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# Preparation of unnatural N-to-N and C-to-C protein fusions

Martin D. Witte<sup>a</sup>, Juan J. Cragnolini<sup>a</sup>, Stephanie K. Dougan<sup>a</sup>, Nicholas C. Yoder<sup>a</sup>, Maximilian W. Popp<sup>a,b</sup>, and Hidde L. Ploegh<sup>a,b,1</sup>

<sup>a</sup>Whitehead Institute for Biomedical Research, Cambridge, MA 02142; and <sup>b</sup>Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142

Edited\* by Gregory A. Petsko, Brandeis University, Waltham, MA, and approved June 14, 2012 (received for review March 30, 2012)

**Standard genetic approaches allow the production of protein composites by fusion of polypeptides in head-to-tail fashion. Some applications would benefit from constructions that are genetically impossible, such as the site-specific linkage of proteins via their N or C termini, when a remaining free terminus is required for biological activity. We developed a method for the production of N-to-N and C-to-C dimers, with full retention of the biological activity of both fusion partners and without inflicting chemical damage on the proteins to be joined. We use sortase A to install on the N or C terminus of proteins of interest the requisite modifications to execute a strain-promoted copper-free cycloaddition and show that the ensuing ligation proceeds efficiently. Applied here to protein–protein fusions, the method reported can be extended to connecting proteins with any entity of interest.**

antibodies | bioorthogonal | engineering | transacylation

The creation of protein–protein fusions, by genetic means, chemically, or enzymatically, has become an important tool to study cell biological and biochemical problems. Genetic fusions with fluorescent proteins are widely used to visualize their (sub)cellular localization in situ and in vivo (1). Coexpression of two orthogonally labeled chimeras allows the study of protein colocalization and dynamics of receptor dimerization. Moreover, protein fusions have been used to evaluate the biological relevance of otherwise transient protein complexes. Fusion or cross-linking of two or more of the interacting proteins can stabilize protein complexes and has been used to explore signaling and (hetero)dimerization of G-protein–coupled receptors (2, 3), chemokines, and cytokines (4–6).

Besides being useful biochemical tools, chimeric proteins are also promising as treatment options for cancer, autoimmune diseases, lysosomal storage diseases, and brain disorders (7–10). Toxins have been conjugated to antibodies, growth factors, and cytokines as a means of delivering these payloads to malignant cells that express the counterstructures recognized by such fusion proteins, to kill tumor cells while minimizing collateral damage (10–12). Bisppecific antibodies, prepared by fusing two single-chain variable fragments (scFV) of immunoglobulins, may combine an antigen-binding domain specific for a tumor cell with a CD3 receptor-binding domain specific for T cells (13). These compounds then allow the T cells to exert cytotoxic activity or cytokine release locally and so elicit the desired antitumor response. Finally, protein fusion strategies have been used to prepare structurally defined biomaterials (14).

The production and purification of fusion proteins remains a biotechnological challenge. To obtain an active product, both domains of the chimera must adopt the native fold, without modification of residues and regions that are required for activity. The standard method to produce such proteins is by genetic fusion of the ORFs of the two proteins or protein fragments. Although recombinant expression often yields the correct product, partly folded and defective products cannot always be avoided.

If it were possible to conjugate by enzymatic means the natively folded, purified proteins by means of a minimal ligation tag, refolding steps could be avoided. Such methods exploit labeling at the C or N terminus of suitably modified protein substrates to produce the adducts of interest, exactly as if one were preparing the corresponding genetic fusions. Sortase-catalyzed transacylation reactions (15) allow such site-specific labeling of proteins, as well as the preparation of head-to-tail protein–protein fusions under native conditions, with excellent specificity and in near-quantitative yields (16, 17). Standard approaches fail to yield protein–protein fusions that are genetically impossible (N terminus to N terminus and C terminus to C terminus), although such unnatural liaisons would have great appeal for the construction of bisppecific antibodies or their fragments. To accomplish constructions of this type, one has to resort to chemical ligation methods. Early chemical conjugation strategies relied on nonspecific cross-linking via amines or sulfhydryls (18). The lack of control over the site and stoichiometry of modification result in the formation of a heterogeneous product, limiting the usefulness of this approach. The rise of bioorthogonal chemistries combined with site-specific mutagenesis, native chemical ligation, intein-based ligation, and amber suppressor pyrrolysine tRNA technology has enabled the synthesis of nonnatural protein fusions, as applied to the production of bivalent and multivalent antibodies (19–21). Structural analogs of ubiquitin dimers were prepared by a combination of intein-based ligation, site-specific mutation, and copper-catalyzed click chemistry (22, 23). Site-specific incorporation of propargyloxyphenylalanine facilitated the synthesis of green fluorescent protein (GFP) dimers (19, 24).

Nonetheless, the synthesis of bisppecifics would benefit from a method that is orthogonal to the published methods and that allows easy access to modified native protein, as well as enables efficient nonnatural conjugation of protein termini. Moreover, the availability of orthogonal methods will make possible the synthesis of protein structures of even greater complexity (heterotrimers and higher-order complexes). We here report a versatile approach that allows the preparation of N-to-N and C-to-C fused proteins under native conditions. Click handles are introduced either N or C terminally, using a sortase-catalyzed reaction. The resulting modified proteins are then conjugated with a strain-promoted cycloaddition in an aqueous environment at neutral pH (Fig. 1).

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Conflict of interest statement: The authors declare that a patent application on the production of N-to-N and C-to-C fusions has been filed.

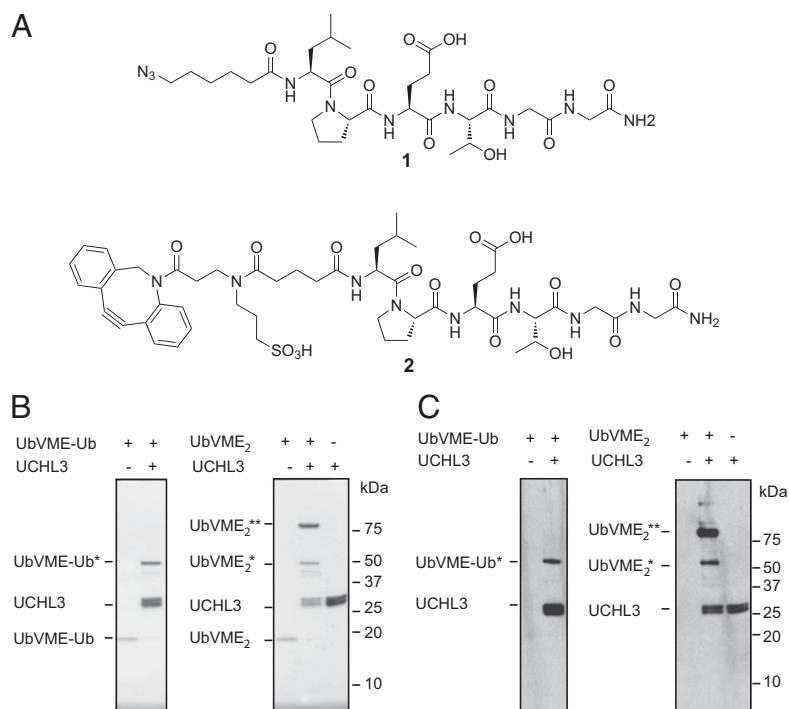
\*This Direct Submission article had a prearranged editor.

<sup>1</sup>To whom correspondence should be addressed. E-mail: ploegh@wi.mit.edu.

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This experiment shows that C-to-C fusion of an antibody fragment, in this case a single VHH domain, is readily achieved



**Fig. 2.** Proof of concept for the synthesis of N-to-N fused proteins. (A) structures of the used N-terminal probes **1** and **2**. (B and C) Labeling of his-tagged UCHL3 with dimeric UbVME. (B) Coomassie brilliant-blue-stained Tris-tricine gel. (C) Immunoblot using anti-His antibody. Ub-UbVME\*, ubiquitin-UbVME bound to a single UCHL3; UbVME<sub>2</sub>\*, dimeric UbVME bound to a single UCHL3 molecule; UbVME<sub>2</sub>\*\*, dimeric UbVME bound to two UCHL3 molecules.

using sortase in combination with click chemistry. Not only is the conversion excellent ( $\sim 90\%$ ), but also the resulting products retain their full function. Because most of the nucleophiles used in the sortase reaction are water soluble, and all necessary functional groups that require harsh and/or nonselective reaction conditions are introduced during the synthesis of the nucleophile, this approach minimizes unwanted side reactions [such as acylation of available amino groups (18) and denaturation of proteins] that might affect biological activity.

We extended the above experiment to include an alpaca-derived VHH that is specific for mouse class II MHC products, VHH7, and linked it to the anti-GFP VHH via their C termini to create a heterobispecific product.

Two adducts were prepared as described above, one containing a tetramethylrhodamine (TAMRA) fluorophore at the junction (using peptide **3**) and the other a nonfluorescent conjugate (using peptide **5**) (see [Fig. S5](#) for structure). The two adducts were purified to obtain the fluorescent and nonfluorescent bispecific VHH preparations ([Fig. S5](#)) and added to mouse lymph node cells, the B cells among which are uniformly positive for class II MHC products. When cells were exposed to the bispecific fluorescent VHH, we observed specific staining of B cells in the TAMRA channel ([Fig. 4A](#)). No staining was detected for the nonfluorescent bispecific antibody ([Fig. S6](#)). We then added GFP to cells exposed to bispecific VHHS, resulting in staining in the GFP channel for both bispecifics. We conclude that in this case, too, each of the fusion partners retains its activity and specificity. Lymph node cells of a MHC class II knockout mouse failed to stain with the bispecific VHHS, demonstrating specificity.

As a proof-of-principle experiment to demonstrate the use of such bispecific antibody derivatives for the creation of a deep tissue reservoir (32), we injected the anti-GFP-VHH7 bispecific construct i.v. with the goal to first target a relevant cell population (B cells) with this reagent. We directly administered a single bolus of recombinant GFP (50  $\mu$ g) either intraperitoneally or 1 h later i.v. and killed the animals 5.5 h later. Splenocytes were harvested

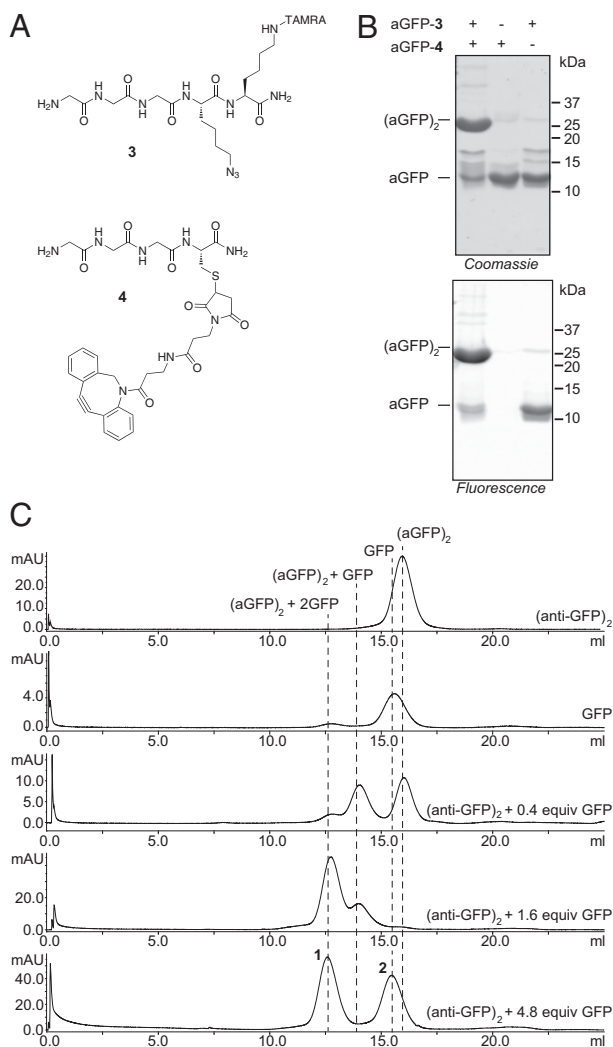
and analyzed by flow cytometry (Fig. 4B). Most CD19<sup>+</sup> cells (B cells) were GFP<sup>+</sup>, indicating successful capture of GFP *in vivo*. Administration of GFP into control animals that had not received the bispecific construct showed no GFP staining on CD19<sup>+</sup> or CD19<sup>-</sup> cells. This experiment thus shows that a bispecific construct can be used to first target a cell population of interest, which can then be addressed with a ligand for the remaining free second binding site. Construction of bispecific reagents of this type would allow the targeted delivery of biologicals in a manner that might avoid acute toxicity, as is observed for systemic interleukin-2 (IL2) administration.

With these tools in hand, it becomes possible, in principle, to connect any entity proved to be a substrate in a sortase reaction and in all possible topologies. We successfully C-terminally conjugated human IL2 and IFN- $\alpha$  to anti-GFP and VHH-7 using this approach (Fig. S7), thus showing the general applicability of these tools.

## Discussion

The ability to fuse proteins via their N or C termini creates immediate opportunities for the production of molecules not accessible by standard genetic means. We show that proteins connected in this manner retain functionality for N-to-N and for C-to-C fusions. As an instructive example, we demonstrate the ability to create C-terminally fused bispecific camelid-derived VHH constructs with full retention of the binding capacity of both fusion partners. Possible applications extend to other fusions as well. For those situations where the desired combination demands that both C or both N termini remain available for proper function, standard genetic approaches fall short. Click chemistry has developed to the point where off-the-shelf reagents suitable for solid-phase peptide synthesis allow ready access to the peptides that enable these types of fusion. Although demonstrated here for protein–protein fusions, further modifications of the click handles used to connect the two proteins allow installation of yet other functionalities, such as fluorophores, or pharmacologically active small molecules. Ease of





**Fig. 3.** C-to-C homodimeric antibodies. (A) Structures of probes 3 and 4. (B) Dimerization of anti-GFP. (C) Size exclusion experiment demonstrating that both anti-GFPs bind GFP. From Top to Bottom: anti-GFP dimer; GFP; anti-GFP dimer + 0.4 equiv GFP; anti-GFP dimer + 1.6 equiv GFP; anti-GFP dimer + 4.8 equiv GFP (excess).

modification of proteins of interest, ready access to recombinant sortases of different origin, and the flexibility afforded in nucleophile design through use of standard peptide synthetic methodology add to the versatility of sortase-mediated transacylation.

Protein fusions not easily accessed by other means are within easy reach using the technology described here. Of note, Hudak et al. described the use of an aldehyde tag in combination with strain-promoted click chemistry to achieve similar goals and produced hIgG fused to human growth hormone and maltose-binding protein (33). This approach is orthogonal to our sort-tagging strategy and immediately suggests the possibility of combining methods such as the aldehyde tag-click chemistry method developed by Hudak et al. with the chemo-enzymatic method developed here to access even more challenging protein-protein fusions.

## Methods

**N-Terminal Sortagging.** Sortase A of *S. aureus* (150  $\mu$ M final concentration, 4.5 $\times$  stock in 50 mM Tris, pH 7.4, 150 mM NaCl) and probe 1 or 2 (0.5 mM final concentration, 10 $\times$  stock) were added to UbVME (58  $\mu$ M final concentration) in sortase buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM  $\text{CaCl}_2$ ). The resulting mixture was incubated at 37  $^\circ\text{C}$  for 3 h. Next, the

solution was acidified with 1% TFA in  $\text{H}_2\text{O}$  and purified by reverse-phase HPLC [30 $\rightarrow$ 45% (vol/vol) B in 20 min, 3 mL/min]. The resulting purified protein was neutralized with saturated aqueous (sat. aq.)  $\text{NaHCO}_3$  concentrated in vacuo, redissolved in  $\text{H}_2\text{O}$ , and quantified by gel electrophoresis. The protein was analyzed by LC/MS:  $\text{N}_3$ -UbVME,  $R_t = 7.70$  min, linear gradient 5 $\rightarrow$ 45% (vol/vol) B in 20 min; ESI (electrospray ionization)/MS,  $m/z = 9,714$  ( $\text{M}+\text{H}$ ) $^+$ ; DIBAC-UbVME,  $R_t = 7.54$  min, linear gradient 5 $\rightarrow$ 45% (vol/vol) B in 20 min; ESI/MS,  $m/z = 9,360$  ( $\text{M}+\text{H}$ ) $^+$ .

**C-Terminal Sortagging.** Sortase A of *S. aureus* (150  $\mu$ M final concentration, 4.5 $\times$  stock in 50 mM Tris, pH 7.4, 150 mM NaCl) and probe (0.5 mM final concentration, 10 $\times$  stock) were added to the VHH (15  $\mu$ M final concentration) in sortase buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM  $\text{CaCl}_2$ ). The resulting mixture was incubated at 25  $^\circ\text{C}$  overnight. The protein was purified by size exclusion on a Superdex 75. The resulting purified protein was concentrated in centrifugal filter units and analyzed by gel electrophoresis and LC/MS. Anti-GFP-3:  $R_t = 6.02$  min, linear gradient 5 $\rightarrow$ 45% (vol/vol) B in 20 min; ESI/MS,  $m/z = 14,330$  ( $\text{M}+\text{H}$ ) $^+$ . Anti GFP-4:  $R_t = 7.90$  min, linear gradient 5 $\rightarrow$ 45% (vol/vol) B in 20 min; ESI/MS,  $m/z = 14,170$  ( $\text{M}+\text{H}$ ) $^+$ . VHH7-3:  $R_t = 7.20$  min, linear gradient 5 $\rightarrow$ 45% (vol/vol) B in 20 min; ESI/MS,  $m/z = 15,549$  ( $\text{M}+\text{H}$ ) $^+$ . VHH7-5:  $R_t = 7.00$  min, linear gradient 5 $\rightarrow$ 45% (vol/vol) B in 20 min; ESI/MS,  $m/z = 15,139$  ( $\text{M}+\text{H}$ ) $^+$ .

**Synthesis of Dimeric UbVME Constructs.** A mixture of azido-modified UbVME (42.5  $\mu$ L, 80  $\mu$ M) and cyclooctyne-modified UbVME (42.5  $\mu$ L, 70  $\mu$ M) was incubated overnight and subsequently purified by reverse-phase HPLC [30 $\rightarrow$ 45% (vol/vol) B in 20 min, 3 mL/min]. After purification, the solution was neutralized with sat. aq.  $\text{NaHCO}_3$  and concentrated in vacuo. Dimeric ubiquitin constructs containing only one reactive vinylmethyl ester were obtained by incubating either azido-modified ubiquitin (42.5  $\mu$ L, 80  $\mu$ M) with cyclooctyne-modified UbVME (42.5  $\mu$ L, 70  $\mu$ M) or azido-modified UbVME (42.5  $\mu$ L, 80  $\mu$ M) with cyclooctyne-modified ubiquitin (42.5  $\mu$ L, 70  $\mu$ M). After dimerization, the proteins were purified and handled as described above.

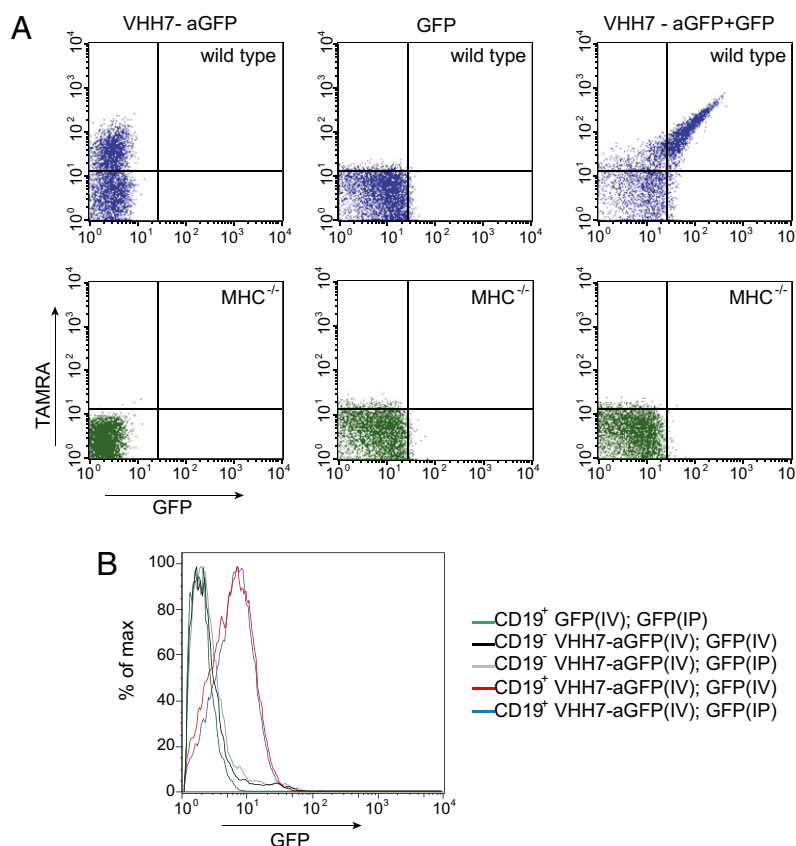
**Labeling of UCHL3 with Dimeric UbVME Constructs.** Purified dimeric constructs (0.5  $\mu$ g, 24.5 pmol) were diluted in 20  $\mu$ L Tris buffer [20 mM Tris, pH 8, 100 mM NaCl, 0.1 mM TCEP (tris(2-carboxyethyl)phosphine)] in the presence or absence of UCHL3 (94 pmol). The resulting mixture was incubated for 2 h, denatured with Laemmli sample buffer (4 $\times$ ), and loaded on a Tris-tricine gel. The proteins were either directly analyzed by Coomassie brilliant-blue staining or transferred to a PVDF membrane. The membrane was blocked with 4% (wt/vol) BSA in PBS/Tween (0.1% vol/vol). Penta-His HRP (1:12,500) was added and the membrane was agitated for 30 min at room temperature. The membrane was washed four times with 0.1% vol/vol Tween in PBS before the proteins were visualized using ECL plus.

**Dimerization of Nanobodies.** Homodimeric anti-GFP nanobody was prepared by incubating anti-GFP-3 (100  $\mu$ L, 80  $\mu$ M) and anti-GFP-4 (100  $\mu$ L, 85  $\mu$ M) overnight at room temperature. Heterodimeric VHH7-3-anti-GFP-4 and VHH7-5-anti-GFP-4 were obtained by reacting either VHH7-3 (200  $\mu$ L of a 20- $\mu$ M solution) or VHH7-5 (200  $\mu$ L of a 60- $\mu$ M solution) with anti-GFP-4 (100  $\mu$ L of a 120- $\mu$ M solution) overnight at 25  $^\circ\text{C}$ . The dimeric nanobodies were purified by size exclusion on a Superdex 75. Fractions were collected and concentrated in centrifugal filter units. The purified dimers were analyzed on 15% SDS/PAGE.

(Anti-GFP) $_2$ :  $R_t = 10.07$  min, linear gradient 5 $\rightarrow$ 45% (vol/vol) B in 20 min; ESI/MS,  $m/z = 28,526$  ( $\text{M}+\text{H}_2\text{O}+\text{H}$ ) $^+$ . VHH7-3-anti-GFP-4:  $R_t = 10.91$  min, linear gradient 5 $\rightarrow$ 45% (vol/vol) B in 20 min; ESI/MS,  $m/z = 29,755$  ( $\text{M}+\text{H}_2\text{O}+\text{H}$ ) $^+$ . VHH7-5-anti-GFP-4:  $R_t = 10.87$  min, linear gradient 5 $\rightarrow$ 45% (vol/vol) B in 20 min; ESI/MS,  $m/z = 29,329$  ( $\text{M}+\text{H}_2\text{O}+\text{H}$ ) $^+$ .

**Functionality Assay of Homodimeric Nanobodies.** Homodimeric anti-GFP nanobody (20  $\mu$ L, 25  $\mu$ M) was incubated with GFP (2.5  $\mu$ L, 10  $\mu$ L, and 30  $\mu$ L of an 80- $\mu$ M solution). The formed nanobody-GFP complex was subjected to size exclusion on a Superdex 200.

**Functionality Assay of Heterodimeric Nanobodies.** Lymph node cells were harvested from C57BL/6 (Jackson Laboratories) or MHCII-deficient mice (Jackson Laboratories), washed, and incubated for 10 min with VHH7-anti-GFP, GFP, and VHH7-anti-GFP+GFP at 4  $^\circ\text{C}$ . The cells were collected by centrifugation, washed with PBS, and analyzed by flow cytometry.



**Fig. 4.** (A) FACS staining of mouse lymph node cells with anti-MHC II-anti-GFP antibodies. (Upper) Staining observed in wild-type cells. (Lower) Staining of MHC class II-deficient cells. (B) In vivo delivery of GFP. Mice were injected with 50  $\mu$ g bispecific antibodies and either received directly intraperitoneally or after 1 h i.v. 50  $\mu$ g GFP. Stained cells were analyzed by flow cytometry.

**In Vivo Delivery Assay.** For the delivery assays, BALB/c mice (Jackson Laboratories) were injected in the tail vein with either the bispecific antibody or GFP (50  $\mu$ g per mouse). The mice receiving the bispecific antibody either directly received GFP (50  $\mu$ g) intraperitoneally or received GFP (50  $\mu$ g) i.v. after 1 h. After 5.5 h, blood was harvested, the mice were killed, and cells were isolated from lymph nodes, thymus, and spleen. Cells were washed with PBS and incubated with anti-CD19-APC (BD Pharmingen) and 7-AAD (Viaprobe; BD) for 10 min at 4 °C. The cells were washed with PBS and analyzed by flow cytometry. Mice were housed at the Whitehead Institute for

Biomedical Research, and all studies were approved by the Massachusetts Institute of Technology Committee on Animal Care.

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