA synthesis to create photolabile peptide or DNA/RNA sequences. Moreover, it also offers a platform to alter some of the photocage's properties such as its aqueous solubility by introducing hydrophilic moieties. One of the next-generation 2-nitrobenzyl

photocages, NPBF[56], has also been modified such that conjugation to two molecules of interest is now possible.

Chapter 5 offers a summary of the above research and some suggestions for future applications. For example, the above-mentioned modified NPBF linker has been applied in the synthesis of a conjugate able to eradicate chemotherapeutic-resistant multiple-myeloma cells *in vitro*. The linker has also been used as a probe that can tag CD8+ T cells in human primary tumor tissue and enables isolation of cells of interest on a single cell resolution, both upon low-dose photo-irradiation.

References

(1) Oosenbrug, T.; van de Graaff, M. J.; Ressing, M. E.; van Kasteren, S. I. Chemical Tools for Studying TLR Signaling Dynamics. *Cell Chem. Biol.* **2017**, *24* (7), 801–812.

(2) Medzhitov, R.; Janeway, CharleS. J. Innate Immune Recognition: Mechanisms and Pathways: Innate Immune Recognition. *Immunol. Rev.* **2000**, *173* (1), 89–97.

(3) Akira, S.; Takeda, K.; Kaisho, T. Toll-like Receptors: Critical Proteins Linking Innate and Acquired Immunity. *Nat. Immunol.* **2001**, *2* (8), 675–680.

(4) Kumagai, Y.; Akira, S. Identification and Functions of Pattern-Recognition Receptors. *J. Allergy Clin. Immunol.* **2010**, *125* (5), 985–992.

(5) Kanneganti, T.-D.; Lamkanfi, M. Intracellular NOD-like Receptors in Host Defense and Disease. *Immunity*, **2007**, *27* (4), 549-559.

(6) Loo, Y.-M.; Gale, M. Immune Signaling by RIG-I-like Receptors. *Immunity*, **2011**, *34* (5), 680–692.

(7) Kingeter, L. M.; Lin, X. C-Type Lectin Receptor-Induced NF-KB Activation in Innate Immune and Inflammatory Responses. *Cell. Mol. Immunol.* **2012**, *9* (2), 105–112.

(8) Fitzgerald, K. A. Toll-like Receptors and the Control of Immunity. *Cell*, **2020**, *180* (6), 1044-1066.

(9) Rahman, A. H.; Taylor, D. K.; Turka, L. A. The Contribution of Direct TLR Signaling to T Cell Responses. *Immunol. Res.* **2009**, *45* (1), 25–36.

(10) Mercier, B. C.; Cottalorda, A.; Coupet, C.-A.; Marvel, J.; Bonnefoy-Bérard, N. TLR2 Engagement on CD8 T Cells Enables Generation of Functional Memory Cells in Response to a Suboptimal TCR Signal. *J. Immunol.* **2009**, *182* (4), 1860–1867.

(11) Ingale, S.; Wolfert, M. A.; Gaekwad, J.; Buskas, T.; Boons, G.-J. Robust Immune Responses Elicited by a Fully Synthetic Three-Component Vaccine. *Nat. Chem. Biol.* **2007**, *3* (10), 663–667.

(12) Moyle, P. M.; Dai, W.; Zhang, Y.; Batzloff, M. R.; Good, M. F.; Toth, I. Site-Specific Incorporation of Three Toll-Like Receptor 2 Targeting Adjuvants into Semisynthetic, Molecularly Defined Nanoparticles: Application to Group A Streptococcal Vaccines. *Bioconjug. Chem.* **2014**, *25* (5), 965–978.

(13) Jones, L. H. Recent Advances in the Molecular Design of Synthetic Vaccines. *Nat. Chem.* **2015**, *7* (12), 952–960.

(14) Zhang, Z.; Ohto, U.; Shibata, T.; Krayukhina, E.; Taoka, M.; Yamauchi, Y.; Tanji, H.; Isobe, T.; Uchiyama, S.; Miyake, K.; Shimizu, T. Structural Analysis Reveals That Toll-

like Receptor 7 Is a Dual Receptor for Guanosine and Single-Stranded RNA. *Immunity* **2016**, *45* (4), 737–748.

(15) Wittmann, A.; Lamprinaki, D.; Bowles, K. M.; Katzenellenbogen, E.; Knirel, Y. A.; Whitfield, C.; Nishimura, T.; Matsumoto, N.; Yamamoto, K.; Iwakura, Y.; Saijo, S.; Kawasaki, N. Dectin-2 Recognizes Mannosylated O-Antigens of Human Opportunistic Pathogens and Augments Lipopolysaccharide Activation of Myeloid Cells. *J. Biol. Chem.* **2016**, *291* (34), 17629–17638.

(16) McGettrick, A. F.; O'Neill, L. A. Localisation and Trafficking of Toll-like Receptors: An Important Mode of Regulation. *Curr. Opin. Immunol.* **2010**, *22* (1), 20–27.

(17) *Exocytosis and Endocytosis*; Ivanov, A. I., Ed.; Methods in molecular biology; Humana Press: Totowa, N.J, **2008**.

(18) Rehli, M. Of Mice and Men: Species Variations of Toll-like Receptor Expression. *Trends Immunol.* **2002**, *23* (8), 375–378.

(19) Hasan, U.; Chaffois, C.; Gaillard, C.; Saulnier, V.; Merck, E.; Tancredi, S.; Guiet, C.; Brière, F.; Vlach, J.; Lebecque, S.; Trinchieri, G.; Bates, E. E. M. Human TLR10 Is a Functional Receptor, Expressed by B Cells and Plasmacytoid Dendritic Cells, Which Activates Gene Transcription through MyD88. *J. Immunol.* **2005**, *174* (5), 2942–2950.

(20) Botos, I.; Segal, D. M.; Davies, D. R. The Structural Biology of Toll-like Receptors. *Structure* **2011**, *19* (4), 447–459.

(21) Jenkins, K. A.; Mansell, A. TIR-Containing Adaptors in Toll-like Receptor Signalling. *Cytokine* **2010**, *49* (3), 237–244.

(22) Kang, J. Y.; Nan, X.; Jin, M. S.; Youn, S.-J.; Ryu, Y. H.; Mah, S.; Han, S. H.; Lee, H.; Paik, S.-G.; Lee, J.-O. Recognition of Lipopeptide Patterns by Toll-like Receptor 2-Toll-like Receptor 6 Heterodimer. *Immunity* **2009**, *31* (6), 873–884.

(23) Jin, M. S.; Kim, S. E.; Heo, J. Y.; Lee, M. E.; Kim, H. M.; Paik, S.-G.; Lee, H.; Lee, J.-O. Crystal Structure of the TLR1-TLR2 Heterodimer Induced by Binding of a Tri-Acylated Lipopeptide. *Cell* **2007**, *130* (6), 1071–1082.

(24) Tseng, P.-H.; Matsuzawa, A.; Zhang, W.; Mino, T.; Vignali, D. A. A.; Karin, M. Different Modes of Ubiquitination of the Adaptor TRAF3 Selectively Activate the Expression of Type I Interferons and Proinflammatory Cytokines. *Nat. Immunol.* **2010**, *11* (1), 70–75.

(25) Li, Q.; Verma, I. M. NF-KB Regulation in the Immune System. *Nat. Rev. Immunol.* **2002**, *2* (10), 725–734.

(26) Honda, K.; Taniguchi, T. IRFs: Master Regulators of Signalling by Toll-like Receptors and Cytosolic Pattern-Recognition Receptors. *Nat. Rev. Immunol.* **2006**, *6* (9), 644–658.

(27) Mancini, R. J.; Stutts, L.; Ryu, K. A.; Tom, J. K.; Esser-Kahn, A. P. Directing the Immune System with Chemical Compounds. *ACS Chem. Biol.* **2014**, *9* (5), 1075–1085.

(28) Barton, G. M. Viral Recognition by Toll-like Receptors. *Semin. Immunol.* **2007**, *19* (1), 33–40.

(29) Thompson, J.; Iwasaki, A. Toll-like Receptors Regulation of Viral Infection and Disease☆. *Adv. Drug Deliv. Rev.* **2008**, *60* (7), 786–794.

(30) Hayashi, F.; Smith, K. D.; Ozinsky, A.; Hawn, T. R.; Yi, E. C.; Goodlett, D. R.; Eng, J. K.; Akira, S. The Innate Immune Response to Bacterial ¯agellin Is Mediated by Toll-like Receptor 5. **2001**, *410*, 5.

(31) Lee, B. L.; Barton, G. M. Trafficking of Endosomal Toll-like Receptors. *Trends Cell Biol.* **2014**, *24* (6), 360–369.
(32) Alexopoulou, L.; Holt, A. C.; Medzhitov, R.; Flavell, R. A. Recognition of Double-Stranded RNA and Activation of NF-KB by Toll-like Receptor 3. *Nature* **2001**, *413* (6857), 732–738.
(33) Heil, F. Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8. *Science* **2004**, *303* (5663), 1526–1529.
(34) Lund, J. M.; Alexopoulou, L.; Sato, A.; Karow, M.; Adams, N. C.; Gale, N. W.; Iwasaki, A.; Flavell, R. A. Recognition of Single-Stranded RNA Viruses by Toll-like Receptor 7. *Proc. Natl. Acad. Sci.* **2004**, *101* (15), 5598–5603.
(35) Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K.; Akira, S. A Toll-like Receptor Recognizes Bacterial DNA. *Nature* **2000**, *408* (6813), 740–745.
(36) Tan, Y.; Kagan, J. C. Microbe-Inducible Trafficking Pathways That Control Toll-like Receptor Signaling: TAN ET AL . *Traffic* **2017**, *18* (1), 6–17.
(37) Kagan, J. C.; Su, T.; Horng, T.; Chow, A.; Akira, S.; Medzhitov, R. TRAM Couples Endocytosis of Toll-like Receptor 4 to the Induction of Interferon-β. *Nat. Immunol.* **2008**, *9* (4), 361–368.
(38) Tatematsu, M.; Yoshida, R.; Morioka, Y.; Ishii, N.; Funami, K.; Watanabe, A.; Saeki, K.; Seya, T.; Matsumoto, M. Raftlin Controls Lipopolysaccharide-Induced TLR4 Internalization and TICAM-1 Signaling in a Cell Type–Specific Manner. *J. Immunol.* **2016**, *196* (9), 3865–3876.
(39) Ryu, K. A.; Stutts, L.; Tom, J. K.; Mancini, R. J.; Esser-Kahn, A. P. Stimulation of Innate Immune Cells by Light-Activated TLR7/8 Agonists. *J. Am. Chem. Soc.* **2014**, *136* (31), 10823–10825.
(40) Mancini, R. J.; Stutts, L.; Moore, T.; Esser-Kahn, A. P. Controlling the Origins of Inflammation with a Photoactive Lipopeptide Immunopotentiator. *Angew. Chem. Int. Ed.* **2015**, *54* (20), 5962–5965.
(41) Stutts, L.; Esser-Kahn, A. P. A Light-Controlled TLR4 Agonist and Selectable Activation of Cell Subpopulations. *ChemBioChem* **2015**, *16* (12), 1744–1748.
(42) Govan, J. M.; Young, D. D.; Lively, M. O.; Deiters, A. Optically Triggered Immune Response through Photocaged Oligonucleotides. *Tetrahedron Lett.* **2015**, *56* (23), 3639–3642.
(43) Chung, S.-J.; Kim, K.-S.; Lin, T.-C.; He, G. S.; Swiatkiewicz, J.; Prasad, P. N. Cooperative Enhancement of Two-Photon Absorption in Multi-Branched Structures. *J. Phys. Chem. B* **1999**, *103* (49), 10741–10745.
(44) Brown, E. B.; Shear, J. B.; Adams, S. R.; Tsien, R. Y.; Webb, W. W. Photolysis of Caged Calcium in Femtoliter Volumes Using Two-Photon Excitation. *Biophys. J.* **1999**, *76* (1), 489–499.
(45) Givens, R. S.; Matuszewski, B.; Athey, P. S.; Stoner, M. R. Photochemistry of Phosphate Esters: An Efficient Method for the Generation of Electrophiles. 6.
(46) Umeda, N.; Takahashi, H.; Kamiya, M.; Ueno, T.; Komatsu, T.; Terai, T.; Hanaoka, K.; Nagano, T.; Urano, Y. Boron Dipyrromethene As a Fluorescent Caging Group for Single-Photon Uncaging with Long-Wavelength Visible Light. *ACS Chem. Biol.* **2014**, *9* (10), 2242–2246.

(47) Li, J.; Yu, J.; Zhao, J.; Wang, J.; Zheng, S.; Lin, S.; Chen, L.; Yang, M.; Jia, S.; Zhang, X.; Chen, P. R. Palladium-Triggered Deprotection Chemistry for Protein Activation in Living Cells. *Nat. Chem.* **2014**, *6* (4), 352–361.

(48) Azoulay, M.; Tuffin, G.; Sallem, W.; Florent, J.-C. A New Drug-Release Method Using the Staudinger Ligation. *Bioorg. Med. Chem. Lett.* **2006**, *16* (12), 3147–3149.

(49) van der Gracht, A. M. F.; de Geus, M. A. R.; Camps, M. G. M.; Ruckwardt, T. J.; Sarris, A. J. C.; Bremmers, J.; Maurits, E.; Pawlak, J. B.; Posthoorn, M. M.; Bonger, K. M.; Filippov, D. V.; Overkleeft, H. S.; Robillard, M. S.; Ossendorp, F.; van Kasteren, S. I. Chemical Control over T-Cell Activation *in Vivo* Using Deprotection of *Trans* -Cyclooctene-Modified Epitopes. *ACS Chem. Biol.* **2018**, *13* (6), 1569–1576.

(50) Tu, J.; Xu, M.; Parvez, S.; Peterson, R. T.; Franzini, R. M. Bioorthogonal Removal of 3-Isocyanopropyl Groups Enables the Controlled Release of Fluorophores and Drugs in Vivo. *J. Am. Chem. Soc.* **2018**, *140* (27), 8410–8414.

(51) Jiménez-Moreno, E.; Guo, Z.; Oliveira, B. L.; Albuquerque, I. S.; Kitowski, A.; Guerreiro, A.; Boutureira, O.; Rodrigues, T.; Jiménez-Osés, G.; Bernardes, G. J. L. Vinyl Ether/Tetrazine Pair for the Traceless Release of Alcohols in Cells. *Angew. Chem. Int. Ed.* **2017**, *56* (1), 243–247.

(52) Neumann, K.; Jain, S.; Gambardella, A.; Walker, S. E.; Valero, E.; Lilienkampf, A.; Bradley, M. Tetrazine-Responsive Self-Immolative Linkers. *ChemBioChem* **2017**, *18* (1), 91–95.

(53) Xu, M.; Tu, J.; Franzini, R. M. Rapid and Efficient Tetrazine-Induced Drug Release from Highly Stable Benzonorbornadiene Derivatives. *Chem. Commun.* **2017**, *53* (46), 6271–6274.

(54) Sheehan, J. C.; Umezawa, K. Phenacyl Photosensitive Blocking Groups. *J. Org. Chem.* **1973**, *38* (21), 3771–3774.

(55) Zayat, L.; Calero, C.; Alborés, P.; Baraldo, L.; Etchenique, R. A New Strategy for Neurochemical Photodelivery: Metal–Ligand Heterolytic Cleavage. *J. Am. Chem. Soc.* **2003**, *125* (4), 882–883.

(56) Komori, N.; Jakkampudi, S.; Motoishi, R.; Abe, M.; Kamada, K.; Furukawa, K.; Katan, C.; Sawada, W.; Takahashi, N.; Kasai, H.; Xue, B.; Kobayashi, T. Design and Synthesis of a New Chromophore, 2-(4-Nitrophenyl)Benzofuran, for Two-Photon Uncaging Using near-IR Light. *Chem. Commun.* **2016**, *52* (2), 331–334.

(57) Momotake, A.; Lindegger, N.; Niggli, E.; Barsotti, R. J.; Ellis-Davies, G. C. R. The Nitrodibenzofuran Chromophore: A New Caging Group for Ultra-Efficient Photolysis in Living Cells. *Nat. Methods* **2006**, *3* (1), 35–40.

(58) Klán, P.; Šolomek, T.; Bochet, C. G.; Blanc, A.; Givens, R.; Rubina, M.; Popik, V.; Kostikov, A.; Wirz, J. Photoremovable Protecting Groups in Chemistry and Biology: Reaction Mechanisms and Efficacy. *Chem. Rev.* **2013**, *113* (1), 119–191.

(59) Bardhan, A.; Deiters, A. Development of Photolabile Protecting Groups and Their Application to the Optochemical Control of Cell Signaling. *Curr. Opin. Struct. Biol.* **2019**, *57*, 164–175.

(60) Il'ichev, Y. V.; Schwörer, M. A.; Wirz, J. Photochemical Reaction Mechanisms of 2-Nitrobenzyl Compounds: Methyl Ethers and Caged ATP. *J. Am. Chem. Soc.* **2004**, *126* (14), 4581–4595.

(61) Sasmal, P. K.; Streu, C. N.; Meggers, E. Metal Complex Catalysis in Living Biological Systems. *Chem Commun* **2013**, *49* (16), 1581–1587.
(62) Zheng, Y.; Ji, X.; Yu, B.; Ji, K.; Gallo, D.; Csizmadia, E.; Zhu, M.; Choudhury, M. R.; De La Cruz, L. K. C.; Chittavong, V.; Pan, Z.; Yuan, Z.; Otterbein, L. E.; Wang, B. Enrichment-Triggered Prodrug Activation Demonstrated through Mitochondria-Targeted Delivery of Doxorubicin and Carbon Monoxide. *Nat. Chem.* **2018**, *10* (7), 787–794.
(63) Lee, R. E. C.; Walker, S. R.; Savery, K.; Frank, D. A.; Gaudet, S. Fold Change of Nuclear NF-KB Determines TNF-Induced Transcription in Single Cells. *Mol. Cell* **2014**, *53* (6), 867–879.
(64) Lee, T. K.; Denny, E. M.; Sanghvi, J. C.; Gaston, J. E.; Maynard, N. D.; Hughey, J. J.; Covert, M. W. A Noisy Paracrine Signal Determines the Cellular NF-KB Response to Lipopolysaccharide. *Science Signaling*, **2009**, *2* (93), ra65.
(65) Blackman, M. L.; Royzen, M.; Fox, J. M. Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels–Alder Reactivity. *J. Am. Chem. Soc.* **2008**, *130* (41), 13518–13519.
(66) Versteegen, R. M.; Rossin, R.; ten Hoeve, W.; Janssen, H. M.; Robillard, M. S. Click to Release: Instantaneous Doxorubicin Elimination upon Tetrazine Ligation. *Angew. Chem. Int. Ed.* **2013**, *52* (52), 14112–14116.
(67) Li, J.; Chen, P. R. Development and Application of Bond Cleavage Reactions in Bioorthogonal Chemistry. *Nat. Chem. Biol.* **2016**, *12* (3), 129–137.
(68) Tu, J.; Xu, M.; Franzini, R. M. Dissociative Bioorthogonal Reactions. *ChemBioChem* **2019**, *20* (13), 1615–1627.
(69) Rossin, R.; Versteegen, R. M.; Wu, J.; Khasanov, A.; Wessels, H. J.; Steenbergen, E. J.; ten Hoeve, W.; Janssen, H. M.; van Onzen, A. H. A. M.; Hudson, P. J.; Robillard, M. S. Chemically Triggered Drug Release from an Antibody-Drug Conjugate Leads to Potent Antitumour Activity in Mice. *Nat. Commun.* **2018**, *9* (1), 1484.
(70) Rossin, R.; van Duijnhoven, S. M. J.; ten Hoeve, W.; Janssen, H. M.; Kleijn, L. H. J.; Hoeben, F. J. M.; Versteegen, R. M.; Robillard, M. S. Triggered Drug Release from an Antibody–Drug Conjugate Using Fast "Click-to-Release" Chemistry in Mice. *Bioconjug. Chem.* **2016**, *27* (7), 1697–1706.
(71) Rossin, R.; Versteegen, R. M.; Wu, J.; Khasanov, A.; Wessels, H. J.; Steenbergen, E. J.; ten Hoeve, W.; Janssen, H. M.; van Onzen, A. H. A. M.; Hudson, P. J.; Robillard, M. S. Chemically Triggered Drug Release from an Antibody-Drug Conjugate Leads to Potent Antitumour Activity in Mice. *Nat. Commun.* **2018**, *9* (1), 1484.